Indiana University
Bloomington, IN

Indiana-Princeton-Tennessee Astrobiology Institute (IPTAI): Detection of Biosustainable Energy and Nutrient Cycling in the Deep Subsurface of Earth and Mars

Principal Investigator:

Lisa Pratt prattl@indiana.edu

Co-Investigators/Collaborators:

At Indiana University:
Ruth Droppo
Michael Jasiak
Doug Pearson
Edward Ripley

Fred Brockman, *Pacific Northwest National Laboratory*
Stephen Clifford, *Universities Space Research Association*
Kimberly Davis, *University of Tennessee, Knoxville*
Charles Dismukes, *Princeton University*
James Fredrickson, *Pacific Northwest National Laboratory*
Terry Hazen, *Lawrence Berkeley National Laboratory*
Duane Moser, *Battelle Pacific Northwest National Laboratory*
Satish Myneni, *Princeton University*
Tullis Onstott, *Princeton University*
Susan Pfiffner, *University of Tennessee*
Tommy Phelps, *Oak Ridge National Laboratory*
Lee Riciputi, *Oak Ridge National Laboratory*
Barbara Sherwood Lollar, *University of Toronto*
David White, *University of Tennessee, Knoxville*

EXECUTIVE SUMMARY

Our decision to seek membership in NASA's Astrobiology Institute is motivated by a desire to bring our expertise in deep subsurface ecosystems to bear on the scientific and technological difficulties that will be encountered during the exploration of life beneath the surface of Mars. Our center will be a consortium composed of senior level investigators representing Indiana University, Princeton University, University of Tennessee, Pacific Northwest National Laboratory, Oak Ridge National Laboratory, Lawrence Berkeley National laboratory, University of Toronto, and the Lunar and Planetary Institute. We believe our expertise in subsurface ecosystems and our access to extraordinary analytical facilities and field sites will enable development of synergistic relationships with other biological, geological and planetary research in the NAI.

Geochemists, chemists, microbiologists, and hydrologists on our team seek to collaboratively investigate physical and chemical limitations on life deep beneath the subsurface of Earth in order to design life detection methods for subsurface exploration of Mars. We have recovered indigenous microbes (Bacteria and Archaea) from ten- to hundred-million year old, highly saline fracture water at depths up to 3200 meters below ground in South African gold mines. Most of these microbes are sulfate reducing bacteria, but indigenous species utilizing other pathways of electron transfer can be detected by ribosomal sequencing. The magnitude of and ergonomic constraints on microbial activity and growth in these environments are poorly understood. The relationship between abiogenic chemical processes, such as radiolysis and Fischer-Tropsch reactions, and biogenic processes that sustain life remain largely enigmatic in the deep subsurface. The identity of specific genes that are critical to the survival of subsurface microorganisms inhabiting these environments has not been established. The evolutionary relationship of such genes to those of surface dwelling microorganisms is not known, but could provide clues at to whether microbial life on Earth originated in the subsurface. Life-forms in the subsurface of other planets presumably concentrate energy from geological sources like terran subsurface ecosystems, but their cellular machinery could be radically different. The fundamental elements and behavior common to all subsurface life forms need to be established in order to design life detection instruments for subsurface planetary probes.

A combination of field and laboratory experiments will be utilized to address the geochemical and genomic signatures of subsurface microbial ecosystems. In addition to continuing work in South African mines, an Arctic field site will be established where brine-containing fractures exist beneath a permafrost cap and provide an environment believed to be analogous to that of Mars. Levels of nutrients and metabolites will be quantified using stable-isotopic and molecular data on gaseous and aqueous species and on cellular and mineral materials collected from boreholes drilled into tunnel walls. Geochemical profiles from these boreholes will be combined with information on the genomic diversity in order to assess potential limiting factors for microbial survival. These results will be compared to results obtained from much warmer South African subsurface sites to determine elements common to microbial life at depths. The development of geophysical and chemical sensors for detecting life will be field tested at these sites.

Laboratory studies will make use of several strains of anaerobic Bacteria and Archaea from deep-subsurface sites including sulfate reducers, thiosulfate/sulfur disproportionators, Fe reducers, Mn reducers, and methanogens. We will design experiments to differentiate between microbial and abiogenic processes that control energy and nutrient cycles in the deep subsurface. These experiments will focus on process response to inferred environmental parameters in the

Martian subsurface, such as low water activity, high salinity, dessication, radiolysis, high CO_2 partial pressure, and clathrate formation. Based upon the results of these microbial experiments, we will develop and deploy borehole instruments to image fractures and biofilms, monitor the expression of genes, detect the presence of metabolites, and identify cellular activity.

The proposed research will address several goals identified in the Astrobiology Roadmap. Recovery of uncontaminated samples from deep-subsurface ecosystems on Earth is directly relevant to exploration for extant life in the subsurface of Mars, Objective 2.1 of Goal 2--Life in Our Solar System. We have accessed deep-subsurface ecosystems at depths greater than 3 kilometers below the ground surface in South African gold mines. The gold bearing conglomerates are late Archean in age and, consequently, characterization of the geochemical, lipid and isotopic signatures preserved in these rocks will enable us to contribute to Goal 4--Earth's Early Biosphere and its Environment. We will explore the evolution, environment and limit of life, Goal 5, by examining the community composition of subsurface ecosystems in different geochemical venues and by performing in situ experiments to see how the community evolves in response to environmental changes. Given high contents of uraninite in the gold-bearing conglomerates, our research is relevant to Objective 5.3--the determination of survival strategies that permit organisms to maintain viability in a radioactive environment for millennia. Finally, our previous research identified isotopic signatures that derive from the presence of subsurface bacterial life, Objective 7.1 of Goal 7--Signature of Life. Our future research plans will evaluate the preservation potential for these bio-signatures in clastic and chemical sediments analogous to regolith deposits on Mars.

Education and Public Outreach (EPO) activities in the proposed Indiana-Princeton-Tennessee Bio-Sustaining Cycles Team are designed around three areas of emphasis. First: educational workshops for undergraduates and high school teachers where participants actively collect and interpret data from laboratory and field experiments. Second: public outreach through a web site with premiere-quality digital media including animations and video that illustrate how and why scientists conduct microbiological research in extreme environments on Earth in preparation for exploration of Mars. Third: mentoring undergraduate and graduate research at Indiana, Princeton, and Tennessee universities. Inclusion of collaborators from the School of Fine Arts, IU Instructional Support Services, and University Information Technology Services at Indiana University is an unusual aspect of this proposal. High-resolution digital video/audio materials will be collected during field experiments and will be use in both research and educational components of the IPTAI. Videos produced by scientists will document research methods in a substantially different way from conventional commercial films. We hope to capture examples of both set-backs and advances in research resulting from unanticipated and challenging conditions in deep mines. Given severe time and logistical constraints in deep mines, digital documentation of physical conditions and instrumental configurations are essential for later interpretation of experimental results.

The proposed collaboration to study Bio-sustainable Energy and Nutrient Cycles in the Deep Sub-surface of Earth and Mars offers unusually high levels of institutional commitment from Indiana University, Princeton University, and the University of Tennessee. The total matching funds from these three institutions is in excess of $1,000,000. The match includes technical staff, graduate student stipends and tuition, academic salary, renovated space for an institutional office, and travel and supplies for E/PO.

TABLE OF CONTENTS

RESEARCH PLAN

INTRODUCTION

Our NAI would focus upon subsurface microbial communities that have been sequestered from the surface photosphere for tens to hundreds of millions of years and the environments that support their in situ activities. These terran ecosystems would represent the closest analogy to what might exist beneath the cryosphere of Mars. We seek to characterize the microbial, mineralogical and geochemical interactions, the isotopic signatures of the organic and inorganic gaseous, aqueous and metallic species, the interspecies and interkingdom communications and interactions, the genomic diversity and capabilities, the proteins expressed and their origin and the metabolites created and exchanged. This information will be used to design life detection approaches that will be tested in well-characterized field locations as a first step towards the design of flight-capable life detection instruments for future Mars drilling missions.

1. WATER ON MARS: A SUMMARY OF CURRENT THINKING

The search for water has been identified as the principal objective and common thread of Mars research – its abundance and distribution having important implications for understanding the geologic, hydrologic, and climatic evolution of the planet; the potential origin and continued survival of life; and the accessibility of a critical in-situ resource for sustaining future human explorers. Of the planet's estimated 0.5 to 1 km global inventory of H_2O, ~90-95% is thought to reside in the subsurface (as ground ice and groundwater), with the remainder stored as ice within the polar layered deposits (PLD).

Evidence for a water-rich Mars is provided by the geomorphic interpretation of a long list of landforms (e.g., (Carr and Schaber, 1977; Lucchitta, 1981; Rossbacher and Judson, 1981; Kuzmin, 1983; Carr, 1986)(Squyres, 1989; Squyres et al., 1992). In particular, it is supported by the existence of the outflow channels, whose distribution, size, and range of ages, suggests that a significant body of groundwater was present on Mars throughout much of its geologic history and may still persist to the present day (Baker, 1982; Carr, 1986; Tanaka, 1986; Tanaka and Scott, 1986; Baker et al., 1992).

Based on a conservative estimate of the discharge required to erode the outflow channels, and the likely extent of their original source region, Carr (Carr, 1996) estimates that Mars may possess a planetary inventory of water equivalent to a global ocean 0.5 to 1 km deep. Of this estimated inventory, ~0.000001% is found in the atmosphere, while ~5-10% is thought to be stored as ice in the perennial polar ice caps and layered deposits. This leaves ~90-95% of this H_2O unaccounted for, the vast bulk of which is believed to reside, as ground ice and groundwater, within the planet's crust.

Expected Nature and Location of Primary Subsurface Reservoirs of H_2O.

The expected distribution and state of subsurface water on Mars, as well as plausible values of the large-scale physical, thermal, and hydraulic properties of its crust, have been discussed by Rossbacher (Rossbacher and Judson, 1981), Clifford (Clifford, 1984, 1993), Squyres (Squyres et al., 1992), Carr (Carr, 1996) and Clifford (Clifford and Parker, 2001). To a first order, subsurface conditions on Mars are expected to resemble those found in cold-climate regions on Earth, particularly the unglaciated, continuous permafrost regions of Antarctica, Siberia, and North America. This similarity is likely to extend to an equivalent level of geologic complexity and spatial variability in such characteristics as lithology, structure, stratigraphy, porosity, volumetric ice content, and mechanical and thermal properties.

Current mean annual surface temperatures on Mars range from ~154^{o}K at the poles to ~218^{o}K at the equator (±5^{o}K), with radiogenic heating expected to result in increasingly warmer temperatures at depth. Consideration of the current best estimates of both the planet's mean geothermal heat flux (~15-45 mW m^{-2}) and the freezing temperature of the most geochemically plausible compositions of groundwater (~252-273^{o}K) suggest that the present depth of frozen ground on Mars, a region also known as the cryosphere (Rossbacher and Judson, 1981; Clifford and Hillel, 1983; Kuzmin, 1983), should vary from ~2.5 – 5 km at the equator to ~6.5 – 13 km at the poles (Fig. 1). However, natural variations in crustal

heat flow, thermal conductivity, and the presence of potent freezing-point depressing salts, are expected to result in significant local departures from these predicted zonally-averaged values (Clifford and Parker, 2001).

At the Martian surface, the low relative humidity of the atmosphere means that ground ice is thermodynamically unstable at latitudes equatorward of ~40^{o} (Leighton and Murray, 1966; Fanale et al., 1986) resulting in its loss by sublimation at a rate that is dependent on the mean annual surface temperature, as well as the local thermal and diffusive properties of the crust (Smoluchowski, 1968; Clifford and Hillel, 1983; Fanale et al., 1986). Depending on the nature of these properties, their variation with depth, and the potential for replenishment from any deeper reservoir of subpermafrost groundwater, these factors may result in local depths of desiccation at low-latitudes that range from centimeters to as much as a kilometer with the potential for significant and complex variations in saturation state beneath the sublimation front (Clifford, 1998). Such uncertainties preclude any reliable theoretical or geomorphic prediction of the present distribution of subsurface H_2O below the seasonal skin-depth.

If the Martian inventory of H_2O exceeds what can be stored as ice within the pore volume of the cryosphere, then the bulk of the excess will be present as a liquid, saturating the lowermost porous regions of the crust (Clifford, 1993). Given a large-scale crustal permeability comparable to that of the Earth, and the lack of any recent rainfall, the influence of gravity should result in a present-day groundwater system that is in effective hydrostatic equilibrium – except where it may be locally perturbed by tectonic, seismic or thermal processes. Because of the low porosity expected at depth, comparatively little water is required to produce a groundwater system of substantial extent. Thus, if a subpermafrost groundwater system is present on Mars, it is expected to underlie much of the planet's surface.

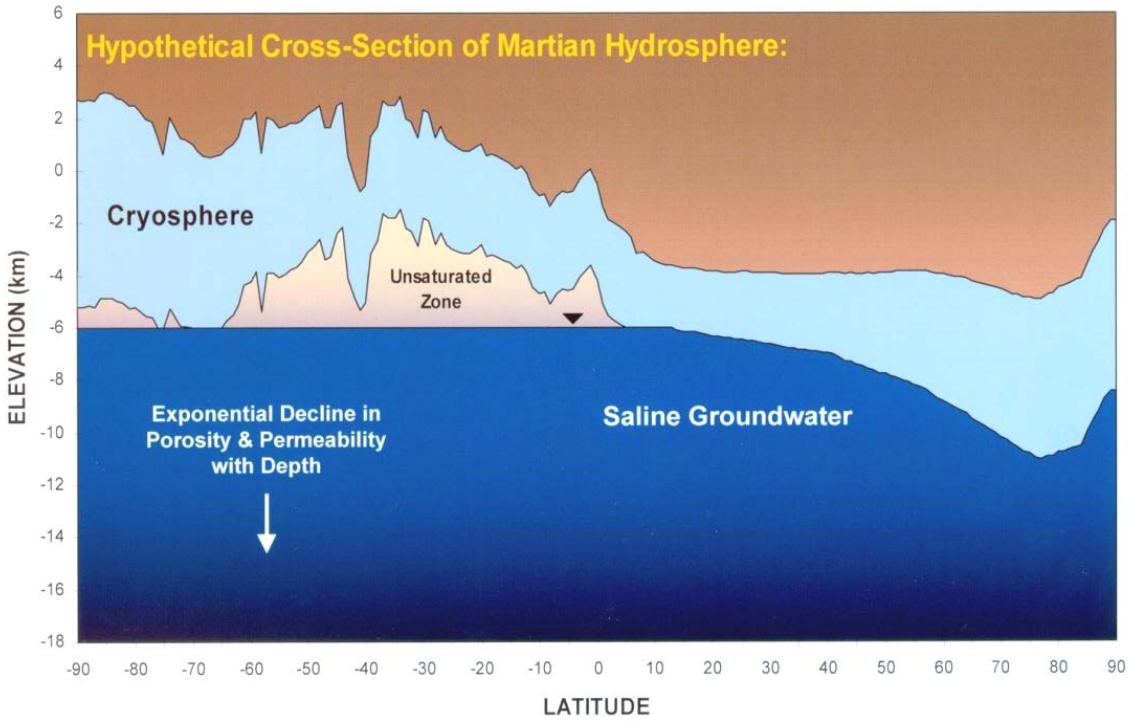


Fig. 1. Hypothetical, meridional, cross section of the Martian hydrosphere showing the distribution of ice, water and water vapor.

The distribution of ground ice is expected to follow the thermal structure of the crust, while the global groundwater table is expected to conform to a surface of constant geopotential. For this reason, the local vertical distance separating these subsurface reservoirs may vary considerably such that the intervening unsaturated zone is maximized in regions of high elevation and minimized (or absent) at lower elevations (Fig. 1). Within the unsaturated zone, the presence of a geothermal gradient is expected to give rise to a low-temperature hydrothermal convection system of rising vapor and descending liquid condensate. Such a system may have lead to the development of perched aquifers and the geochemical evolution of the underlying groundwater into a highly mineralized brine (Clifford, 1991, 1993). The liquid stability field of the brine encompasses much of the range in martian surface P-T conditions (Bodnar, 2001) and could be present just below a frozen duricrust.

Although there is evidence that Mars once possessed a sizable inventory of subpermafrost groundwater, it is also possible that such a inventory may no longer survive, outside of that which may be locally and transiently produced by the melting of ground ice from local geothermal activity. Such a state could well result from the progressive cold-trapping of a once large inventory of groundwater into the pore volume of the thickening cryosphere, as the planet's internal heat flow declined with time (Clifford and Parker, 2001).

The belief that any sizable body of liquid water on Mars must reside at depths of several kilometers or more (e.g., Figure 1) has recently been challenged by Malin and Edgett (Malin and Edgett, 2000), who have identified features in high-resolution Mars Orbiter Camera (MOC) images that they interpret as evidence of recent (and possibly contemporary) discharges of liquid water from near-surface aquifers (~100-500 m). However, given the enormous difficulty of reconciling the shallow aquifer hypothesis with both plausible environmental conditions and the need to explain the various enigmatic characteristics of the gullies (e.g., their restriction to mid- to high-latitudes and preferential occurrence on poleward-facing slopes), significant doubts have subsequently been raised about the uniqueness and plausibility of this interpretation.

Currently, there is only one explanation for the origin of the gullies that appears to satisfy the most serious environmental and observational constraints. The Martian obliquity is known to be chaotic on a time scale of $\sim 10^7$ years, varying from $\sim 0^o$ - 60^o (Laskar and Robutel, 1993). For obliquities $\geq 45^o$, the peak insolation on poleward facing slopes at mid- to high-latitudes can yield summertime surface temperatures that easily exceed the melting point for continuous periods that range from hours to many months (Pathare and Paige, 1998; Costard et al., 2002; Paige, 2002). Under these conditions, large amounts of water ice are expected to sublime and melt from the summer polar ice cap – increasing the atmospheric vapor pressure of H_2O sufficiently to allow liquid water to flow readily across the surface. Under such conditions, formerly stable near-surface ice deposits could conceivably melt and produce sufficient run-off to form the gullies (Costard et al., 2002; Paige, 2002).

Potential Occurrence and Distribution of Gas Hydrates and Liquid CO_2.

In addition to the potential presence of such well-recognized volatile reservoirs as ground ice and groundwater, subsurface H_2O may also be present in the form of physical compounds of water ice and various gases, known as gas hydrates. Hydrates form when CO_2 and other gases (like CH_4 and H_2S), are concentrated under conditions of high pressure and low temperature in the presence of H_2O, where they can become stabilized by Van der Waals bonding within the cubic crystalline lattice of water ice molecules.

A number of investigators have argued that substantial amounts of CO_2 hydrate may be present in the Martian subsurface, most probably formed by the progressive cooling and freezing of CO_2-saturated groundwater (Miller and Smythe, 1970; Milton, 1974; Kargel et al., 2000; Komatsu et al., 2000). At 200^oK, CO_2 hydrate is stable at depths as shallow as ~5 m (corresponding to a confining pressure of ~50 kPa) and may remain so down to a maximum depth defined by the location of the 283^oK isotherm (Sloan, 1997). It is also possible that CH_4, has been produced on Mars by both biotic (Farmer, 1996; Fisk and

Giovannoni, 1999; Max and Clifford, 2000; Max and Clifford, 2001) and abiotic (Wallendahl and Treimann, 1999) processes resulting in local concentrations ranging from a dispersed contaminant, to massive deposits (Max and Clifford, 2000; Max and Clifford, 2001). The stability field of CH_4 hydrate is similar, but not identical, to that of its CO_2 counterpart extending from a depth as shallow as ~15 m (corresponding to a confining pressure of ~140 kPa) at 200^{o}K, to a maximum depth of as much as much as a kilometer below the base of the cryosphere. As the internal heat flow of Mars has declined with time, the resulting downward propagation of the freezing-front at the base of the cryosphere may have incorporated both CO_2 and CH_4 hydrate in concentrations ranging from a dispersed contaminant, to massive deposits (Max and Clifford, 2000).

In addition to gas hydrates, several recent studies have argued that the pressure and temperature conditions expected in the Martian subsurface may also permit the stable existence of liquid CO_2 in amounts that may range from small inclusions to aquifer-like reservoirs that may have played a role in the genesis of the outflow channels (Hoffman, 2000). While it is possible to identify a P-T environment in the Martian subsurface where liquid CO_2 would be stable if emplaced after that environment had formed, it appears difficult to imagine an evolutionary scenario by which aquifers of liquid CO_2 could have initially formed and survived to the present day (Stewart and Nimmo, 2002). If, however, liquid CO_2 is currently present in the subsurface, it appears that it will most likely occur as inclusions and localized pockets.

In terms of subsurface martian ecosystems, therefore, three classes of environments exist; 1) the water ice and CO_2/CH_4 clathrate of the cryosphere with briny interstices; 2) a deep vadose zone dominated by either CO_2 or possibly CH_4 gas; and 3) a deep, saline, fractured aquifer potentially saturated with either CO_2 or CH_4 gas. These environments should be stable on the time frame of ~10^7 years, perturbed by changes in martian obliquity and the occasional meteorite impact. Our recent LExEn-supported research focuses on a terran subsurface ecosystem that is most analogous to the third class of martian environments.

2. BIOSUSTAINABLE DEEP SUBSURFACE TERRAN ECOSYSTEMS

For the past two decades an increasing number of microbiologists, molecular biologists, geochemists and mineralogists have been examining subsurface microbial ecosystems to understand their biodiversity, origins, impact on ground water chemistry and aquifer mineralogy and to remediate contaminated aquifers (Pedersen, 1993; Whitman et al., 1998). Only few of these studies have examined the microbial biomass and community structure at a depth greater than a few hundred meters primarily because of the lack of accessibility and the difficulties of contamination during drilling (Colwell et al., 1997). Most of these studies rely upon groundwater samples pumped from wells or a few cores (Phillips and Lappin-Scot, 1997; Onstott et al., 1998b).

Inferences about the subsurface microbial communities present in the ocean crust have been based in part on ODP sediment cores (Parkes et al., 1994), on observations and measurements of ocean ridge hydrothermal vents (Deming and Baross, 1993) and off axis vents and on fluids emanating from boreholes drilled into the ocean floor (Cowen et al., 2002). More recently, however, our group has been able to obtain saline water samples and rock samples from depths up to 3.3 kilometers below the surface in the deep mines in South Africa (Takai et al., 2001a; Takai et al., 2001b; Mormile et al., 2003). The attribute that sets this deep subsurface site apart from the other studies is that the saline water has been isolated for up to ~100 myrs. from the surface and are encased in highly impermeable, 2.9 Ga rock strata. Fluid flux in these environments are 10^4 times slower than those studied in the marine environment and any residual organic matter is confined to rare horizons and has been metamorphosed to lower greenschist faces. Yet, these isolated fluid-filled fractures yield intact cells with DNA suggesting that their survival has depended upon chemical energy sources and growth substrates tapped by a combination of gas/water/rock/microbial interactions. A number of recent studies have speculated that H_2 producing water-rock interactions may provide the energy for subsurface microbial metabolism deep in the earth's crust (Lilley et al., 2001) and may be possible terrestrial analogues for subsurface microbial ecosystems

under reducing conditions on other planets or moons (McCollum, 1999; Chapelle et al., 2002). But many questions still remain including:

- Would these communities, having survived for tens of millions of years, continue to survive *ad infinitum* barring a geological cataclysm? Barring the invention of time travel the only way to address this question is to begin studying the electron fluxes, genetic, lipid and protein composition of both the planktonic and sessile microbial communities trapped within these deep fractures.
- Are autotrophic and acetogenic microorganisms key marker species to these types of communities? Stevens and McKinley(Stevens and McKinley, 1995), reported the presences of these phenotypes at ~1 kmbls in the 14 Ma Columbia River Basalt aquifer. This observation combined with H_2 and $\delta^{13}C$ analyses led them to propose that oxidation of Fe-bearing minerals in the basalt generates H_2 that is consumed by methanogens and acetogens. The latter then provide an organic carbon substrate for heterotrophic microorganisms. Although this hypothesis, referred to as SLIME's, has been hotly debated (Anderson 1998), the fundamental assumptions still appear to be tenable (Chapelle et al., 2002) and the presence of methanogens and a gram-positive homoacetogen in a granitic aquifer at depths up to 420 m has been cited as evidence in support of this concept (Kotelnikova and Pedersen, 1997; Pedersen, 1997).
- Are these microorganisms still relying upon an organic nutrient source, tenuous though it may be, that was first formed photosynthetically in the Archean, deposited, metamorphosed and in some cases radiolytically altered? The extent to which heterotrophs in subsurface environments such as the Columbia River Basalt aquifer utilize acetate generated by homoacetogens or upon dissolved organic substrates exuded by the sediments inter-layered between the volcanic horizons was never established. With one notable exception, the heterotrophic members of subsurface microbial communities typically outnumber the autotrophs even within a Precambrian granite aquifer (Ekendahl and Pedersen, 1994). To answer this question will require detailed, compound specific ^{13}C analyses of the major organic species present and of the microorganisms themselves. One also has to look to the origins of other essential nutrients including N and P.
- If not, have these microorganisms developed mechanisms or a strategy for enhancing, manipulating and efficiently utilizing in situ chemical energy fluxes, sequestering key nutrients and energy polymers and efficiently repairing DNA, proteins and cell membranes so that they are truly independent of any surface biosphere? In terran marine and lake environments, photosynthetic autotrophic communities appear to mitigate the variations in C, N, and P such that they follow the Redfield ratios or the average C, N and P composition of the plankton. In subsurface ecosystems that have been isolated for geologic time intervals do similar ratios define covariation in C, N and P?
- Just how long can a single cell maintain its viability without dividing? Geochemical analyses combined with ^{14}C dating of inorganic carbon in groundwater and determination of the concentration of "viable" biomass from the Middendorf aquifer indicates cell turnover times on the order of thousands of years (Phelps et al., 1994). Based on the estimates for nM yr^{-1} methanogenic activity given below and on previous investigations by members of our team, it is clear that growth in the deep terrestrial subsurface is a very slow process with estimated doubling times for heterotrophic organisms in deep Atlantic coastal

plains sediments on the order of hundreds of years (Phelps et al., 1994). Estimates of respiration rates in ground water from this same environment range from 10 to 10^4 nM of CH_2O oxidized per year, depending on ground water age and electron acceptor (Murphy and Schramke, 1998). Geomicrobial investigations of a number of deep terrestrial environments with relatively low permeability and hydraulic conductivity suggest that they are inhabited by populations or descendants of populations that were present during the original depositional events millions to tens of millions ago (Fredrickson et al., 1995; Fredrickson et al., 1997). Thus, it is reasonable to hypothesize that most members of deep subsurface microbial communities are highly adapted to low nutrient conditions with very slow or infrequent cell division, and the utilization of energy sources for maintenance rather than cell division, are the norm. Although this finding is provocative, programmed cell death is not known for bacteria and hence no reason exists to preclude Methuselahian microorganisms. ^{14}C analyses of the microorganisms, lipids and dissolved organic matter will be required to directly tests this hypothesis, however.

The last question has been partially answered by the recent successful recovery and resuscitation of bacteria and Archaea trapped in salt crystals. The age of entrapment is ascertained from a combination of radiometric analyses and petrofabric relationships. The answer appears to be that at least in spore form, bacteria can survive trapped within crystals for 250 myr (Vreeland et al., 2000) and Archaea for at least 97 kyr (Mormile et al., 2003).

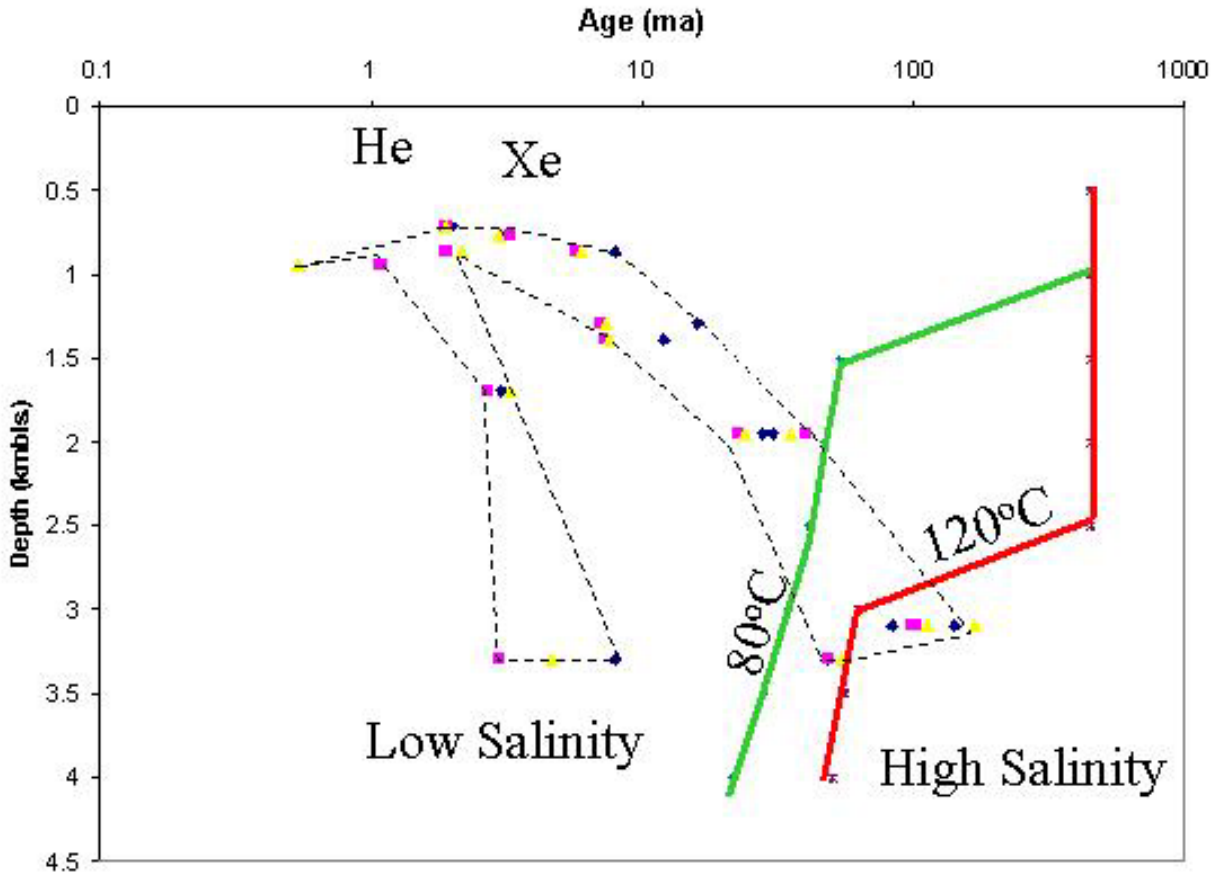
Long-term hydrological isolation

The highly saline fluid-filled fractures intersected in deep mines are arguably larger scale versions of mineral fluid inclusions, but more open to gas transport and storage, matrix diffusion and mineral surface reactions (Guha and Kanwar, 1987). Determining the age of entrapment, however, is far more difficult than in the case of mineral fluid inclusions and we have relied upon the following three approaches:

1. Fission track apatite dating-Because the partial annealing zone overlaps the maximum verifiable temperature of microorganisms (~<120^{o}C) and the optimum growth temperature of many thermophiles (~>80^{o}C) fission track apatite length distributions delineate the thermal history relevant to colonization of rock strata providing a *maximum* age for the indigenous community (Omar et al., 2003). As these age are younger at greater depths, the fission track apatite ages for rock strata at a few kilometers depth that have been the targets of microbial studies have ranged from 1.5 to 160 Ma (Tseng et al., 1995; Colwell et al., 1997). Much of the Precambrian/Paleozoic continental crust reflect the Mesozoic thermal overprinting or uplift associated with the breakup of Pangea with the exception of fission track apatite dates reported from the western Canadian Shield that range back to 650 Ma.
2. Noble gas isotope analyses-Because the saline fluids collected in South Africa yield $^{36}Cl/Cl$ values consistent with in situ nucleogenic production they are older than 1.5 myr. and their age beyond the reach of cosmogenic isotopes (Lippmann et al., 2003). Analyses of the $^3He/^4He$, $^{22}Ne/^{20}Ne$, $^{40}Ar/^{36}Ar$, $^{134}Xe/^{132}Xe$ and $^{136}Xe/^{132}Xe$ and the noble gas concentrations of the saline fluids and the pore waters in the rock strata and the chemical composition and porosity of the rocks can determine the flux of the radiogenic noble gases from their to the fluid filled fracture and the total subsurface residence time and unlike cosmogenic isotopes has no maximum age limit (Andrews, 1987; Andrews et al., 1989). The subsurface residence time represents a *minimum* age for the microbial communities since microorganisms strongly adhere to the mineral surfaces given the high ionic strength of the saline fluids and their migration through the crust should be strongly retarded with respect to that of the water.

3. $\delta^{18}O$ and $\delta^{2}H$ of H_2O and fracture minerals-The $\delta^{18}O$ and $\delta^{2}H$ of the water determines both its origin and its pathway, e.g. whether it has interacted with rock at low or high temperatures. In the case of low water/rock the $\delta^{18}O$ and $\delta^{2}H$ can determine how much fluid has been lost to the formation of hydrous minerals and how much mineral dissolution has occurred (Pearson, 1987). They provide valuable checks to the noble gas model ages in method 2. The $\delta^{18}O$ of the fracture minerals in conjunction with fluid inclusion analyses can determine the $\delta^{18}O$ of the fluid from which the mineral precipitated and the when tied to the fission track thermal history provides an additional constraint on the age and origin of the fluid and hence the origin of the microbial community.

In the case of the deep subsurface of South Africa the thermal history derived from fission track apatite length distributions (Omar et al., 2003) and noble gas isotopic analyses (Lippmann et al., 2003) constrain the maximum and minimum ages for microbial occupation and isolation as a function of depth

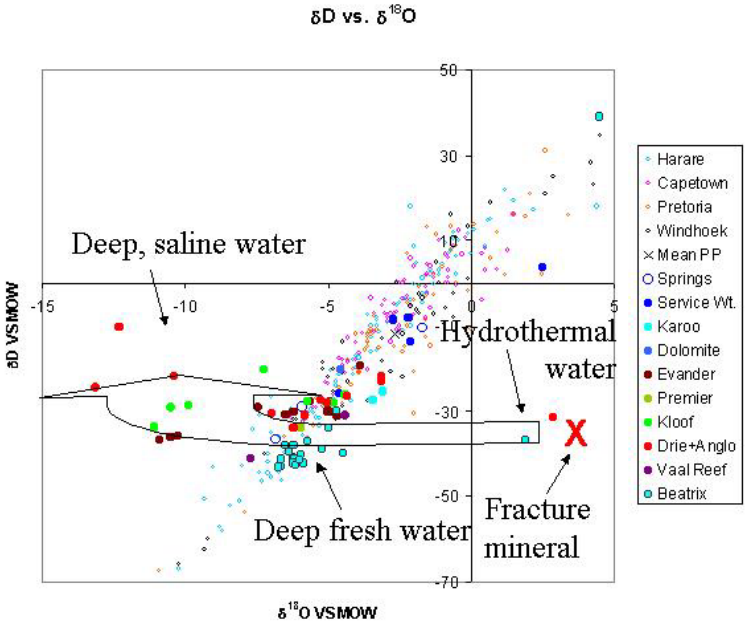


(Fig. 2).

Fig. 2. He and Xe ages for fissure water and 80°C and 120°C thermochrons as a function of depth for Witwatersrand basin, S. Africa. The thermochrons are based upon fission track length distribution data from a 3.7 kmbls apatite-bearing sample.

The maximum and minimum age estimates converge at a depth of ~3 kmbls. indicating that our deepest sites were quickly colonized following cooling below 120°C. Isotopic analyses of fracture coating calcite when compared to the $\delta^{18}O$ of the saline fluid indicates that the saline fluid must have changed its isotopic composition during this time either by flushing or by mineral/water exchange during slow cooling. Given that the present day fluid is distinctly non-meteoric (Fig. 3) the latter explanation is more likely, but requires a more detailed examination of the fracture minerals.

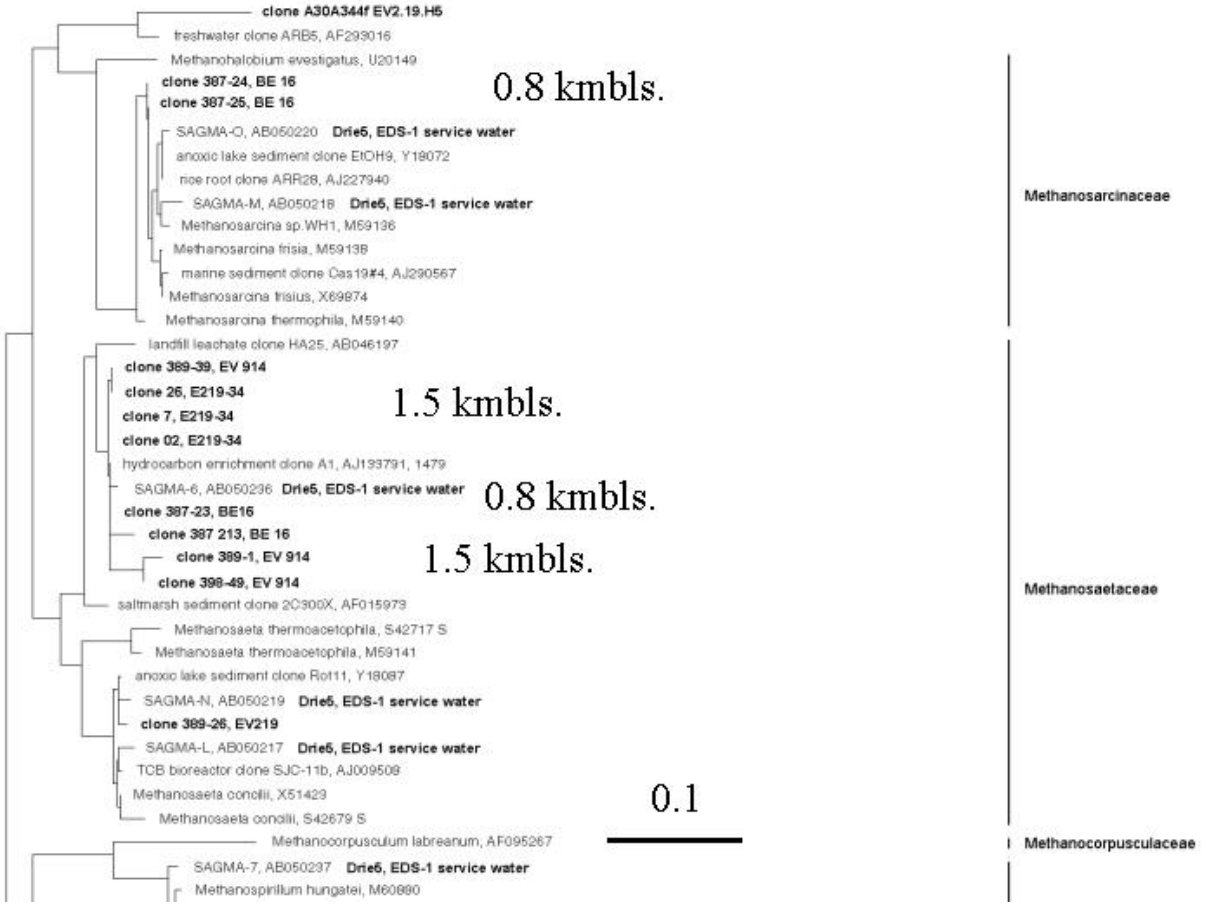
Fig. 3. $\delta^{2}H$ versus $\delta^{18}O$ of fissure water from various mines within the Witwatersrand Basin compared to precipitation, surface water and hot springs and carbonate crystals located along a fracture zone.



Autotrophs versus Heterotrophs-16S rDNA data

The identity of the microbial occupants on fluid-filled fractures has been determined by sequencing of the 16S rDNA gene of DNA extracted from filtered water samples and from thermophilic and mesophilic microbial enrichments (Takai et al., 2001a; Takai et al., 2001b; Moser et al., 2003; Onstott et al., 2003). Members of the bacterial and archaeal kingdoms are present and the similarity of some of the sequences to those in the RDP database for microorganisms with know physiology has permitted the identification of some of the potential metabolic processes in the deep subsurface. Methanogens have been detected in fracture fluids from depths up to 1.6 kmbls. and from the circulation water used for mining, service water, (Fig. 4), but appear to be absent from the deeper, hotter and more saline fracture fluids. A distinct cluster of Crenarchaeota have been detected in the service water and in water samples of the ~1 km thick, 2.5 Ga dolomitic aquifer that caps the Au-bearing formations. Sequences similar to those found within this clade have just been reported for Fe and Mn oxyhydroxide crusts in Lechuguilla cave (Northrum pers. comm. 2002). S and thiosulfate oxidizing autotrophs are also associated with the dolomite water, but are absent in the deeper saline environment. Of the 16S rDNA sequences recovered from the latter environments, the ones that are similar to the database match dissimilatory sulfate-reducing bacteria, SRB's, in the δ-Proteobacteria and gram positive (or Firmicutes) divisions (Fig. 5 and 6), members of the genus Thermus and Clostridia, potential thermophilic and hyperthermophilic Euryarchaeota (Fig. 7). Quantitative PCR indicates that the archaeal portion of the microbial community is minor compared to the bacterial portion. Some of the sequences associated with the genus Clostridia are suggestive of thermophilic homoacetogens (Fig. 8), but over 90% of the ~500 16S rDNA sequences recovered to date are novel and are too dissimilar from those in the database to unambiguously infer their environmental significance. In some cases, the metabolic significance of a novel 16S rDNA is inferred from the media used to enrich the

microorganism, as in the case of the gram positive DR504, which grew up in a Fe(III) reducing media



with only CH_4 compounds as electron donors (Fig. 8).

Fig. 4. Euryarcheota divisional tree showing positions of clades associated with Methanogens and clones related to hydrothermal vents rDNA sequences.

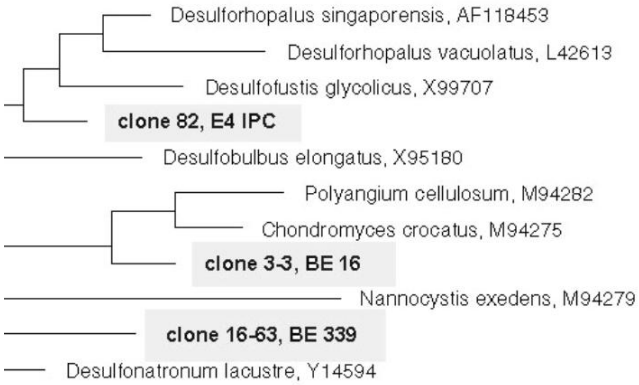


Fig. 5. δ Proteobacteria division, sulfate-reducing bacteria.

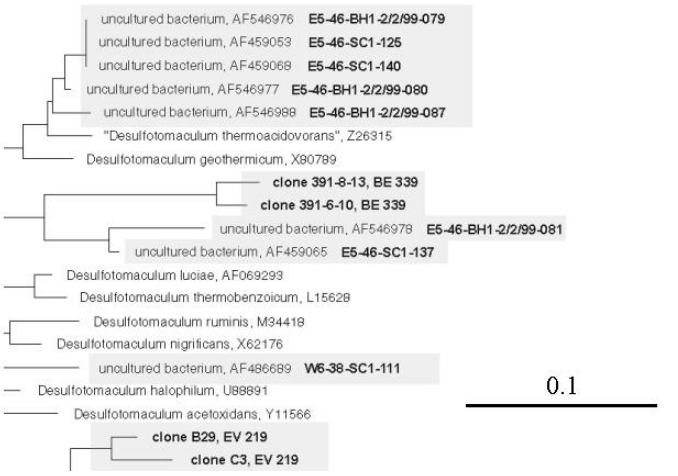


Fig. 6. Firmicutes or gram positive phyla.

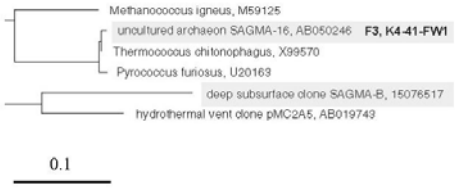


Fig. 7. Hyperthermophilic Euryarchaeota from 3.2 kmbls. (Takai et al., 2000)

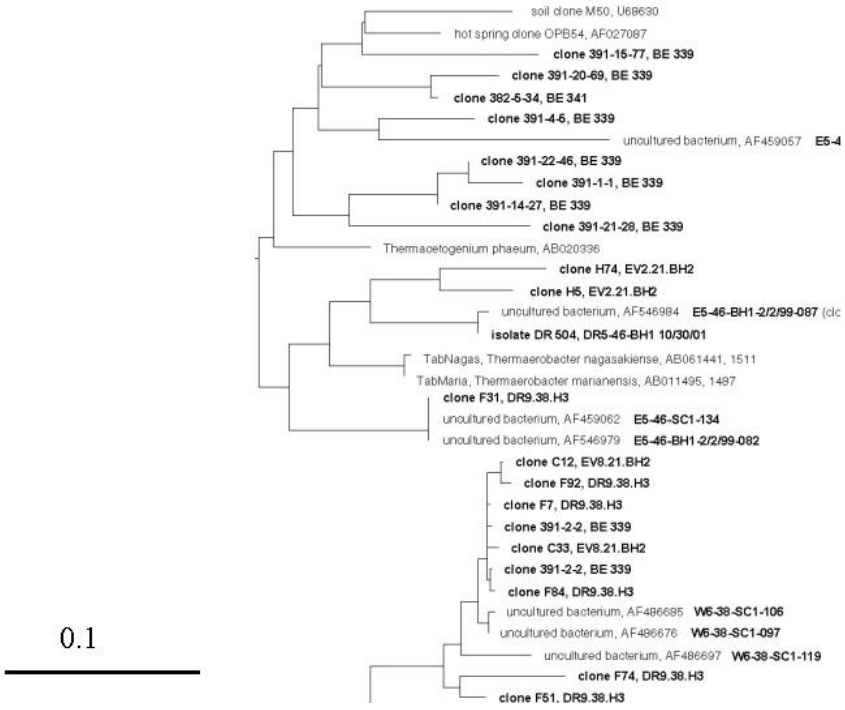


Fig. 8. Novel thermophilic? Firmicutes, including DR504 which was enriched at 50°C with CH_4 as the electron donor and Fe(III) oxide as the electron acceptor.

Autotrophs versus Heterotrophs-Stable Isotope Signatures

Because so many of the 16S rDNA sequences form unique clades, determination of whether or not autotrophs or heterotrophs dominate the communities relies upon stable isotope analyses of the dissolved gases and aqueous species. The $\delta^{13}C$ and $\delta^{2}H$ of the C_{1-4} compounds from the deeper, saline environments are similar to those reported for Canadian and Scandinavian shield brines and distinct from those of thermogenic hydrocarbons (Sherwood-Lollar et al., 1993; Sherwood Lollar et al., 2002). The Canadian Shield rock strata, a 2.7 Ga metamorphosed ultramafic/mafic hydrothermal complex, are distinct from the 3.0 Ga siliclastic and basaltic andesite strata of the Wits basin, yet the reaction mechanisms responsible for formation of the large quantities hydrocarbons and H_2 gas (up to 50% by volume with flow rates of 1-30 L gas/minute) appear similar.

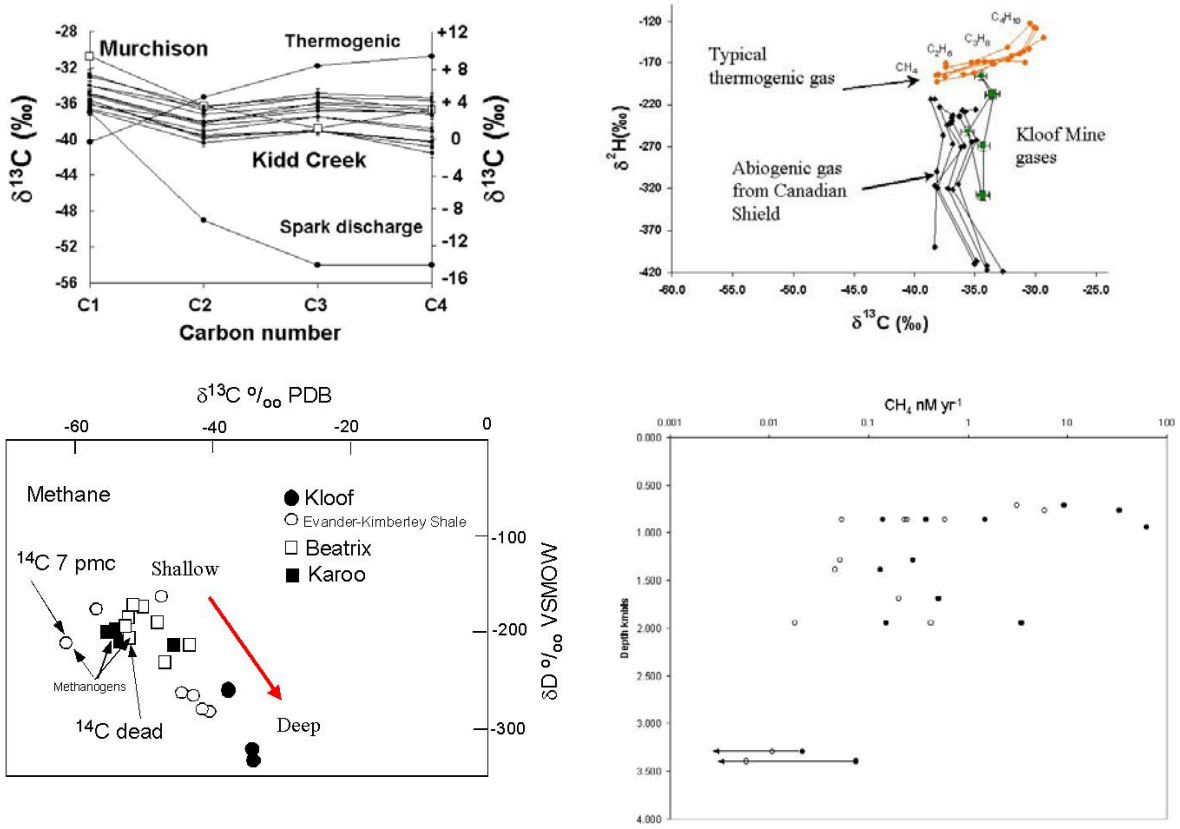


Fig. 9. From top left to lower right. a) The $\delta^{13}C$ for C_{1-4} compounds from Canadian Shield versus C number compared to those reported for the Murchison meteorite and hydrothermal experiments. b) δD and $\delta^{13}C$ for C_{1-4} compounds from Canadian Shield and South African brines. c) δD and $\delta^{13}C$ for CH_4 from South African fissure water. ^{14}C values reported for biogenic CH_4. d) The methane production rate as a function of depth in South Africa.

Sherwood Lollar et al. (Sherwood Lollar et al., 2002) demonstrated that the abiogenic reactions that may have generated prebiotic organic molecules in the early earth and on other planets may be the same reactions involved in the production of hydrocarbons in Precambrian Shield crystalline rocks. The most distinctive feature of these gases is the unusual pattern of ^{13}C values between the C_1-C_4 alkanes. Thermogenic hydrocarbons have been shown empirically and experimentally to have a characteristic

isotope distribution pattern whereby the C_{1-4} alkanes become more enriched in ^{13}C (less negative $\delta^{13}C$ values) with increasing molecular weight. This orderly isotopic distribution results from kinetic fractionation effects whereby alkyl groups separating from source organic matter cleave preferentially at weaker ^{12}C-^{12}C rather than ^{12}C-^{13}C bonds (Des Marais et al., 1981). In contrast, the Canadian and South Africa C_1-C_4 alkanes reveal a significant depletion in ^{13}C for C_2-C_4 with respect to C_1. No thermogenic or bacterial formation reaction or post-genetic alteration process is known to produce such a pattern (Fig. 9a).

Such a pattern can be generated, however, when hydrocarbons are produced from CH_4 in an abiogenic reaction. This was first demonstrated in a spark discharge experiment (Des Marais et al., 1981). The isotope pattern results during kinetically controlled synthesis of higher molecular weight homologues from lower ones due to the fact that $^{12}CH_4$ reacts faster than $^{13}CH_4$ to form chains, so that ^{12}C is more likely to be incorporated into larger hydrocarbon chains. The Canadian Shield brines were the first reported terrestrial samples to demonstrate this consistent depletion of C_2-C_4 with respect to C_1. Only values of $\delta^{13}C$ obtained for C_2-C_4 n-alkanes for the Murchison meteorite yield a similar pattern, also attributed to formation by abiotic polymerization reactions (Fig. 9a; Yuen et al., 1990). The absolute $\delta^{13}C$ values of the hydrocarbons from the Murchison are significantly more enriched in ^{13}C than the Canadian brines, reflecting their extraterrestrial origin. Nonetheless, the Murchison meteorite and the Canadian brines are remarkably similar in the isotopic pattern of the C_1-C_4 n-alkanes (Fig. 9a).

The δ^2H values for the C_1-C_4 gases provide additional support for an abiogenic origin. The distribution of $\delta^{13}C$ and δ^2H values is distinctly different than for thermogenic gas (Fig. 9b), and the inverse correlation of $\delta^{13}C$ and δ^2H values between C_1 and C_2 supports the participation of these compounds in an abiogenic polymerization reaction. (Des Marais et al., 1981) proposed that in a kinetically controlled synthesis of higher molecular weight homologues from lower ones, the lighter isotope (^{12}C) will react faster than the heavy isotope (^{13}C) to form a 2-carbon chain and the resulting C_2H_6 would be depleted in ^{13}C versus the CH_4 precursor. The reaction will also proceed more rapidly if the weaker ^{12}C-^{1}H bond versus the ^{12}C-^{2}H bond is cleaved, thus preferentially eliminating the lighter ^{1}H. The C_2H_6 should be isotopically enriched in ^{2}H and isotopically depleted in ^{13}C with respect to the CH_4 precursor as observed in Canadian and South African brine samples.

The $\delta^{13}C$ and δ^2H of the CH_4 (Fig. 9c) and the C_{2-4} compounds reveal that the CH_4 from the environments < 2.0 kmbls. does appear to have been produced by methanogens, many of which appear to be aceticlastic (Gelwicks et al., 1994), not CO_2 reducing autotrophs (Fuchs et al., 1979) on the basis of the 16SrDNA (Fig. 4). The biogenic CH_4 has no detectable ^{14}C, with one exception, suggesting that the source acetate is > 50,000 years old. The proportion of this biogenic CH_4 decreases with depth and based upon the Xe age of the fissure water (Fig. 2) the biogenic CH_4 production rated decrease exponentially with depth from 10 nM yr^{-1} at 0.6 kmbls to <0.01 nM yr^{-1} at 3.2 kmbls. Based upon estimated methanogenic biomass in the fissure water of these samples by flow cytometry and qPCR, these rates correspond to a cell turnover time a few decades at 0.6 kmbls. to hundreds of years at 2.0 kmbls.

The $\delta^{13}C$ of the dissolved inorganic carbon, DIC, doesn't follow the enrichment trend reported by (Stevens and McKinley, 1995) for the Columbia River Basalt aquifer and that expected for an autotrophic dominated environment, but instead yields isotopically light values more consistent with heterotrophic respiration of dissolved organic matter (Fig. 10). The S isotopes suggest that one of the principal electron acceptors to this respiration is sulfate. The difference in the $\delta^{34}S$ of sulfate and sulfide from the deep saline water is consistent with that produced by microbial fractionation by SRB's in lab cultures (Fig. 11)(Kemp and Thode, 1968). This fractionation is present in saline water with a sulfate concentration of 50 μM, lower than has yet to be reported for lab cultures and suggestive of very slow sulfate reduction rates

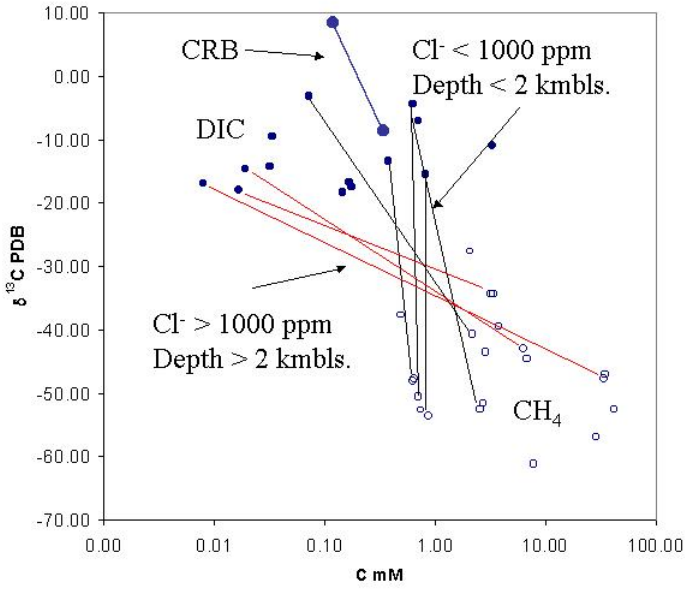


Fig. 10. $\delta^{13}C$ of CH_4 and DIC for South African fissure water and $\delta^{13}C$ of DIC Columbia River Basalt aquifer (Stevens and McKinley, 1995).

(Harrison and Thode, 1957). Dissimilatory sulfite reductase, DSR, genes recovered from two saline water samples yield some sequences that closely align with those recovered from the Columbia River Basalt aquifer and other sequences that are unique and deeply rooted in the DSR tree (Fig. 12; Baker et al., 2003). This may indicate that some SRB carry reductive enzymes that are uniquely adapted to deep subsurface environments, but until these enzymes are characterized this remains speculation. The acetate, sulfate and sulfide concentrations yield a free energy of ~28 kJ/mole sulfate, sufficient to support the formation of ATP (Schink, 1997).

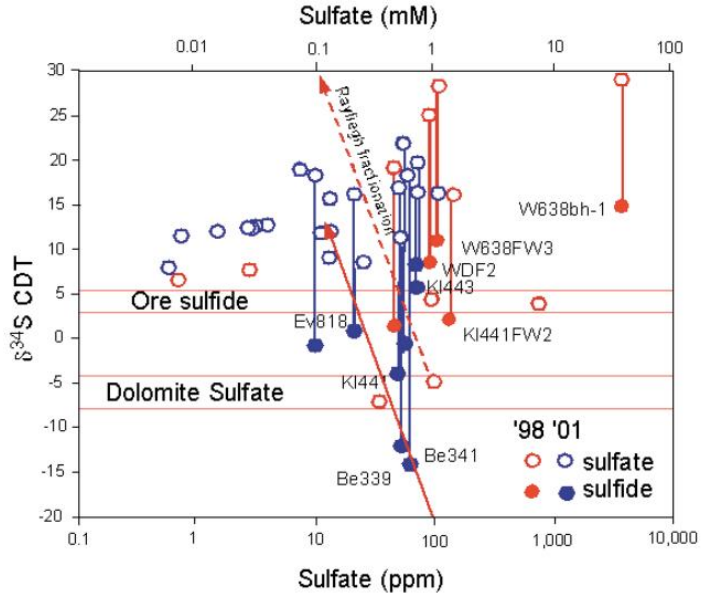


Fig. 11. $\delta^{34}S$ of sulfate and sulfide from fissure water in Witwatersrand Basin, S. Africa.

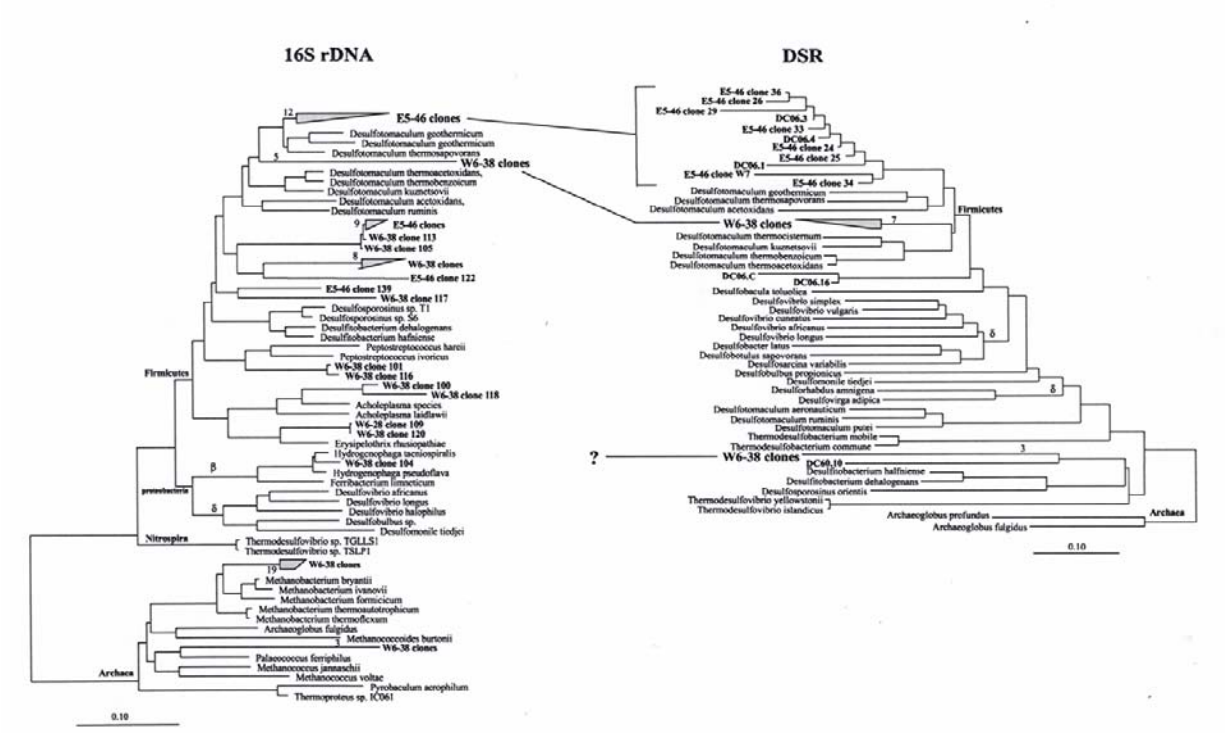
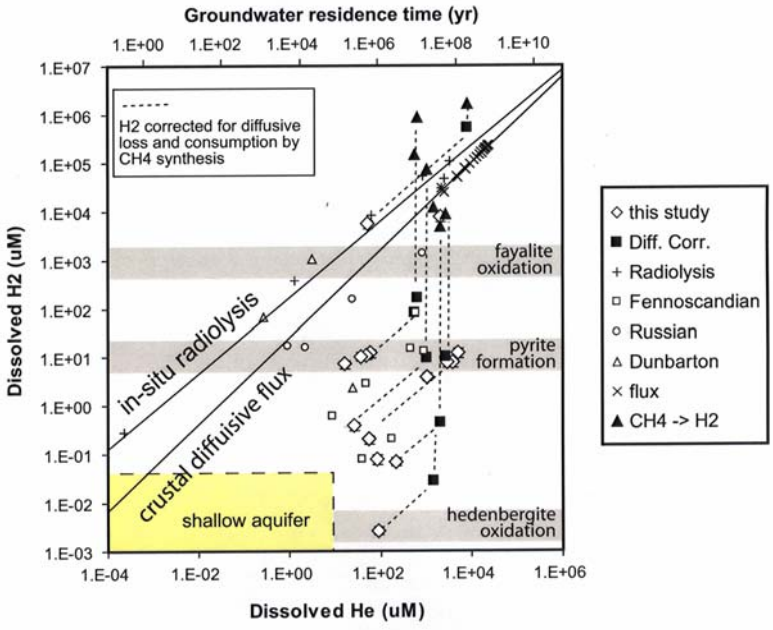


Fig. 12. 16SrDNA (left) and DSR (right) tree for two deep saline water samples in the Witwatersrand Basin, S. Africa.

The H_2, sulfate and sulfide concentrations yield a greater free energy, ~56 kJ/mole sulfate, suggesting that most of the electron flow is generated from H_2. The H_2 concentrations in the South African fissures span 6 orders of magnitude and are as high as 1 mM (Fig. 13)[1]. The lack of appropriate Fe-bearing



[1]Fig. 13. H_2 vs. He for fissure water from South Africa, Fennoscandian shield, Ukranian Shield and North American Triassic basin. Dashed line represents predicted radiolytic yield as a function of time and He concentration.

mineral phases and thermodynamic calculations indicate that this H_2 cannot result from water-rock interactions (horizontal bars in Fig. 13). Measurements of the TOC and carboxylic acid concentrations indicate that the Gibbs free energies of fermentation reactions are positive for all samples with the exception of one, so fermentation cannot explain the high H_2. Nor are ultramafic rock present in the lower crust underlying the Wits basin as can been seen in the crustal section exposed ~40 km south in the Vredefort impact structure. Given the concentrations of radiogenic elements in the strata, the porosity and the ^4He residence times (Fig. 2), the calculated H_2 concentration agrees with that of the highest measured values (dashed line in Fig. 13). The mostly likely source of the H_2, therefore, is radiolytic reactions (dashed line in Fig. 13; Lin et al., 2003b). Furthermore, if one assumes that the abiogenic CH_4 utilized radiolytic H_2 and correct for this loss, then many more of the corrected H_2 concentrations overlap the radiolytic production line (solid triangles in Fig. 13). Data from other Precambrian shields and from the Triassic basin in eastern North America have yielded similar H_2 versus He distributions. Unlike shallow aquifers where the H_2 concentration (lower rectangle in Fig. 13) has been used as a yardstick to infer the principal microbial electron receptor for respiration (Lovley and Goodwin, 1988; Jakobsen et al., 1998), the deep subsurface H_2 concentration appears not to be solely mitigated by microbial activity, but reflects a balance between abiotic production, abiotic hydrocarbon generation reactions and nutrient-limited microbial consumption. The microbial footprint appears more pronounced in the shallower depths, particularly < 2.0 kmbls. Similar behavior may be found for other metabolites and nutrients.

The elevated concentrations of H_2 also indicate that microbial respiration is not efficiently extracting all of the available chemical energy for growth. Other factors that might be limiting both microbial metabolism and growth are:

- Lack of electron acceptors-Onstott et al. (Onstott et al., 1998b) observed that the deep subsurface environment of a Triassic rift basin was electron donor rich compared to available electron acceptors. A similar discrepancy between electron equivalents of electron donor and electron acceptor compounds exists for South African deep subsurface samples. Most of this excess electron donor capacity is present in the form of CH_4. The only electron acceptor that could redress this imbalance is Fe(III) and if it was being utilized by or was available to anaerobic CH_4 oxidizers then the donor/acceptor balance might be restored.
- Lack of organic C. Unlike marine sediments, continental and ocean crustal rocks may not have sufficient organic carbon. This possibility seems less likely given the recent reports of long chain hydrocarbons from hydrothermal vent fluids and the abiogenically produced light hydrocarbons in Precambrian shield rocks. Hydrothermal redox catalyzed reactions may generate light-weight organics at deeper levels in the crust and they may diffuse upwards to nourish the subsurface biosphere as suggested by Tommy Gold (Gold, 1992). The 10^3 ml^{-1} biomass concentrations observed in deep saline water from South Africa are far less that would be expected from extrapolation of biomass concentrations from shallower, marine sediment environments (Fig. 15) and less than that expected given the 1 μM, minimum measured concentrations of carboxylic acids (Fig. 16).
- Lack of N. When dissolved organic carbon (DOC) and total inorganic N (NH_4^+ and NO_3^-) concentrations are compared to the C:N values for bacteria, some of the deep saline water exhibit a deficiency in N relative to DOC (Fig. 16). The minimum N concentrations of 0.1 nM correspond to a cellular concentration of 10^3 ml^{-1} and thus could be limiting. Given the surfeit of dissolved N_2 in these fissures, N_2 fixation would alleviate this deprivation, but only if sufficient energy was present to sustain this energetically costly enterprise.
- Lack of phosphate. The C:N:P values for the South African deep fissures indicate a relative paucity in P (Fig. 17a), which could account for the low levels of biomass. Dissolution of P bearing phases such as apatite could replenish this nutrient, but phosphate is very much oversaturated with respect hydroxyapatite (Fig. 17b) in these systems. The mechanism responsible for the apparently elevated phosphate concentrations has not been resolved.

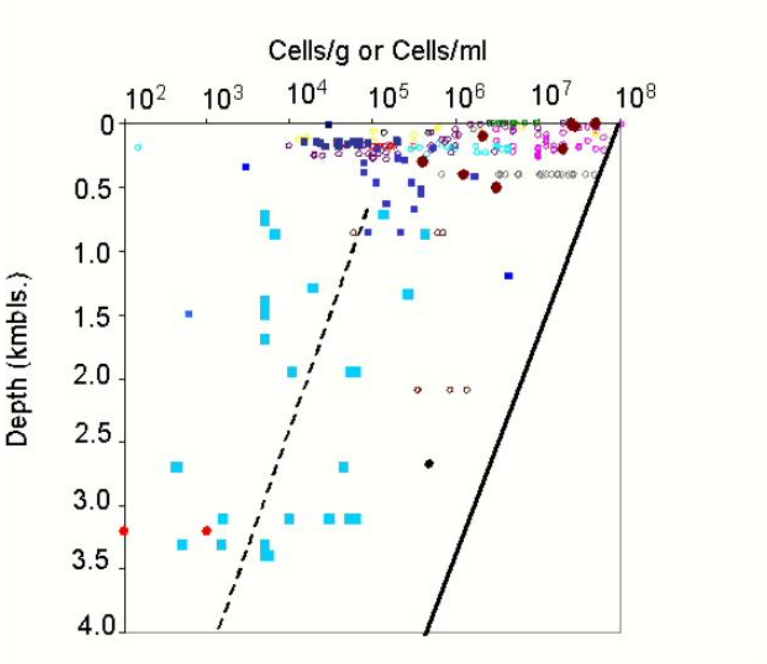


Fig. 15. Summary of biomass concentrations based upon concentrations of PLFA or direct cell counts per gram of sediment (open symbols) and per ml of groundwater (closed symbols). Data from (Onstott et al., 1998a), Hall (Pers. comm., 2003) and Pfiffner (Pers. comm. 2002). Red dots are biomass estimates for quartzite and organic-rich ore zone (Onstott et al., 2003). Solid line is based upon extrapolation of biomass values determined for deep-sea sediments (Parkes et al., 1994). Our results suggest three orders of magnitude lower subsurface biomass in continental crust.

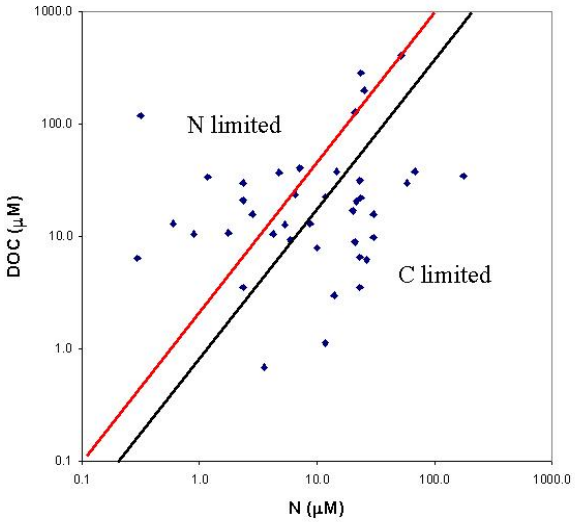


Fig. 16. Dissolved Organic Carbon (DOC) versus total inorganic N (NH_4^+ and NO_3^-) for South African deep groundwater. Red and black lines represent the C:N values measured on stationary and exponential phase bacteria, respectively, grown in lab (Vrede et al., 2002).

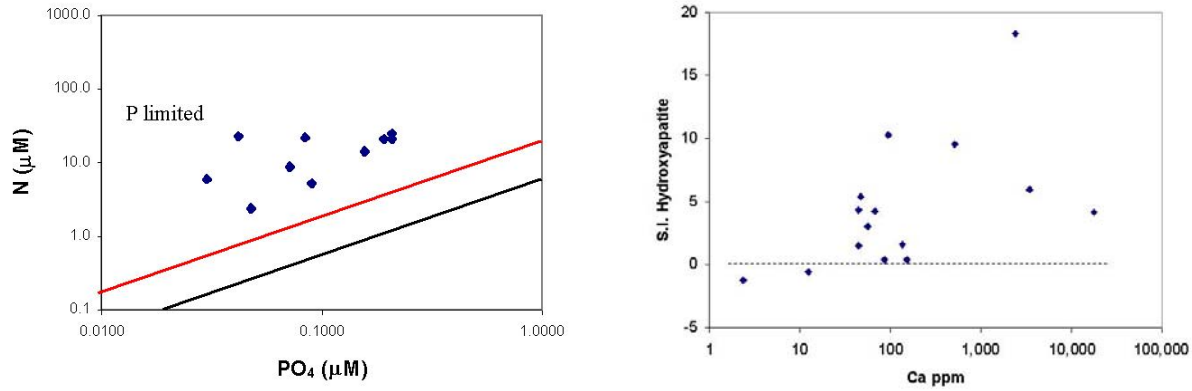


Fig. 17. Left-a) Total inorganic N versus PO_4^{2-}. Red and black lines represent the C:N values measured on stationary and exponential phase bacteria (Vrede et al., 2002). Right-b) Saturation Index of hydroxyapatite and calcite versus Ca concentration for South African deep groundwater.

- Lack of trace metals. In South Africa, fissure water <1.5 kmbls. tends to be bicarbonate rich, whereas, fissure water deeper than 1.5 kmbls. tend to be saline and sulfidic. Trace metals tend to have different valence states and form different anionic complexes for these to systems with many metals precipitating as sulfides. The lack of Fe, Ni and other transition metals may limit the total abundance of enzymes such as hydrogenase and CO dehydrogenase, which in turn may limit H_2 utilization and growth.

3. IMPLICATIONS FOR BIOSUSTAINABLE DEEP SUBSURFACE MARTIAN ECOSYSTEMS

Our research to date indicates that abiogenic hydrocarbons in Precambrian Shield geologic environments are a globally and volumetrically significant phenomenon, hitherto significantly underestimated. It has to been largely assumed that after the evolution of life on earth, biologically mediated reactions overprinted evidence of the pre-biotic abiogenic chemistry of the earth. This is the first indication that abiogenic reactions that contributed to the formation of primary organic molecules on the early earth continue to play a significant role in the isolated deep subsurface environments. Although our South African research indicates that some of these environments were inoculated with microorganisms, they do not appear to dominate the C or H cycle in the deepest, most saline fissure water. These isolated brines may host other abiogenic and even prebiotic reactions involving N, S and C compounds that have yet to be detected.

Based upon the radiogenic element concentration of SNC's, the hydrological model of Clifford (Clifford, 1993) and our results, substantial reservoirs of radiolytically derived H_2 and abiogenic CH_4 would likely exist in the martian subsurface, the former as trapped gas diffusing upwards through the cryosphere towards the surface and the latter as a clathrates within the cryosphere (Max and Clifford, 2000). The isotopic composition of the H_2 should be distinct from that of the martian atmospheric H_2. Light hydrocarbons may be trapped in residual saline brine as the cryosphere base deepens with temporal cooling of Mars and could provide potent electron donors and C substrate.

The abundance of S and Fe in martian crust estimated from the martian surface measurements (Clark et al., 1982; Reider et al., 1997) and from SNC's (Wanke and Dreibus, 1988) and especially of the bioavailable forms of sorbed sulfate, S in smectite and ferrihydrite in SNC fractures (Treiman et al., 1993)

suggest that sulfate and Fe(III) reduction could be two principal electron acceptor reactions. In this case, radiolysis would renew these resources from their biologically reduced mineral equivalents. The concentration of P is high compared to that of terrestrial rocks (Banin et al., 1992; Dreibus et al., 1999) suggesting that unlike terran deep subsurface systems, P limitation would not be a serious concern. Bioavailable N, however, is a serious dilemma given that N_2 abundance of the martian atmosphere is 5000 times less than that of earth and only trace amounts of nitrate have been reported in SNC's (Clark, 1998). Our results have detected microorganisms that are capable of N_2 fixation, but this function is energy-intensive. The high abundance of N_2 in some saline fissure water and its absence in others suggest that some mechanism is removing N_2 in these deep environments. Nitrate reducers have been found in the deep saline fissure water, but whether they are reducing nitrate in situ is difficult to assess given the absence of nitrate.

Our observations of a deep subsurface terran ecosystem that has been tectonically quiescent and isolated from the surface for ~10^8 years, therefore, carries implications about long term potential energy and nutrient flux in a martian subsurface environment that differ from previous assessments. Fisk and Giovannani (Fisk and Giovannoni, 1999) propose oxidants, such as dissolved O_2 from the martian atmosphere enter the subsurface through basal melting of the polar cap (see Fig. 1) and downward recharge to interact with H_2 produced by water dissociation from oxidation of fayalite. Assuming the polar ice traps dissolved O_2 at a concentration of 10-20 nM, a melt recharge rate of $10^{10\text{-}12}$ L yr^{-1} (Clifford, 1993) and a subsurface brine volume equivalent to 10 to 250 m global ocean, the O_2 recharge rate is 10^{-5} to 10^{-9} nM yr^{-1}, which is 8 to 12 orders of magnitude LESS than the oxidant production rate predicted by radiolysis (see Fig. 19 below).

PROPOSED RESEARCH

The mechanisms that are at work in deep biosustainable habitats in South Africa could clearly play critical roles in biosustainable environments beneath the surface of Mars. Our information doesn't however address the other two potential martian habitats mentioned above, the cryosphere and deep vadose zone. Nor do our results provide insight energy and nutrient cycling in saline aquifers where CO_2 is a dominant gas phase, but they do imply that over a period of at least ~10^8 years this CO_2 could be converted to CH_4 and light hydrocarbons.

Our proposed research program, therefore, has four major themes:

1. Abiotic Microcosm Experiments-Abiotic geochemical and radiolytic experiments designed to mimic the processes inferred to be taking place in the deep subsurface of South Africa and Canadian Shield, to quantify the resulting energy and nutrient fluxes, determine the stable isotopic fractionation and to develop simple aqueous geochemical and isotopic models that can be applied to martian hydrogeological conditions.
2. Biotic Microcosm Experiments-Biotic experiments similar to the abiotic experiments, but inoculated with selected subsurface microbial isolates that cycle C, N and S. These results will be used to develop models for martian biosustainable deep subsurface habitats. The chemical and stable isotopic composition of the metabolites will be compared to that of the abiotic experiments and from this comparison we will begin designing life detection tools that can be utilized in the field and potentially on mars missions.
3. Subsurface Simulator Stress Experiments-Both abiotic and biotic microcosm experiments will examine the effects of dessication to the limit of thin, saline films on mineral surfaces and the effects of CO_2 and CH_4 clathrate formation. These stresses are designed to examine the impact of these processes on energy and nutrient flux and on the chemical and isotopic composition of abiogenic species and biogenic metabolites. To examine the effect upon a subsurface microbial community field rock and water samples will be placed into large volume subsurface simulators.

This information will also be incorporated into models for martian subsurface habitats and in the design of potential life detection instruments.

4. In situ Activity and Substrate Utilization Experiments-Geochemical, isotopic and microbial analyses and in situ experimentation at a field site where a relatively shallow (< 1km), brine that has been isolated from the surface by permafrost for ~10^7 years is accessible through mining operations. Several candidate sites exist in northern Canada and the results from this field site will be compared to our observations from the deep subsurface of South Africa, which has experienced a much different surface climate history. The results of the experimental studies will be compared to the results from both field studies as an initial step towards scaling up biogeochemical modeling. Finally, the results from the northern Canada site will be compared to those of Artic permafrost deposits to determine if any evidence for biological or chemical transport between the two habitats exists.

1. ABIOTIC ENERGY AND NUTRIENT SOURCES

(Onstott, Myneni, Dismukes, Sherwood-Lollar, Pratt and Lehman)

Radiolysis

Background-Dissolved H_2 and He measurements indicate that radiolysis is the simplest explanation for the elevated H_2 concentrations reported for Precambrian crustal aquifers (Lin et al., 2003b). Radiolysis also generates strong oxidants, H_2O_2 and O_2 (Spinks and Woods, 1990) and the production rate of all three species depends upon the fluid-filled porosity and radiogenic element composition and concentration (Hoffman, 1992). The occurrence of H_2 and O_2 in fluid inclusions within quartz demonstrates the stability of these radiolytic products over the geological periods in the absence of variable valence elements (Dubessy et al., 1988). This has been confirmed by laboratory irradiation of complex brines at PNNL (Gray and Simonson, 1984). The relative production rates of H_2O_2 and O_2 depend upon the relative α, β and γ dosage and upon the salinity of the fluid since Cl inhibits H_2O_2 production (Lin et al., 2003a). H_2O_2 and O_2 will react with reduced metal oxides, organic matter and sulfides to generate potential electron acceptors, Fe(III), CO, CO_2, S^o, $S_2O_3^{2-}$, SO_4^{2-} and or with NH_4^+ to produce NO_2^- or NO_3.

Published estimates of radiolytic yields rely almost entirely upon irradiation of homogenous water solutions. The efficiency of radiolytic decomposition of water is increased in heterogenous water/mineral solutions since the ephemeral products, such OH, H, and e-, interact with the mineral surface before they have a chance to recombine into H_2O. The radiolytic of H_2 for thin films of water absorbed to oxides is ~15-50 times that of pure H_2O (Vovk, 1987a). In the presence of gas hydrates, the rate of radiolytic decomposition is 3 to 4 times that of pure H_2O (Vovk, 1987a). Laboratory irradiation of aerobic, organic-rich sediments has yielded both CH_4 and CO_2 with the production of H_2, the consumption of O_2 and surprisingly the consumption of N_2 (Vovk, 1987a).

Since most of the fluid filled porosity in deep crustal rock is associated with an effective porosity with sub-micron pore throats or fluid inclusions and not within the fractures (Nordstrom and Olsson, 1987), most of the radiolytic production takes place in the rock matrix. As a result, much of the Fe(III)-bearing oxyhydroxides and S^o precipitated during radiolytic reactions may not be bioavailable and the soluble electron acceptors, SO_4^{2-}, $S_2O_3^{2-}$, NO_2^- and NO_3^-, will diffuse from the matrix to the fracture zone along with the H_2. The oxidation reactions that take place within the matrix reduce the pH of the pore fluid thereby promoting the dissolution/precipitation of clays, feldspars and quartz and elevate the Eh thereby promoting the mobilization of other redox sensitive metal species, such as U. Pore water and fissure

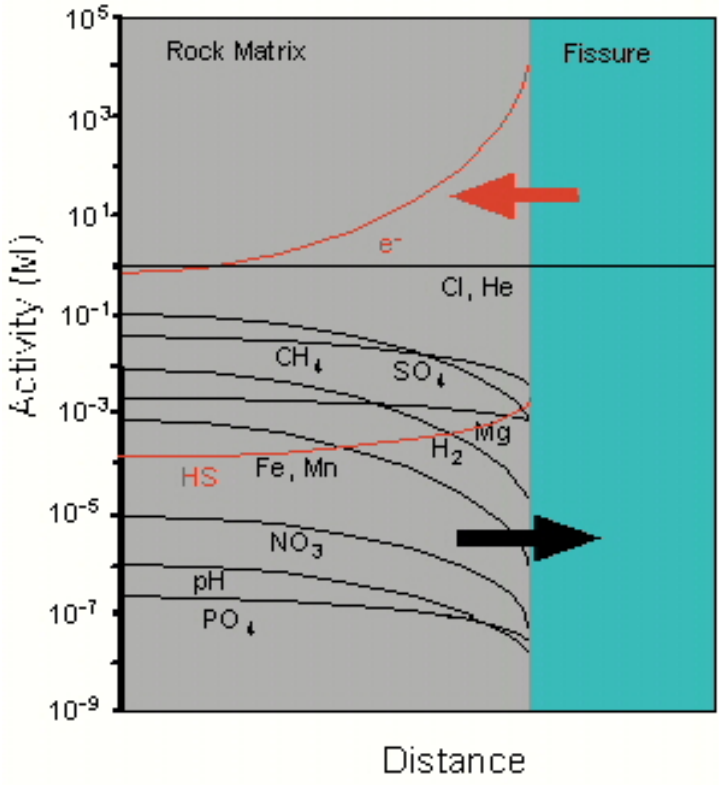


Fig. 19. Diagrammatic representation of observed concentrations gradients between rock pore water and sulfidic fissure water. Vertical scale is activity in M. Horizontal distance is diffusion controlled. Not included are mineral precipitating reactions on fracture face, such as sulfide or U oxide formation. Also not shown are coupled microbial redox reactions, e.g. reduction of nitrate that is diffusing outward by HS.

water geochemical results from South Africa and geochemical modeling have delineated some of these gradients (Fig. 19). SO_4^{2-} appears to be the principal electron acceptor diffusing into the fracture explaining the wide spread occurrence of SRB's. By reducing the SO_4^{2-} to HS^- the SRB's enhance the diffusive flux and their overall metabolic rate. The HS^- can either react with the metals diffusing from the matrix to form sulfides or act as an electron donor to other electron acceptors, such as NO_3^-. This redox couple can be utilized by microorganisms, such as *Thiobacillus denitrificans*, as an energy source, producing N_2 as a waste product (Fig. 19).

The potential chemical power made available to chemolithotropic microbial communities depends upon the relative volume of the fracture to matrix rock, the diffusivities of H_2 and the electron acceptors and the age of the fracture itself. A newly formed fracture in an ancient rock will immediately yield the maximum chemical power, which approaches steady state over a period of 10,000 to 100,000 years (see Fig. 20). Because of the tiny facture volume typical of deep crustal rocks (~0.001-0.01%; (Nordstrom and Olsson, 1987), the potential biosustainable chemical power production is greater than that present in Atlantic coastal plain aquifers that support a large diversity of microbial species.

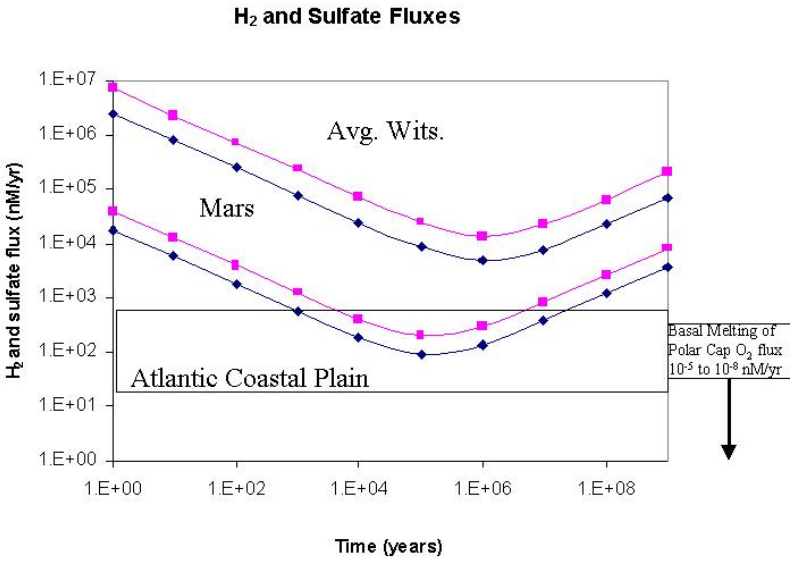


Fig. 20. Chemolithotrophically available power as determined by maximum production rates for H_2 and SO_4^{2-} in nM yr^{-1} based upon instantaneous and steady-state solution of diffusion equation for a semi-infinite medium, measured pore water concentrations and estimated radiolytic productions rates using experimentally determined yields for H_2 and assuming that all radiolytically produced O_2 and H_2O_2 oxidizes sulfide to SO_4^{2-}. The production rates for H_2 and SO_4^{2-} for Mars are based upon the radiogenic content of SNC meteorites. Shown for comparison is the chemical fluxes of H_2 and SO_4^{2-} for Atlantic Coastal Plain aquifers from (Lin et al., 2003b) and (Chapelle and McMahon, 1991) and also the redox power availiability for martian hydrosphere based upon basal melting of polar caps as the sole source of recharging oxidant (Fisk and Giovannoni, 1999).

Several questions need to be resolved, however, in order to determine whether this mechanism alone can sustain subsurface life.

- Does the presence of mineral surfaces enhance radiolytic yields and alter redox chemistry?

Several studies from the late 60's to early 80's reported these effects (Vovk, 1987a) but experiments utilizing modern surface spectroscopic techniques have not been performed. One would anticipate that different aqueous surface complexes whose type and abundance depend upon the pH and mineral would influence both the radiolytic yield and the surface reaction. The surface reaction would entail both dissolution/precipitation and oxidation/reduction reactions.

- How do dissolved organic and inorganic C and N species behave in this system?

Only a few studies have reported the effect of radiation on organic matter and of those most focus on kerogen and bitumen associated with U ore deposits where the calculated α dosage is ~10^{13}-10^{14} rads (Leventhal and Threlkeld, 1978; Leventhal et al., 1987). Aromatic carbon complexes that are stable to γ irradiation and depleted in H and O relative to immature organic matter typify these deposits (Zumberge et al., 1978) or sometimes enriched in O (Dubessy et al., 1988). The reported loss of N_2 during sediment irradiation experiments (Vovk, 1987a) the susceptibility of NH_3 to UV photolysis and the reported correlations between N_2 and He in natural gas fields (Ballentine and Sherwood-Lollar, 2002) are all very suggestive, but nothing is known about radiolysis and N cycling.

- What S species are formed by radiolysis of sulfide/saline anaerobic water?

Radiolytic production rates for SO_4^{2-} by γ and β irradiation of S^o in water ice (nonsaline and microaerophilic) were recently reported by (Carlson et al., 2002). Under these conditions SO_4^{2-} was the principal S species, but further experimentation is required as oxidation of sulfide by H_2O_2 typically

yields a coating of microcrystalline S^o and pore water analyses of U-bearing ore zones in South Africa also detected the presence of $S_2O_3^{2-}$. Because the $S_2O_3^{2-}$ can be simultaneously oxidized to SO_4^{2-} and reduced to S^{2-} its radiolytic production and subsequent destruction could potentially cause large values in the $\Delta^{34}S$ of SO_4^{2-} and S^{2-} (Jorgensen, 1990).

- Are there any isotopic or molecular signatures that are indicative of radiolytic processes?

The $\delta^{13}C$ of the solid phase organic matter in U ores has ranged from –17 to –44$^o/_{oo}$PDB (Leventhal and Threlkeld, 1978; Zumberge et al., 1978; Leventhal et al., 1987) and, in one case, that of occluded CH_4 and CO_2 was –44 and –35$^o/_{oo}$ PDB, respectively (Leventhal et al., 1987). The light isotopic values and petrographic observations have fueled speculation that some of the organic matter formed by radiation-induced condensation of CH_4 and or CO_2 (Robb et al., 1994). To date, no compound specific stable isotopic analyses have been performed. Although the δ^2H of radiolytically produced H_2 is isotopically light, its signature will equilibrate with that of water too quickly to be preserved (Lin et al., 2003a). The same argument may apply to the $\delta^{18}O$ of H_2O_2 and O_2 formed by radiolysis and preserved in the SO_4^{2-} formed by oxidation sulfide bearing rock strata, but no measurements have been performed to date to test this.

Energy Flux for Thin Films on Minerals

Background-It is well known that heterogeneous reactions at the mineral-water interfaces modify the energetics of several reactions on the surface of the Earth (Brown et al., 1999). The energetics of redox reactions mediated by mineral surfaces are vastly different from that of homogeneous aqueous phase reactions. Our recent vibrational spectroscopy studies indicate that water sorbed on mineral surfaces (thin films of water on surfaces) interacts with mineral surface hydroxyls and modify their H-bonding environment significantly (Myneni, unpublished). In addition, these H-bonding interactions vary as a function of water film thickness. This suggests that the interfacial reactions involving minerals do not entirely depend on the type of metal coordination of surface hydroxyls but on the H-bonding environment of solvated water at the interfaces. In unsaturated subsurface environments including the deep martian vadose environment, most of the water exists as thin films on mineral surfaces, and it is expected that the thickness of water films, the composition of the vapor phase, e.g. CH_4 and CO_2 and with N_2, mineral substrate composition and interfacial chemistry would play a critical role in various geochemical reactions, including the radiolysis of water. Total radiolytic yield of H_2 should diminish with decreasing abundance of H_2O, but the yield of O_2 and H_2 O_2 could be quite different. Very little information is currently available, however, on redox reactions and water radiolysis in thin film conditions.

We propose to investigate the role of mineral surfaces on the radiolytic yields of different chemical species. Since the H-bonding environment at mineral-water interfaces changes significantly as a function of pH (modifies the point of zero-charge and thus the interaction of different ions at interfaces), water film thickness (modifies the coordination of water and the strength of H-bonding environment at interfaces), solution composition (ionic strength and composition of fluid phase modifies the overall distribution of charged and uncharged species at interfaces) and substrate composition, we would like to explore the influence of these important variables on the radiolysis of water. Using the vibrational and the soft X-ray spectroscopy accessories that our group has built in the past few years, we would like to explore the chemical state of water on mineral surfaces under different conditions stated above, and probe the radiolytic breakdown of water and its reaction products. The OH stretching and bending vibrations of water and the 1s electronic transitions of O in water are sensitive to the H-bonding environment of water molecules in aqueous solutions and at interfaces (Myneni et al., 2002).

Hydrocarbon Yielding Water/Mineral Reactions

To date we have been able to identify C and H isotopic fractionation patterns consistent with abiogenic versus thermogenic and bacterial processes for C1-C4 hydrocarbons, but we have not been able to distinguish between different potential abiotic pathways and reactions. An additional goal of the proposed research is elucidate the specific reaction mechanisms responsible for the production and fate of

hydrogen and hydrocarbon gases in this geological setting, taking advantage of the enormous potential that isotopic signatures have as diagnostic indicators of reaction mechanisms. Several reactions have been suggested in the literature as potential mechanisms for production of abiogenic gases including:

1. CH_4 from dissolved bicarbonate (HCO_3^-) under hydrothermal conditions with metal catalysts (Horita and Berndt, 1999).
2. CH_4 and higher hydrocarbons via the Fischer-Tropsch synthesis (Yuen et al., 1990; Charlou and Donval, 1993; Hu et al., 1998; Lilley et al., 2001).
3. CH_4 and higher hydrocarbons via serpentinization of ultramafic rocks (Charlou and Donval, 1993; Berndt et al., 1996; Kelley, 1996).

While some work has been done to establish C isotope fractionation effects associated with CH_4 production, a paucity of information exists on formation of the higher hydrocarbons and on H isotope effects. The objective of the proposed research is to undertake a focused experimental program to determine both the C and H isotope fractionation associated with these important abiogenic reactions and to extend the information base we have developed to incorporate higher molecular weight hydrocarbons in order to address the following questions:

- To what extent do the different potential abiogenic gas formation reactions have distinct isotopic fractionation associated with them?
- If distinct isotopic differences do exist between these reactions are they observed in field samples?
- If they are observed in the field to what extent can the inferred reaction be related to the geologic setting and geochemical history of the locale as provided by fluid inclusion analyses?
- If these gases occur in ore bearing rocks does this association reflect a casual relationship whereby the dispersed metals act as catalysts to facilitate the conversion of inorganic carbon to hydrocarbons?

It will be particularly important to distinguish whether the gases are primarily controlled by geological/geochemical features of the host rock or are controlled by structural features, implying migration from the point of origin. If the former, then the differences in gases observed in different geological settings will yield important information on potential reaction mechanisms and the production of gases via water-rock interaction.

Experimental Approach

Objectives-The focus of research into radiolysis will be to perform laboratory irradiation experiments on homogenous fluid and heterogeneous fluid/mineral systems to obtain radiolytic production rates, to identify the reactive species forming on the mineral surfaces and to quantify any isotopic fractionation effect associated with the radiolytic products. Both homogenous and heterogenous fluid experiments will utilize anaerobic, Na-Ca-Cl solutions as these best represent the bulk composition of deep subsurface brine. Heterogeneous experiments will also use pyrite, magnetite, olivine, pyroxene and quartz. The initial pH of the solutions will vary from 3 to 10 in order to examine the effects of surface species and at 4, 25, 45 and 80^{o}C. To examine the effects of mineral surface reactions, the experiments will be performed without mineral substrates, with an equal volume of mineral and saline solution and just with minerals whose surface is saturated with a thin film of saline solution. Total dosage from the ^{60}Co γ source used at Columbia University will vary from 0 to 10^4 Gy. The surfaces of the minerals will be examined with XAFS to identify surface adsorbed aqueous complexes. Finally, the heterogeneous experiments will be performed without irradiation, but with low levels of H_2O_2 and O_2 to see if the

kinetics and products of these reactions can approximate that of the irradiation experiments. If so, this will greatly facilitate incorporation of radiolytic effects into standard geochemical modeling codes.

Tasks-The experiments will focus on H, C, N and S cycles.

1. H cycle-The production rates of H_2, H_2O_2 and O_2 will be determined as a function of temperature, pH, water saturation and Fe concentration in the mineral. The non-irradiated control experiments will be used to further quantify the effect of H_2 production via oxidation of Fe in mineral phases.
2. S cycle-For irradiation experiments utilizing FeS_2, the aqueous S species will be measured by ICMS and the surface absorbed S species by XAFS. The production rates and species distribution will be determined as a function of temperature, pH and water saturation. The S isotope composition of the various products will be determined.
3. N cycle-Anaerobic saline water with NH_4^+ and Ar and with N_2 will be irradiated to determine the stability of both phases during irradiation and whether oxidized N species, NO_2^- and NO_3^-, are produced. The aqueous N species will be measured by ICMS and the surface absorbed species by XAFS. The N and O isotopic composition of the different species will also be determined. The homogenous experiments will be compared with the heterogeneous experiments to determine the effects of temperature, pH, water saturation and Fe concentration in the mineral.
4. C cycle- Anaerobic saline water with CH_4 and CO_2 and with N_2 will be irradiated to determine the stability of the C phases during irradiation and whether oxidized or reduced C species, such as low molecular weight hydrocarbons or carboxylic acids, are produced. The aqueous C species will be measured by ICMS, HPLC and the surface absorbed C species by XAFS. The C and H isotope composition of the different species will also be determined. The homogenous experiments will be compared with the heterogeneous experiments to determine the effects of temperature, pH, water saturation and Fe concentration in the mineral.

New Gas Detection Methods

In the past decade, Cavity Ring Down Spectroscopy (CRDS) has become a widely used optical spectroscopic technique for detection of very weak absorption, including by trace species(Busch and Busch, 1999). In this method, absorption of a gaseous sample is detected by a change in the decay rate of an optical cavity former by very high reflectivity mirrors when the sample concentration between the mirrors is changed. Lehmann (1996)(Lehmann, 1996) and (Romanini et al., 1995; Romanini et al., 1997) have pioneered the application of a continuous wave laser to excite the optical cavity. Using such lasers, optical absorptions as small as one part per billion per pass of the cell can be detected. The methods developed at Lehman's laboratory at Princeton University have led to introduction of the first commercial trace gas analyzer based the CRDS method (Dudek et al., 2003).

This instrument can detect concentrations of moisture in a wide range of gases at a concentration of 100 ppt by volume. The laboratory prototype had a noise equivalent concentration ~10x more sensitive. With minor modifications, this instrument can detect other of simple hydride species, including HCl, HF, CH_4, NH_3, and C_2H_2 with detection sensitivities within one order of magnitude of the moisture sensitivity. The detection is capable of isotopic analyses and is also quite fast, limited primarily by the time required to exchange the sample in the cell. The method is most compatible with a slow, continuous flow of sample through the detection cell.

In order to apply this technique to problems relevant to Astrobiology, we propose several developments for the instrument. By minimizing volumes of the cell and transfer line, we should be able to reduce the size of the sample to be analyzed to about one standard cc of gas. With a detection sensitivity of 100 ppt, this would correspond to a detection limit of about 100 femtogram of analyte or ~0.006 nM of CH_4 as an example. This is about 100 to 1000x more sensitive than analytical techniques

employing GC-IRMS but less sensitive than those utilizing ^{14}C. The second direction is to combine this detection technology with chemical transformation methods to selectively convert other chemical species into ones we can detect. For example, both CO and CO_2 can be quantitatively converted with a methanizer into CH_4, which can then be analyzed. We will also explore methods to pre concentrate larger samples to allow similar absolute quantity sensitivity. Finally, we will determine the concentrations required for obtaining C, H and N stable isotope precisions of ~1‰. The distinct advantage of this approach is that it conceivably can be adapted to fly on Mars drilling missions.

2. BIOTIC ENERGY AND NUTRIENT CYCLING

(Onstott, Myneni, Dismukes, Lehman, Pratt, Sherwood-Lollar, Hazen, Moser)

Introduction

The research program will focus on two main, long-term objectives:

1. To determine the distribution between abiogenic and microbial C, H, N and S metabolites and the geological, structural, geochemical and microbiological controls on the transition between these two regimes in deep subsurface brines.
2. To determine whether the abiogenically derived C, H, N and S compounds act as substrate(s) supporting the microbial communities.

Although these two objectives may appear contraindicative, i.e. if microbial communities are utilizing abiogenically derived C, H, N and S compounds wouldn't they be transformed into microbial metabolites and erasing their former abiogenic signature? To successfully distinguish between abiogenic source and microbially produced metabolites we will rely heavily upon compound specific stable isotope analyses and isotopically labeled substrates. These methodological approaches in concert with laboratory microbial microcosm and macrocosm experiments, with a field site that offers a range in environments with more or less microbial activity and with in situ experiments performed in boreholes will enable us to document the processes that operate to sustain subsurface microbial communities for 10^7 to 10^8 of years.

Evolution of the C Cycle From Abiotic to Biotic Processes

Background-The $\delta^{13}C$ and $\delta^{2}H$ analysis of CH_4 clearly detected biogenic methane production in our deep subsurface systems in South Africa (Fig. 9a) and in the Canadian Shield (Doig, 1994). The absence of ^{14}C in some of this CH_4 suggests it is the product of indigenous, deep subsurface methanogens. The 16SrDNA data indicate that aceticlastic methanogens are responsible for most of the biogenic CH_4. The challenge now is to characterize the interface between these two major gas-producing geochemical/microbial systems in the complex and dynamic field settings encountered the deep biosustainable geological environments.

- What is the degree of chemical/biogeochemical interaction between the abiogenic hydrocarbon gases and the deep subsurface microbial community?
- To what extent do indigenous anaerobic microbial communities utilize these compounds as substrates?
- To what extent do indigenous anaerobic microbial communities utilize these compounds as electron donors for energy and convert them back into CO_2 and cycling the C compounds between the abiotic and biotic systems?
- If C_{1-4} utilizing, anaerobic microorganisms exist, then how do they alter the C and H composition of the C_{1-4} compounds?

Field sites will be sampled extensively to determine the nature and spatial distribution of gases within the mine. Sampling will be targeted to cover different depths, geologic formations and structural features. A key feature of this research is close coordination with the mine geologists and exploration drilling programs to sample both from newly completed boreholes and to re-sample these boreholes over time to

determine temporal as well as spatial variability in measured parameters. This has never been systematically accomplished in the mines in Canada, South Africa or Finland and will allow us to resolve whether the microbial hydrocarbons are stored over long times periods in the host rock (as are the abiogenic gases) or are a phenomenon that appears post-borehole completion. If the latter, it will be essential to determine if this is due to mixing with *in situ* native microorganisms or due to colonization of boreholes with surface microorganisms associated with mining contamination. The goal will be to establish both the contribution of microbial processes to the groundwater and gases, and also the substrates (DIC, DOC, H_2, CH_4, CO) that the *in situ* microorganisms are utilizing as a basis for this deep subsurface community.

Samples will be collected for compositional and isotopic analysis of CHN-bearing gases and dissolved organic compounds as well as associated saline groundwater and brines. Concentration data, as well as stable C and H isotope signatures, have traditionally been used to identify microbial gases mixing with non-microbial end-members (Bernard et al., 1977; Schoell, 1988). Sherwood-Lollar et al.(Sherwood Lollar et al., 2002) have now established a model for isotopic fractionation patterns in abiogenically-produced gases that can be used to identify this novel end-member and provide constraints to determine the relative contribution of the end-members to each sample. Once the distribution pattern of microbial and abiogenic hydrocarbons is established for these systems, radiocarbon analysis (^{14}C) of the hydrocarbon gases from selected systems will be undertaken to determine if the microbially-produced CH_4 is young (and potentially related to mining contamination of the deep subsurface), or geologically old, representative of an *in situ* microbial community.

Prior to this, determination of substrate utilization will be done by collecting microbial samples, isolating them in laboratories by conventional or Gel Microdroplet techniques and culturing them under the same suite of substrates with natural isotopic abundances and optimal and in situ growth conditions. The isotopic fractionation associated with substrate utilization will be determined from these experiments. While valuable, extrapolating this type of result back to the field is uncertain. While such tracer experiments to evaluate substrate utilization have not to our knowledge been attempted systematically before in the challenging environment of the deep subsurface, the results will provide critical data for resolving the question of substrate utilization in these unique geological and microbiological systems.

Evolution of the N cycle from abiotic to biotic?

Background-In conjunction with our research on biogeochemical cycling of C in deep subsurface biosustainable systems, the role of N cycling must be addressed. Our work in the Canadian Shield and South Africa has demonstrated that N_2 is often the second major component (ranging in concentration from 5% to in some cases as high as 60 vol.%), but the origin of this important component has yet to be resolved. The South African mine geochemical and dissolved gas samples have revealed some high N_2 gas partial pressures (P_i ~72 bars) and NH_3 concentrations as high as 200 μM and undetectable NO_3^- concentrations (<1 μM). Non-atmospheric N in geologic systems is predominantly derived from four sources: 1) the thermal deamination of organic material within sediments; 2) N held as NH_4^+ in the lattice structure of silicate minerals within metasediments; 3) N associated with recent magmatism, or of an igneous/mantle origin; and, 4) hydrothermal reduction of N_2 to NH_3 (Brandes et al., 1998). The absence of ^3He in both the Canadian Shield (Sherwood-Lollar et al., 1993) and South African (Lippmann et al., 2003) gas occurrences rules out a mantle origin. Nor is "recent" magmatism a potential candidiate. In the 3.0 Ga siliclastic/volcanic rocks of the Witswatersrand basin clays might provide a source of NH_4^+ although its bioavailability has not been tested. Only one thin, marine, shale unit, the Kimberly Shale, might represent a potential source of bioavailable NH_3. The N_2 (0.1 – 1 M), NH_4^+ (30 to 90 mM) and NO_3^- (up to 50 mM) present in fluid inclusions of hydrothermal veins cutting the Witswatersrand rock strata (Drennan et al., 1999); Frimmel et al., 1999) and the NO_3^- in the leachates of crushed ore-bearing rock (Onstott et al., 2003) suggests that fluid inclusion may provide a source for all three N species.

Since the variation in N_2 isotopic signatures for these different sources is overlapping, N_2 isotope systematics alone cannot conclusively resolve all possible origins of N_2, but stable isotope studies do have

the potential to distinguish between atmospheric-derived N and nitrogen-derived form crustal or mantle processes. Unfortunately, compared to the subsurface C cycle, isotopic studies of the subsurface N Cycle are in their infancy. Sherwood-Lollar et al. (Sherwood-Lollar et al., 1993) have observed $\delta^{15}N$ values for N_2 that increase toward a heavy signature with increasing concentration, consistent with the type of signatures expected for N_2 derived during devolatilization reactions associated with metamorphism (Haendel et al., 1986; Bebout and Fogel, 1992)). Ballentine and Sherwood-Lollar (Ballentine and Sherwood-Lollar, 2002) find the N_2 and ^4He concentrations are correlated suggesting a crustal source. No information on the $\delta^{15}N$ values of NH_4^+, N_2, NO_3^-, NH_3 or NO_2^- in the aqueous or solid phases have been reported, however, from either of these deep subsurface environments.

In the case of our South African research, nitrifying and dissimilatory denitrifying bacteria have been detected by 16s rDNA analyses and thermophilic, nitrate reducing bacteria have been isolated (Kieft et al., 1999), but which of these are mining contaminants or indigenous subsurface habitants is currently being resolved.

Objectives-Like the C cycle objective 1, N cycle research will focus on characterizing abiogenic and biogenic components of N in the deep subsurface of South Africa and Canada. This will involve the N isotopic analyses, N species analyses by Ion chromatography mass spectrometry, DNA extraction, amplification and sequencing of the 16S rDNA gene, and possibly functional genes, analyses of deep groundwater. The N:C and the $\delta^{15}N$ and $\delta^{13}C$ of the microbial biomass will be analyzed. Finally the N:C concentration of $\delta^{15}N$ and $\delta^{13}C$ of the Kimberly shales and of the ore-associated kerogen and bitumen and the $\delta^{15}N$ of the siliclastic and volcanic units enveloping the fissure water will be analyzed.

The research will examine the source of the various components of the N cycle, including the excess N_2 gas. Probable pathways for N_2 formation will be addressed by looking at the typical isotope signatures observed for such pathways and comparing that with the field data, by looking for certain functional genes for the processes of nitrification and denitrification within our environmental microbial population, and by examining the fluid inclusions to determine the chemical composition, isotopic composition, and the source of the mineralizing fluid. In addition to the field component, some (high-temperature, high-pressure) laboratory experiments to explore certain abiotic possibilities for sources and sinks of N, such as mineral-catalysed reduction of NO_3^-, NO_2^-, and N_2 to NH_3. The fundamental database derived will be used to compare N isotopic results to that of other Archean sediments to address the question of correlating the modern terrestrial N cycle to that of the Archean. The research will address four major questions:

- What are the $\delta^{15}N$ values for the solid phase NH_4^+ and the N_2 gas in the rock strata? What is the $\delta^{15}N$ value of the organic-rich Kimberly shales? What are the $\delta^{15}N$ and $\delta^{18}O$ values for the NO_3^- and the $\delta^{15}N$ for the NH_4^+ of the pore water and the fluid inclusions? What are the $\delta^{15}N$ and $\delta^{18}O$ values for the NO_3^- and the $\delta^{15}N$ for the NH_4^+ of the mining water contaminated with explosive residues? Do these isotopic values correlate with other geochemical, isotopic, microbial or geological parameters? Is there any suggestion of an abiotic source for the N_2?
- Do we see evidence in the microbial community of nitrifiers, N_2 fixers and denitrifiers by the presence of 16S rDNA or certain functional genes?
- What are the $\delta^{15}N$ and $\delta^{13}C$ values of the microbial biomass? Do these values reflect the source of N? Are the indigenous microorganisms deriving their N from an abiogenic source?

Nitrate and ammonium isotope analyses-The isotope measurements on NO_3^- will be made using the 'denitrifier method', in which the NO_3^- sample is quantitatively converted to N_2O, which is then measured by gas chromatography-isotope ratio mass spectrometry [Sigman et al., 2001]. This method offers several improvements relative to previous methods that are important to this study. It is extremely sensitive (down to 10 nmol N and 0.5 μM NO_3^-), reducing the constraints and burdens associated with sample concentration and volume. It is completely free of cross-contamination by dissolved organic N or NH_4^+.

Finally, it measures both N and O isotope ratios of NO_3^- in a much more sensitive and time-efficient way than previously available methods [Casciotti et al., 2002].

The NH_4^+ isotope measurements will be made using an adaptation of the 'passive NH_4^+ diffusion' that is optimized for natural abundance isotope work [Sigman et al., 1997]. This method is not as sensitive as the denitrifier method but is more sensitive than other NH_4^+ methods, and the abundance of NH_3/NH_4^+ in many of the systems being studied makes this less of an issue.

Both the NO_3^- and NH_4^+ isotope measurements will be made in Sigman's lab at the Department of Geosciences at Princeton University. The N_2 isotopic analyses will be performed in the Sherwood-Lollar's lab at the University of Toronto.

Evolution of the S Cycle From Abiotic to Biotic Geochemistry

Background-Sedimentary sulfide minerals are typically depleted in ^{34}S due to their formation from sulfide (H_2S or HS^-) produced during bacterial sulfate reduction. The extent of ^{34}S-depletion recorded by the sulfide is dependent primarily on the rate of sulfate advection or diffusion to the site of sulfate reduction but factors such as the concentration of sulfate, pH, temperature, substrate availability, bacterial species, and general growth conditions are important secondary controls. Laboratory studies have shown that the kinetic isotope effect associated with dissimilatory bacterial sulfate reduction produces sulfide that is depleted in ^{34}S by 5 to 46‰ relative to the source sulfate (e.g.,(Kaplan and Rittenberg, 1964; Habicht and Canfield, 1996). Measured $\delta^{34}S$ values of marine sulfides may show values as low as -50‰ (70‰ depletion relative to a 20‰ sulfate, e.g., (Canfield and Teske, 1996). The additional depletion in sulfide is likely due to isotopic fractionation produced during bacterial disproportionation of S^0, SO_3^{2-} (sulfite), and $S_2O_3^{2-}$ (thiosulfate) (e.g., Habicht et al., 1998)). Intermediate sulfur species are produced without isotope fractionation during biotic and abiotic re-oxidation of sulfide that is not sequestered as metal sulfide minerals and is, therefore, available for reactions in sediment pore water (Habicht et al., 1998). Both biotic and abiotic pathways readily transform these intermediate compounds. Consequently, study of sulfur isotope signatures is crucial for distinguishing between chemical gradients that sustain microbial life and chemical gradients that are abiogenic. At temperatures in the range of -5 to 110°C, biotic isotope fractionation of sulfur allows for rapid establishment of substantial ^{34}S depletion in sulfides while abiotic isotope exchange is minimal due to slow reaction rates. If abiotic equilibrium were reached in this temperature range, however, the isotopic partitioning between sulfate and sulfide would be 70 to 80‰.

In any type of hydrothermal environment, circulating geothermal waters may react with, and dissolve, sedimentary sulfides with strongly negative $\delta^{34}S$ values. If the waters are strongly oxidizing, sulfate with anomalously low $\delta^{34}S$ values may result. If the waters are reducing (but still sufficiently acidic to dissolve sulfide minerals), sulfide with low $\delta^{34}S$ values will result. If the water is characterized by a fO_2 value near the sulfate/sulfide boundary then an equilibrium fractionation between aqueous sulfide and sulfate species may result ($\Delta^{SO_4}_{H_2S}$ values ~15 to 25‰ at temperatures between 450 and 250°C). Sulfide $\delta^{34}S$ values more negative than those of the sedimentary sulfide source may coexist with ^{34}S-depleted sulfate under these conditions. A similar scenario would apply to leaching of virtually any type of sulfide (mantle or sedimentary) with a $\delta^{34}S$ value less than ~ 20‰. Depending on the fO_2 conditions and produced SO_4^{2-}/H_2S ratio, plus the degree of isotopic re-equilibration during cooling of the hydrothermal fluid, $\delta^{34}S$ values of sulfide could range from strongly negative to highly positive.

A third method for producing sulfides with low or variable $\delta^{34}S$ values is through low-temperature mineral-fluid interaction that leads to the production of intermediate S species such as SO_3^{2-} and $S_2O_3^{2-}$. Rapid, but abiotic, disproportionation to sulfate and sulfide has been proposed by (Warren, 1972) to explain ^{34}S-depleted pyrite in the ore-zone of roll-front-type sandstone-hosted uranium deposits. Laboratory reactions have not confirmed abiotic disproportionation reactions involving intermediate S species produced as oxygenated groundwater interacted with disseminated sedimentary pyrite in the reduced, down flow, portions of the sandstone. In light of recent discoveries, it is likely that microbial

communities are associated with these ore-producing chemical gradients and that microbial reduction and disproportionation are responsible for ^{34}S-depleted pyrite in these types of U deposits.

The above model for sulfide oxidation and the production of unstable sulfur intermediaries depends on the availability of large volumes of oxygenated water. If such water is absent from the geologic environment in question then the proposed reactions cannot proceed. (Vovk, 1987b) has proposed that in the absence of oxygenated groundwater, radiolysis of H_2O by α, β, or γ rays may produce oxygen-equivalent oxidants (primarily H_2O_2 and OH). In sandstone-type U deposit the interaction between a radiation source and oxygen-poor interstitial water may produce radiolytically generated oxidants that would be similar in abundance to those derived from an atmospheric source. Radiation oxidation of sulfides in low-O_2 water results in the formation of both SO_3^{2-} and $S_2O_3^{2-}$ (DellaGuardia and Johnston, 1980). Vovk ((Vovk, 1987b)) suggests that the large spread in the ^{34}S values of pyrite and groundwater sulfate in roll-front-type U deposits are produced through a radiolytic process of oxidation followed by disproportionation of the intermediate S species. As noted previously, microbial processes may influence pyrite associated with uraninite because laboratory experiments have not demonstrated low-temperature S isotope fractionation during abiotic disproportionation of SO_3^{2-} and $S_2O_3^{2-}$.

Objectives-We propose to further evaluate the possible effectiveness of radiolytic sulfide oxidation in the production of $\delta^{34}S$ values that resemble those produced by bacterially mediated sulfide oxidation and disproportionation reactions. Initial experiments will involve ultra-pure water, freshly cleaved pyrite, H_2O_2, and H_2 sealed in quartz tubes. ^{34}S values will be determined for residual pyrite and aqueous S species, and monitored with respect to the progress of oxidation reactions. Follow-up experiments will involve a low-level radiation source (e.g., Polonium-210 foil), ultra-pure water, and pyrite. Results will be used to evaluate the possible role of disseminated uraninite in the radiolytic oxidation of sulfide minerals and the sulfur isotopic systematics that accompany this process. Results of these experiments have far-reaching consequences for understanding how biosustaining chemical gradients can be established in the subsurface.

Sealed tube experiments will be performed to evaluate the S and O isotopic effects associated with the oxidation of FeS_2 by potential products of the radiolysis of H_2O. Initial experiments will track the changes in FeS_2 ^{34}S values, the sulfur isotopic composition of the aqueous phase, and isotopic variations in headspace an N_2:H_2. Pyrite in the experiments will be from hydrothermal veins in Park City, Utah.

Finely ground FeS_2 (< 20 μm) will be placed into silica glass reaction tubes and 5 ml of 1 and 10 mM H_2O_2 solution will be added. The water and H_2O_2 will be frozen into the tubes using a liquid N_2 cold trap and the sample tubes will then be evacuated. The mixture will then be thawed and the process repeated to ensure that all O_2 has been eliminated from the H_2O_2 solution. Although oxidation of FeS_2 by H_2O_2 does not normally yield H_2, an amount of H_2 equal to $1/100^{th}$ the molar concentration of H_2O_2 will be added to the tubes to evaluate H_2 isotopic exchange during the reaction. The reaction tube will then be sealed and placed into a horizontal resistance furnace controlled to 1^oC. Experiments will be conducted at 60, 80, 100, and 110^oC, with run durations ranging from 100 to 700 hours.

We anticipate that at the temperatures and run durations of these experiments only partial isotopic exchange will be achieved. We will monitor the extent of FeS_2 oxidation by determining the SO_4^{2-} and $S_2O_3^2$ concentrations in the aqueous phase. S species produced will be determined via spectrophotometric methods. The S isotopic compositions of residual FeS_2 and aqueous species (converted to barite) will be measured using elemental analyzer-continuous flow isotope ratio monitoring mass spectrometry. S isotopic fractionation between the residual FeS_2 and aqueous S species will be evaluated as functions of time, temperature, and extent of oxidation. The H_2 isotopic exchange and fractionation will be evaluated by measuring δ^2H on initial H_2O, H_2O_2, and H_2 followed by measuring δ^2H on final H_2O and H_2 gas. Similarly, O isotopic exchange and fractionation will be evaluated by measuring $\delta^{18}O$ on initial H_2O and H_2O_2 followed by measuring $\delta^{18}O$ on final H_2O and SO_4^{2-} and $S_2O_3^2$. If equilibrium is not attained in the longer duration runs we will be able to accurately estimate these values from the partial exchange results. Dissolution and precipitation of new minerals on the FeS_2 surfaces will be monitored using soft X-ray

spectroscopy and SIMS (Riciputi at ORNL). For experiments where the degree of S isotopic exchange has been small, it will be necessary to measure $\delta^{34}S$ values at grain margins on a 20 to 30:m scale. Ion microprobe $\delta^{34}S$ analyses will be required for these samples. Ion microprobe data will allow us to better model S isotopic exchange that may occur prior to the conversion of FeS_2 to aqueous S species and will allow detection of grain-to-grain isotope variability in experiments where sulfide oxidation is rapid.

Using the experience gained from the sealed-tube experiments, we will design an experimental reactor utilizing only ultra-pure water, FeS_2, and α radiation from a Polonium-210 foil. The configuration of the experimental reactor will allow for continuous monitoring of the chemical gradients across a 1-2 mm aqueous layer between the α source and the FeS_2 target. We will use the microelectrode chemical array (pH, oxygen, sulfide) currently in use at Indiana University for studies of sub-millimeter chemical gradients in algal/bacterial mats. All S, O and H species described in the sealed-tube experiments will be isotopically monitored for exchange and fractionation. After doing sterile radiolysis experiments, we will inoculate the reactor with sulfate reducing bacteria with and without growth nutrients and monitor the chemical gradient between source and target.

The common association of uraninite and pyrite in upper Archean and lower Proterozoic sedimentary rocks from Australia, South Africa and Canada (see references in Kimberley (1978)) suggests the intriguing possibility that biosustaining radiolysis facilitated the transition from anaerobic to aerobic ecosystems by allowing microbes in some sedimentary basins to evolve oxygen tolerance prior to oxygenation of the atmosphere.

Experimental Approach

Objectives-The focus of this research will be to perform biotic versions of the abiotic laboratory experiments, with and without irradiation, on heterogeneous fluid/mineral systems to obtain radiolytic production rates and biological utilization rates. The nonirradiated experiments will provide some indications as to whether certain microorganism can modify their environments to enhance microbial activity. The microorganisms selected will come either from our current set of subsurface isolates or from the ATCC culture collection yielding an isolate with the appropriate physiology and 16SrDNA most similar to our environmental 16S rDNA results. The physiologies to be included will be: an autotrophic methanogen, acetogen, aceticlastic methanogen, an H_2- and acetate-utilizing sulfate-reducing bacteria, a hydrocarbon-utilizing sulfate-reducing bacteria, an H_2-utilizing Fe(III)-reducing bacteria, an N_2 fixer, a nitrifier and denitrifier. Electron donor and acceptor concentrations will be measured by IC-MS and growth by flow cyotmetry. Depending upon the success of the Polonium-210 experiment described above, this approach will be used to establish a micrometer scale redox gradient that should prevent radiation damage of the selected microbial species. The reactive species forming on the mineral surfaces and to quantify any isotopic fractionation effect associated with the radiolytic products. These heterogenous fluid experiments will utilize anaerobic, Na-Ca-Cl solutions used in the abiotic experiments. Heterogeneous experiments will also use pyrite, magnetite, olivine, pyroxene and quartz. The pH and T of the solutions will be set at the optimal growth of the selected microorganisms and will be set at the pH and T of the fissure water if these two do not coincide (as they often do not). A mixture of inorganic salts will be used to buffer the solution. To examine the effects of dessication on microbial activity, the experiments will be performed with an equal volume of mineral and saline solution and then allowed to evaporate into an anaerobic glove bag over the course of several weeks until the mineral surface and Polonium-210 foil is saturated with just a thin film of saline solution. The surfaces of the minerals will be examined with XAFS to identify surface adsorbed aqueous complexes and by SR-FTIR to record changes in the metabolic state of the microorganisms. Finally, the heterogeneous experiments will be performed without irradiation, but with low levels of H_2O_2 and O_2 to see if the kinetics and products of these reactions can approximate that of the irradiation experiments. If so, this will greatly facilitate incorporation of radiolytic effects into standard biogeochemical modeling codes.

Tasks-The experiments will focus on H, C, N and S cycles.

1. H cycle-The production rates of H_2, H_2O_2 and O_2 will be determined as a function of temperature, pH, water saturation and Fe concentration in the mineral. The non-irradiated control experiments will be used to further quantify the effect of H_2 production via oxidation of Fe in mineral phases.
2. S cycle-see experimental description above.
3. N cycle-Anaerobic saline water with NH_4^+ and Ar and with N_2 will be irradiated to determine the stability of both phases during irradiation and whether oxidized N species, NO_2^- and NO_3^-, are produced. The aqueous N species will be measured by ICMS and the surface absorbed species by XAFS. The N and O isotopic composition of the different species will also be determined.
4. C cycle-Anaerobic saline water with CH_4 and CO_2 and with N_2 will be irradiated to determine the stability of the C phases during irradiation and whether oxidized or reduced C species, such as low molecular weight hydrocarbons or carboxylic acids, are produced. The aqueous C species will be measured by ICMS, HPLC and the surface absorbed C species by XAFS. The C and H isotope composition of the different species will also be determined. The homogenous experiments will be compared with the heterogeneous experiments to determine the effects of temperature, pH, water saturation and Fe concentration in the mineral.

Synchrotron FTIR Direct Analysis of Stress Changes in Living Cells.

The ability to image and characterize microbial responses to stresses in complex and extremely heterogeneous and transient natural environments has been one of the most challenging areas. Recent work from the Holman group (Holman et al., 1999; Holman et al., 2000; Holman et al., 2002) suggests that one can meet this challenge by using synchrotron-radiation-based Fourier transform infrared (SR-FTIR) spectromicroscopy, especially by combining SR-FTIR results with information from other bioanalytical and imaging techniques. Here, we propose to use this as a real-time technique to characterize microbial resistance to stress.

Synchrotron radiation is utilized because the infrared photons extracted from the radiation source can be focused to a spot less than 10 μm (in diameter). The LBNL Advanced Light Source, the brightest radiation source in the world, routinely obtains high infrared intensities with a signal-to-noise ratio that is at least 100 times better than any infrared thermal source at a spatial resolution of 3 to 10 μm (Lumppio et al., 2001). This unique property allows very sensitive discrimination of IR spectra, even on geological materials that inherently have very low infrared reflective surfaces and at the same time often have a spatial variation of typically 3 to 10 μm. More recently, Holman's group has proved experimentally that although the infrared light from ALS's sychrotron radiation source is very intense, it neither heats the sample nor affects the physiology of a living biological system (Holman et al., in press). Unlike other surface chemistry microprobe techniques (e.g., UV or x-ray microprobe), the SR-FTIR spectromicroprobe is truly a nondestructive and noninvasive technique that provides for direct interrogation of the same location of geological materials with microorganisms. This provides researchers with an extremely beneficial opportunity to quickly track and image the fine-scale (3–10 μm) chemical changes in different compartments of a living biofilm on surfaces of geological materials without the need for staining procedures or chemical tags (see Fig. 21).

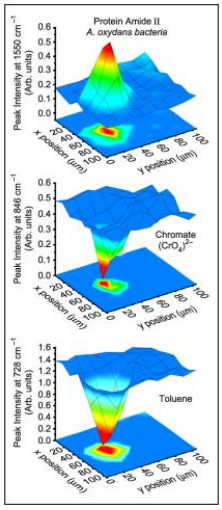


Fig. 21. SR-FTIR mapping showing *Arthrobacter oxydans* bioremediating mixtures of Cr^{6+} and toluene on a mineral surface (Krupa, 1999).

The microcosm experiments, such as microbial response to dessication and formation of thin H_2O films can be observed directly with SR-FTIR. The DSPS and in situ experiments (discussed below) will provide polished rock chips from the sampling site can be inserted into and removed from the chambers and reactors at different points to monitor biofilms on the surface using the SR-FTIR technique. This way, the resistance to stress in these systems can be measured, IR signature changes can be documented, and population changes can be imaged in the biofilm on the chip, all in real-time. This alternate method for measuring stress changes in the cell can also cross-verify the expression analysis studies for the stress regulation pathways in the environmental simulators and chemostats.

Thus although geological materials are inherently heterogeneous and have low reflectivity surfaces, the high brightness of the synchrotron beam enables one to measure, identify, and locate key compounds undergoing changes, as well as microbes involved in the changes. The technique has been successfully used to identify a new pathway of chromate reduction on surfaces of geological materials (Holman et al., 1999) and to provide direct insight into the relationship between localization of chromate reduction, toluene transformation, and *Arthrobacter oxydans* bacteria distribution on surfaces of mixed-valence iron oxides (Fig. 21).

3. LABORATORY SIMULATION OF SUBSURFACE MICROBIAL COMMUNITIES

(***Phelps, Pfiffner, Pratt, Sherwood-Lollar and Hazen)***

SPS Operations Overview of Seafloor- and Deep Subsurface- Process Simulators (SPS and DSPS)

Background-Recent technological developments enable examination of biogeochemical interactions at temperatures and pressures of native deep biosphere environments. Temperature controlled high pressure test facilities at ORNL are available through this research to perform experiments examining the biogeochemical interactions between life processes and the physical environment typical of those several km below land surface or beneath seafloors at temperatures >100^{o}C and pressures greater than 300 bars.

Laboratory experiments typically accomplished with small test tube-sized vessels differ fundamentally from field experiments because of scaling, poor visualization, insufficient monitoring, lack of detailed sampling and wall effects. These limitations make it difficult to extrapolate from small-scale experiments to natural systems. Scaling of dimensions, appropriate for detailed scrutiny with precisely controlled meso-scale experiments, can be accomplished with the ORNL Seafloor Process Simulator (SPS; Fig. 22) a large (72-L usable volume, maximum linear dimension 0.9 m) corrosion-resistant Hastelloy pressure vessel located in an explosion proof cold room, as described in Phelps et al. (2001)).

Characteristics of the SPS include operating ranges of -2 to 20^{o}C (with the addition of a heating element can go to >50^{o}C) at pressures of 0.1 to 20 MPa that covers the relevant regions of the seafloor hydrate phase diagrams, most near surface systems including regions of supercritical CO_2. The SPS also features the ability to extensively sample without significantly altering pressures or volumes. Sampling will be accomplished through a series of high-pressure ball valves connected to 1/4 -3/8 high-pressure stainless steel tubing that is again sealed with high-pressure ball valves and a vent needle valve. Accordingly, by sequentially operating the ball valves 1-50 ml (g) aliquots can be transferred to and from the vessel. Six sapphire-viewing windows (3/4 to 2 in diameter) facilitate visualizations and video or digital photography. A Saphwire-windowed boroscope provides a 10 μm size resolution. Laser-Raman spectroscopy is also available through the boroscope or through sapphire windows. Injections into the SPS include gaseous, liquid injections into the liquid, and headspace or sediment phases through.

Capable of even higher temperatures and pressures is the newly established Deep Subsurface Process Simulator (DSPS; Fig. 23), a Monel-alloy high-pressure apparatus capable of >325 bar at >100^{o}C. This system was designed for evaluating deep terrestrial carbon sequestration and is capable of evaluating various fluids including; aqueous, brines, hydrocarbons and super critical carbon dioxide in native subsurface solid matrices. In this system the subsurface materials are placed into 10-50m length columns of a nominal 3/8" internal diameter. Fluids and or gases are then injected into any of a dozen Monel pressure chambers (50-500 ml volumes) where they are equilibrated at pressure and temperature prior to being pumped into and or through the pre-packed sediment columns. Effluents from the test apparatus automatically pass into an autosampler that can aliquot samples into fraction collectors, subsamplers, a GC and or an HPLC. Similar to the SPS the system is computer operated and data logged by LabView.

Both temperature-controlled, high pressure systems represent unique experimental facilities, ideally suited to the determination of biological, biogeochemical, kinetic, thermophysical, and mechanical properties of the deep subsurface environment and martian hydrosphere while permitting direct observations of processes, and investigation of how these processes are influenced by of partial pressures, temperature, salinity, gas or liquid phases, or biogeochemical transformations. Through the use of natural rock fragments in our experiments, we can investigate the response of microbial communities to high gas/liquid ratios where the saline fluid will "wet" the surfaces and internal pores spaces of the rock fragments.

Objectives-The overall objective of the proposed task is to gain a basic understanding of complex biogeochemical effects of perturbations such as changes in temperature, pressure, nutrient bioavailability and their impacts on the diversity, abundance, activities and metabolomics of subsurface derived microbial trophic groups.

1. Quantify key biogeochemical consequences of perturbations;
2. Evaluate the changes in microbial community composition and assess the functional gene diversity and metabolomics of the microbial community; and
3. Identify potential molecular markers from the microbial community that can be used for the detection and assessment of biogeochemical perturbations and associated consequences.

Experiments- Experiments will rely on rock fragments from cores of the 2.7 Ga Ventersdorp volcanic formation and 2.9 Ga ore-bearing Witwatersrand quartzite/conglomerate collected in South Africa and rock fragments collected from cores obtained from the new field site in northern Canada, complemented by sediment gathered from Ocean Drilling Program (ODP) Legs 204 and 201. Phelps was affiliated with Leg 204 ("Hydrate Ridge") that collected and preserved >30 meters of methane hydrate-rich sediments off Oregon in Summer 2002 archived under in situ CH_4 partial pressures at the ODP TAMU site, College Station, TX.

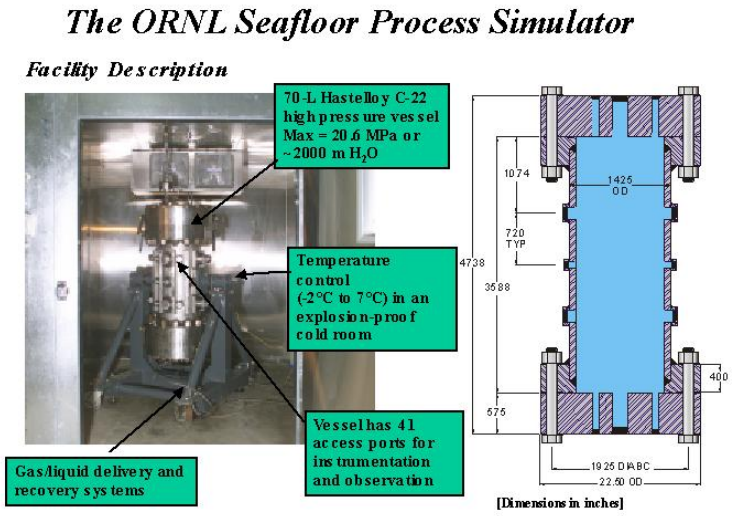


Fig. 22. SPS-Seafloor Process Simulator is constructed of corrosion resistant C-22 Hastoloy with a 72 L internal volume. The 32 cm I.D. x 90 cm length is capable of operation at any angle. 41 access ports with ¾" to 2" NPT fittings. Six sapphire windows. Influent and effluent flow meters. Flow rates can be accurately metered from 0.1 ml/min. to 10 ml/min. Simulator resides in an explosion proof cold room maintained from –2°C to 20°C (type K thermocouples +/- 0.2°C). Fluid pH monitored by 6 high-pressure probes. Visual records obtained with digital camera and boroscope. Internal pressures are measured using a series of piezoelectric transducers with a working range of 0 to 200 bars. Temperatures within the vessel are measured using a series of Hastelloy-sheathed type K (Riestenberg et al., 2003) thermocouples - which are calibrated to an accuracy of +/- 0.2°C. Six pH probes are also currently adopted for internal use. Data are collected in time sequences of 10 - 60 seconds using a data acquisition system consisting of National Instruments Field-point modules connected to a PC using LabView v5.1.

Early experiments will be used to develop the baseline operation of all injectors and sample withdrawal equipment. Liters of sediment will be added to the SPS (or <kgm volumes to the DSPS) vessel in the upright position and rotated ~120 degrees to the horizontal position to spread the sediments uniformly across the curved bottom of the SPS. If necessary, an additional 3 to 5 liters of rock fragments may also be added or a window unit removed to facilitate the spreading of rock fragments via raking to provide adequate coverage. Six liters of rock fragments should provide ~5 cm depth at the deepest location throughout the 0.9 m length of the vessel. It is estimated that 12 liters of sediment would provide a maximum depth of nearly 8-cm, the length of the SPS. The vessel will then be nearly filled with injected, artificial aqueous solution of fresh or sea water (previously sterilized and cooled), such that the headspace is less than 1 liter (eliminating headspace gas is unlikely in horizontal SPS operations) and equilibrated overnight at ~5°C and at pressures ~70% of those experimentally desired. For this project, we anticipate experimental temperatures of ~5°C and pressures of ~100 bar, which it the anticipated formation T and P at 1000-m depth in northernmost Canada. Previous experiments demonstrated that after 12hr of equilibration the vessel contents varied less than 1°C and after 16 hr vessel contents vary less than 0.2°C, which is near the accuracy of thermocouples and thermistors.

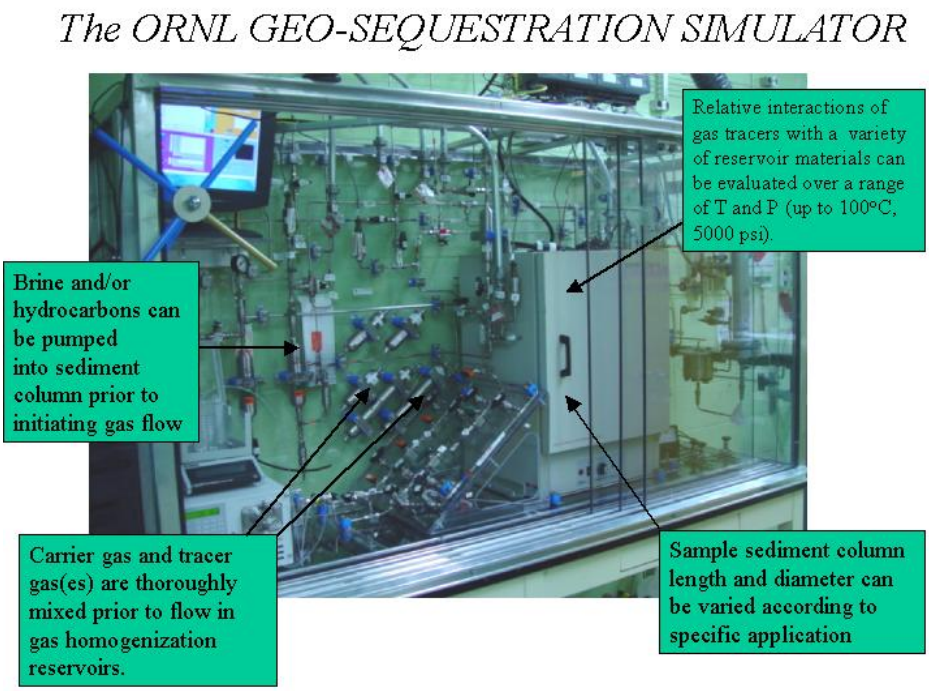


Fig. 23. Constructed of corrosion resistant Monel (brine injection areas) and 2507 stainless steel with Swagelock valves and tubing. Working pressure range up to 344 bars. System can operate with CO_2 as a gas, liquid, or supercritical fluid. Working temperature up to 100°C. Temperature measurements by type T thermocouples. Pressure measurements using piezoelectric transducers (0-6000psi). Data continuously measured using Labview System can be remotely operated via internet connection. Metered flow rates from 0.01ml/min to 10ml/min. Multiple gas homogenization reservoirs (75ml, 225ml, 50ml) allow for continuous sampling. Valco sample valve inject gaseous effluent directly into HP gas chromatograph with an electron capture detector. Conservative tracers include stable isotopes, noble gases, nonreactive salts, and perfluorocarbonsCapable of measuring fate and transport of biogeochemical parameters including varied gas, aqueous, and solid phases under reactive subsurface media

Upon equilibration (SPS or DSPS), the final pressures will be adjusted through additions of subsurface saline water or brine, N_2, CO_2 or liquid CO_2 or CH_4 and allowed to equilibrate for approximately 4 hrs. Upon secondary equilibration completion, samples (time =0) will be withdrawn and analyzed, as described later below. All sediment and aqueous subsamples will be retrieved using the high-pressure transfer chambers. These post depressurization subsamples will be microbiologically, geochemically, and mineralogically characterized during time course perturbations. Throughout the course of each experiment, temperature, pressure, and pH measurements will be monitored from three or more locations. Once quasi-steady state has been established (~6-9 days into an experiment), the vessel will undergo one or more perturbations (liquid or gas) via injection into the aqueous or porous rock phase. Experiments planned for the first year will pursue end member analyses of highly perturbed levels of pCO_2, pCH_4, pH_2, pH, redox, and nutrient amendments, on microbial metabolism and biogeochemistry. Such highly perturbed end member experiments will allow us to fine-tune the boundary conditions for SPS/DSPS operations. Second and third year experiments will focus more on biogeochemical interactions over time. The experiments will be iterative in that lessons learned will be incorporated and factored into designs. Time course characterization will include assessments of changes in the microbial community composition over time, assessment of the changes in metabolic diversity in response to altered nutrient bioavailability, and perturbations and environmental biogeochemistry alterations with time.

Analyses-Total C, N, and S will be determined on rock samples using a Leco dry combustion furnace. Rock fragment surfaces from time-course sampling will also be mineralogically characterized SEM with

energy dispersive X-ray. A JSM-35CF scanning electron microscope (SEM), utilizing both secondary electron imaging and backscattered electron imaging in conjunction with energy dispersive and wavelength x-ray spectrometer, will be used for the analysis of morphology, particle size, and elemental distributions on rock fragment surfaces.

Analysis of the pore fluids and overlying water phase will be important to constrain mass balances and subtle chemical changes in the rock system that may not be measurable by solid phase techniques. In addition to measuring the chemistry of these aqueous phases (e.g., Ca) by ICP-AES and ICP-MS, major anions will be analyzed by IC. $Fe^{2+/3+}$ and HS^- will be measured colorimetrically. Methane, inorganic carbon, and volatile fatty acids will be determined by GC (TCD/FID). H_2 will be measured as a headspace gas using GC-thermal conductivity detection (TCD). Reduced metal precipitates will be assayed mineralogically as described below. After dramatic shifts in bioprocessing are noted, ^{13}C-substrates will be added to select experiments with the distribution of the stable carbon isotopes followed over time into microbial biomass, lipids, and end products such as acetate, methane and VFAs determined by GC-MS. When radioactive ^{14}C or ^{3}H are used in time course metabolomic studies the radioactive end products will be determined by GC-gas proportional counting (GC-GPC) as described previously or by newer procedures being demonstrated by the PI's ((Cochran, 2001)). Microbial lipids and metabolic functions will be analyzed by personnel at UTK under the direction of White/Pfiffner/Peacock (see section 4 below).

DNA extraction, amplification and sequencing will follow the protocols of Qui et al. ((Qiu et al., 2001)). The sequences obtained will be compared with reference sequences from GeneBank and aligned with ClustalW and compared with reference sequences ((Thompson et al., 1994)). Phylogenetic and molecular evolutionary analyses will be conducted using MEGA version 2.1 ((Kumar et al., 2001)), and phylogenetic trees constructed with distance matrices and the neighbor-joining method.

The functional gene diversity has been assessed for a variety of environments and has been shown to serve as an indicator of biochemical capacity and stability. In conjunction with metabolimic and phylogenetic analyses, gene microarrays will be used to assess the response of the community activity to the perturbations during time course experiments. These microarrays have been fabricated for the DOE-GTL program with thousands of gene probes on a small surface area so that genes from an entire community can be monitored. Accordingly, no new microarrays will be developed for this work, rather accessing existing microarrays developed by DOE-funded research to provide additional insights into functional diversity of extreme environments. In addition to high throughput and parallel analysis, microarrays offer a number of other advantages over conventional molecular methods including rapid detection, high sensitivity, multi-color detection for differential display, lower cost, automation, and high signal to background detection (Shalon, 1996). Both DNA and oligonucleotide microarrays have been successfully used for monitoring gene expression and detecting differentially expressed genes in bacteria (Tao, 1999; Peterson, 2000; Ye et al., 2000). These studies demonstrate that microarrays allow researchers to switch from the very focused single gene view to a global view of entire genomes and communities. In addition, the applicability of this method has been tested for studying microbial community mRNA and DNA isolated from soils and marine sediments (Wu et al., 2001). The functional gene array (FGA) will contain many genes of interest as well as the following controls: 16S rRNA genes as positive controls and five yeast genes as negative controls. DNA samples will be arrayed with a single pin (ChipMaker 3) using a PixSys 5500 robotic printer (Cartesian Technologies, Inc.) on silane-modified slides (Cel Associates). The slides will be post-processed as previously described (Wu et al., 2001). We will also use denaturing gradient gel electrophoresis (DGGE) to monitor changes in the microbial composition as previously described (Ferris et al., 1996).

4. FIELD GEOCHEMICAL, ISOTOPIC AND MICROBIAL ANALYSES AND IN SITU EXPERIMENTS

(Onstott, Moser, Sherwood-Lollar, Brockman, Fredrickson, Pratt, Pfiffner, Phelps, van Heerden and Hazen)

Field Sites for Biosustainable Subsurface Environments

South Africa-As a result of our previous characterization studies in South Africa, we have at our disposal five flowing boreholes distributed in depth from 0.95 kmbls. to 3.2 kmbls. and ranging in salinity from 0.01 M to 0.5M and temperatures up to 60^{o}C. The most saline of these boreholes has a $\delta^{2}H$ and $\delta^{18}O$ values that lie off the meteoric water line (Fig. 3) and He-Xe ages of 40-80 Myr (Fig. 2). The lipid and 16SrDNA analyses of these boreholes are complete and reveal a diverse population of Archaea and bacteria, isotopic evidence of sulfate reduction and abiogenic C_{1-4} formation. This large diameter borehole inclines downward at 60^{o} and intersects a couple of fractures at depths >2.0 kmbls. As such, it is ideal for using a multi-level sampler designed by our team for determining substrate incorporation and quantifying in situ respiration.

Northern Canada-The brines encountered by Con Au mine near Yellowknife, NWT have been the subject of numerous previous publications (see, for instance, Frape and Fritz, 1987). The mine contains numerous boreholes emanating brine at various depths. The origin of the brine has been somewhat controversial, but recent ^{129}I analyses yield a minimum residence time of 80 Myr (Bottomley et al., 2002). This is considered by the authors to be a minimum subsurface age as their preferred interpretation for the brines origin is that it represents ancient seawater, trapped in these metamorphosed mafic rocks during the Middle Devonian at which time the Canadian Shield was covered by seawater (Bottomley et al., 1999). Bein and Arad (1991) suggest that the brines could also be created by freezing of seawater and infiltration beneath the continental ice sheet during the Pleistocene at ~ 1 Myr. Isotopic data are most consistent with the former interpretation and indicate these subsurface brines have been beneath a frozen permafrost cap for ~10^{7} years until quite recently. In this respect, their origin and hydrogeological history is very distinct from that of the saline groundwater in South Africa, which originated as meteoric water infiltrating from the surface, as this part of South Africa has not been covered by seawater since 2500 Myr. Given the different origins of these brines and the lower formation temperatures at Con Au mine, it will be of interest to know whether the subsurface microbial communities exhibit similar dominant species and activities. The isotopic signatures of CH_4 from Con Au mine is similar to that of Kidd Creek suggesting an abiogenic origin. The antiquity of this brine qualifies it as a biosustainable subsurface environment, if we find evidence of indigenous microorganisms. The chief geologist at Con Au mine has offered their assistance in providing access to these boreholes (see letter of collaboration).

Nearby, the Ekati diamond mine has started developing an underground mine, which should be available to our team within a year or two. The 50 million year old kimberlite cuts quartz-feldspathic basement and brine has already been encountered at 0.5 kbmls. in an exploration borehole that can be sampled by our team. As fresh brine intersections will be encountered during the lifetime of this mine, it represents an opportunity to obtain both core samples and to monitor drilling contamination during these intersections. Ekati mine is accessed from Yellowknife and like Con Au mine is logistically the most convenient and least costly.

The disadvantage of Ekati and Con mines is that they no longer have permafrost caps. Lupin Au mine and Nanisivik Pb-Zn mine in Baffin Island do have permafrost caps and underground works that would potentially provide an opportunity to examine the relationships of subsurface brine communities and the freezing front. Contacts with mine geologists have been made and access is being discussed (see email correspondence).

Finally, our team has contacted Kidd Creek Au mine in Timmins, Ontario and the Pt mine in Sudbury as potential brine sampling sites given their isotopic and geochemical similarity to Con Au mine, but distinctly different rock types and depths.

Regardless of which mine becomes the host of the proposed research, reconnaissance sampling of brine emanating boreholes will be performed in year 1. Like our South African site, a suite of isotopic and geochemical water and gas samples will be collected along with filters of the water for microbial analyses by 16SrDNA and microbial enrichments. For Con Au mine brines, because so much work has already been completed on the isotopic signature of the water, its age and its major anions and cations and because Clark's research group at Univ. of Ottawa is already performing noble gas isotope analyses, our team will focus be able to focus on the redox metal chemistry, the microbial and DNA analyses, the $\delta^{34}S$ of the sulfate and sulfide and the $\delta^{2}H$ and $\delta^{13}C$ of the C_{1-4} compounds. The $\delta^{33}S$ and of $\delta^{36}S$ sulfate will also be measured for the mines in Archean rock since their sulfide should preserve an anomalous signature of mass independent fractionation (Farquhar et al., 2000). The presence of mass independent signatures in the sulfate would support local derivation by subsurface oxidation and not transport from overlying post-Archean formations.

The fissure water sample collected from mine boreholes in South Africa typically have temperatures ranging 50 to 60^{o}C, which discourages the growth of mesophilic contaminants. For the Canadian mines with their lower formation temperatures, however, much greater care will be required to avoid mining contaminants. Field data such as salinity, sulfide and O_2 concentrations will be used to target brine samples and minimize the probability of contamination. Associated core samples, if available, will be frozen and processed back at the lab, paying particular attention to segments corresponding to water-bearing features. With this information and by working closely with mine geologists, it should be possible to collect water from fresh intersections within hours or days of a water strike and before contaminating microbes have had a chance to bloom within the flowing borehole. Flowing boreholes will be sealed and valved using a well-tested telescopic packer and sampling manifold design (Moser *et. al* 2002) and with stainless steel cartridge filters with pressure relief valves and check valves, which allow for the aseptic and anaerobic sampling of boreholes.

Based upon these results obtained on these samples we will select a mine in year 2 for follow up study that focuses on Gel microdroplet (GMD) enrichments and in situ experiments.

HomeStake Au mine-A new National Underground Science Laboratory that will house higher sensitivity neutrino detectors at 2.5 kmbls. at Homestake Au Mine in Lead, South Dakota. As part of this intiative, a new multimillion dollar NSF program called EarthLab is being submitted to NSF next year to establish an underground laboratory for geomicrobiological investigations. The facility will undertake coring down to 6 kmbls. reaching formation temperatures of >100^{o}C to search for subsurface hyperthermophiles in uncontaminated rock formations. Fracture networks will be instrumented with boreholes, geophysically imaged and used for in situ experiments and microbial transport experiments. Hydrofracturing experiments will investigate the effects of rock fracturing on nutrient supply to subsurface microbial communities. A deep recharge experiment will instrument one fracture zone from 0.1 kmbls. to 2.7 kmbls. to examine interactions of photosphere microbial communities and metabolites with deep subsurface communities. This underground lab will provide opportunities that will not be attainable at a working mine. The time frame for the construction of this laboratory will overlap with the 5 year duration of this proposal and conceivably will present new opportunites and capabilities for investigating deep subsurface biosustainable habitats.

Gel Microdroplet Enrichment

Background-Traditional procedures for cultivation of microorganisms from environmental samples rely on the use of solid, semisolid, or liquid medium containing relatively high concentrations of nutrients to encourage relatively rapid growth or microorganisms to high cell densities. The success of such techniques is often equated to the formation of visible colonies on plates or extensive increases in turbidity of the medium. Yet, the concentrations of cells required to detect a change in turbidity by spectroscopy is ~10^{6} cells mL^{-1}, much higher than that found in our deep subsurface fractures (Fig. 15). Visible colonies on plates requires a microcolony of at least 10^{5} cells and strongly selects for microbes that are fast-growing, resistant to high concentrations of nutrients, and able to grow in isolation. We

argue that these strategies are counter to the normal growth habit of most deep subsurface microorganisms and a major reason why most microorganisms in subsurface environments appear to be uncultivable. The 16S rDNA gene sequencing results from the South African samples record a considerable prokaryotic phylogenetic diversity including members of the *Archaea* and novel lineages within the *Euryarchaeota* (figs. 4-8; (Takai et al., 2001a), but only a few living and archived isolates have been obtained to date. This is despite the fact that our team utilized filter-sterilized fissure water for enrichment media preparation in order to exactly replicate the major element chemistry. Our continued lack of success in attempts to transfer multiple times microorganisms from positive enrichments of fissure water into fresh media comprised of artificial fissure water is attributed our requirement to obtain ~10^6 cells mL^{-1}. Another complicating factor with dilution and transfer approaches is that it requires the target physiology to grow in isolation. In the fracture environment, however, the planktonic species are probably associated with a much larger microbial community that has colonized the fracture surfaces. Because of the difficulty of obtaining fresh, uncontaminated samples of these fracture surfaces our only strategy to sample this part of the population is to immerse into the borehole or pass the emanating fissure water through cartridges containing sand or crushed formation rock in the hopes that the sessile species will colonize these surfaces. The enrichment strategy then employs a mixture of this sand or rock and its pore water where multiple species can grow in proximity to each other.

Objectives-The objective of this task is to apply a novel technology for the cultivation of previously uncultivable microbes with an aim towards their subsequent characterization by genomic and physiologic methods and eventual use in laboratory microcosm experiments. This technology, developed by and to be conducted by Diversa Corporation, is based on encapsulation of single (or a small number) of cells within individual gel microdroplets (GMD's) followed by reconstitution of the starting microbial community by loading the GMD's into a column. The community is grown in the column under very low nutrient flux conditions using filter-sterilized water from the sampling site. Over time, each cell capable of growth under the conditions in the column forms a microcolony within the its GMD. A high-speed flow cytometer and cell sorter is then able to identify GMD's containing microcolonies of >100 cells using intrinsic forward and side scatter and sort each positive GMD into a well of a microtiter plate. The individual cultures can then be: grown further by adding effluent collected from the growth column and performing metabolic analyses, studied by FISH with various gene probes or used for partial genomic sequencing in combination with rolling circle amplification.

Although the use of dilution culture has been successfully applied to the cultivation of "oligobacteria" from natural aquatic assemblages (Button et al., 1993) it has not, to our knowledge, been applied to the cultivation of deep terrestrial microorganisms. The Diversa approach extends Button's oligotrophic medium-limiting dilution approach by replacing the glass tubes with microscopic "polymer cages" (i.e., the GMD). This confers several advantages for cultivating microbes. First, the GMD's allow the microbial community to be grown together versus individually in glass tubes, yet allows each "caged microcolony" to be later separated and analyzed. Second, microbes are grown in a flowing open system that simulates natural environments, where microbes are exposed continually to a very low concentration of nutrients. This is in contrast to a closed batch system where microbes receive a high concentration of nutrients at one point in time and metabolic byproducts can build up to unnaturally high and inhibitory concentrations. The low concentration of nutrients also minimizes overgrowth of fast-growing organisms, thereby allowing propagation of microbes with extremely slow growth rates and/or that only grow to low cell densities. Third, the ability to reconstitute the community within the GMD's of the column allows for diffusive cross-feeding of metabolites and other molecules (e.g. regulatory molecules) between members of the community. This feature also simulates the natural environment, and thus preserves some of the community interactions and other specific requirements that may be needed for successful cultivation.

The Diversa approach has been shown to enable cultivation and isolation of previously uncultivated microbes ((Zengler et al., 2002)). Methanogens have been successfully encapsulated, grown, sorted by flow cytometry, and grown post-sorting, demonstrating the technology can be combined with standard

anaerobic methods to grow strict anaerobes. In addition, recent data has shown fluorescent nucleic acid probes will hybridize to microcolonies within gel microdroplets, enabling flow cytometric screening and sorting based on nucleic acid probes or antibodies. In summary, we believe this novel culturing approach will result in the ability to culture and study a much greater number of organisms from the ultra-deep South Africa subsurface environment.

Cell concentration-Because of the low cellular concentration in the South African fissure water several cell concentration approaches have been developed and will be used in parallel to assure the availability of undamaged, cells of a suitable density for encapsulation. Cells will be concentrated *in situ* behind 0.1 μm MediaKap hollow fiber filtration units, such that particulates from hundreds or thousands of liters will be concentrated into a small volume (ca. 150 ml). These filters will be back-flushed in the field into sterile, Ar-filled serum vials and transported to surface on ice. An alternative method will involve tangential flow filtration (TFF), which has been used in preparation for gel encapsulation of planktonic microbes (Zengler et al., 2002) and a field-ready technique for the strictly anaerobic concentration of cells by TFF has already been successfully demonstrated in previous sampling in the mines. Another approach will be to use high surface area cartridge filters in stainless steel containers. Like the Mediakap hollow fiber filters they will be directly attached to the borehole and will filter tens of thousands of liters, but can handle greater flow rates and pressures. They can then be sealed and removed from the borehole without exposure to air and the canister with filter and ~1 L of filtered water and several sealed canisters of the filtered water will be shipped to the U.S.

Encapsulation-As few as 10^4 cells in 0.1 ml can be encapsulated at ~ 10% efficiency, and efficiencies improve to >90% at $>10^6$ cells per 0.1 ml. Cells concentrated from fissure water will be encapsulated directly, while those from rock samples will be first concentrated by nycodenz gradient centrifugation (Zengler et al., 2002) prior to encapsulation. Singly occupied GMD's, or multiply-occupied if multiple cells are attached to one another) of approximately 50 microns in diameter will be generated using a microdrop maker licensed to Diversa. Only a very small percentage of the total GMD's contain a cell. The encapsulation procedure will be carried out in an anaerobic glove bag.

Growth columns-To minimize the need for collecting and shipping large quantities of groundwater, the cultivation system will be a closed, recirculating loop (Fig. 24). Since the media is pumped through the column at a low rate (10 mL/hr), we propose to use 3 L of media for each column assembly. At this rate, the water reservoir will have a turn over time of 9.6 days. Each assembly will consist of a growth column and an upstream cartridge containing crushed rock chips, sourced from as near the fissure zone water source as possible. These chips will provide fracture surfaces, which may generate soluble weathering products potentially required by the microbes for energy or nutrition. It may also be possible to seed the growth column with milled and cleaned 0.1 to 1 mm-diameter rock from host rock from the location fissure water was sampled. In this case rock particles would be carefully removed by anaerobic centrifugation prior to sorting of GMD's. The GMD's (10 million per column) will be dispensed into sterile chromatography columns equipped with a 0.1 um membrane filter at the inlet and a 8 um filter at the outlet. A second 0.1 um filter will be placed immediately upstream of the media reservoir return. This prevents cells from contaminating the media reservoir and retains GMD's in the column while allowing any free-living cells to be washed out.

Preliminary work at Diversa indicates the GMD formulation can be modified to generate microdroplets that are stable at elevated temperatures. Diversa's cultivation system allows for different medium conditions in different growth columns. Individual columns are snapped into place on a rack and connected to media reservoirs via quick-connects. Individual columns can also be temperature controlled. Incubation of growth columns will be in an anaerobic glove bag.

Growth media-As in the past, we will use on-site filter-sterilized mine water as a basal medium rather than synthetic basal medium or simulated mine water and does seem to work better than using strictly artificial media (Rappe et al., 2002; Zengler et al., 2002).

Deep fissure waters contain high concentrations of various gases. While readily available, for safety-related reasons, we do not feel that the collection and transport of mine gas in quantity is advisable. Nor will it be possible to infuse the cultivation system with gases at the 10's of mPa pressures expected in the deep subsurface. Rather, the entire cultivation system will be infused with a custom gas mix that approximates the composition noted in the mines and maintained at ca. 2 bar (Fig. 24). Infusion with reduced gases may be important, since it is possible that they serve as respiratory electron donors for certain microbes in this environment. A typical brine gas mix might contain, in decreasing abundance, CH_4/ N_2/He/H_2/C_2H_6/C_3H_8/CO. Previous N and P analysis have shown many brine samples with NH_4^+ at 0.1 to <0.0001 μM and PO_4^{3-} at 0.5 to <0.05 μM. Geochemical analyses of these trace elements and trace metals will be used to determine if any amendments are required to ensure adequate availability of these compounds at <0.1 μM levels.

Our general philosophy follows that of others (Bruns et al., 2002; Janssen et al., 2002) who simulated key chemical aspects of an environment to improve the cultivation of microbes from that environment. Cells will be grown with filter-sterile and gas-charged groundwater under 4 different anaerobic electron donor-acceptor combinations at or near the *in situ* temperatures of the samples (Table 1). The addition of electron donors and acceptors simulate the known geochemistry and biogeochemical processes of the waters and are used in different combinations to stimulate growth of different physiologies in the growth columns. One assembly will be maintained without supplementation other than the headspace gas mix. In three other assemblies, alternative energy substrates will be tested. This may vary as the work progresses, but initially we propose to test alternative electron acceptors coupled with, in addition to the dissolved gases, supplemental organic carbon sources. In case the fissure water contains adequate sulfate to support sulfate reduction (Takai et al., 2001a; Moser et al., 2003), one column assembly will be supplemented with only organic carbon sources. The acetate may also stimulate acetoclastic methanogenesis. In some cases, saline fissure water already contains as much as 100 μM of acetate or formate. In this case an alternative organic acid, e.g. benzoate, will be used to select for fermenters or aromatic oxidizers. To stimulate microbial Fe(III) reduction, another will be supplemented with organic substrates and Fe(III)NTA. Both of the column assemblies with organic substrates added may also support lactate fermentation and propionate-oxidizing syntrophs. Finally, one column assembly will be established with an N_2/CO_2 headspace and supplemented with thiosulfate ($S_2O_3^{-2}$). In this case, a small amount of MnO_2 will be injected into the media reservoir to react with the sulfide and regenerate thiosulfate to enhance favorable thermodynamic conditions for thiosulfate disproportionation.

The flow cytometer is able to analyze 5,000 GMDs per second, which is important because the vast majority of GMD's do not receive a cell during the encapsulation procedure. The flow cytometer can easily distinguish between the light scattering properties of unencapsulated single cells, empty or singly occupied GMD's, and GMD's containing a microcolony (Zengler et al., 2002). The cell sorter function of the instrument will distribute microcolony-containing GMD's into separate wells of 96-well microtiter plates.

Analysis of microcolonies-Microcolony-containing GMD's will be grown in microtiter wells by adding effluent (to a total of 0.5 ml) collected from the parent growth column, and/or by addition of simulated fissure water derived from geochemical analyses of the sampled water. Prolonged growth of the 0.5 ml cultures typically leads to rupture of the GMD (Zengler et al., 2002) and provides biomass sufficient for multiple types of analyses. Cultures will undergo metabolite and 16S rDNA sequence analysis, and also be archived. For metabolite analysis, culture supernatant will be flash frozen for subsequent metabolite analysis by GC and HPLC. An aliquot of each culture will be archived for potential future growth studies by adding glycerol and flash freezing with liquid N_2.

As noted above, DNA will be extracted from the cartridge filter produced during purification of the fissure water used in cultivations. This DNA will be amplified with Archaeal and Bacterial 16S rDNA-targeted primers, followed by the generation and sequencing of clone libraries. This will establish a baseline microbial community structure that will be compared to the community cultivated in the GMD growth columns.

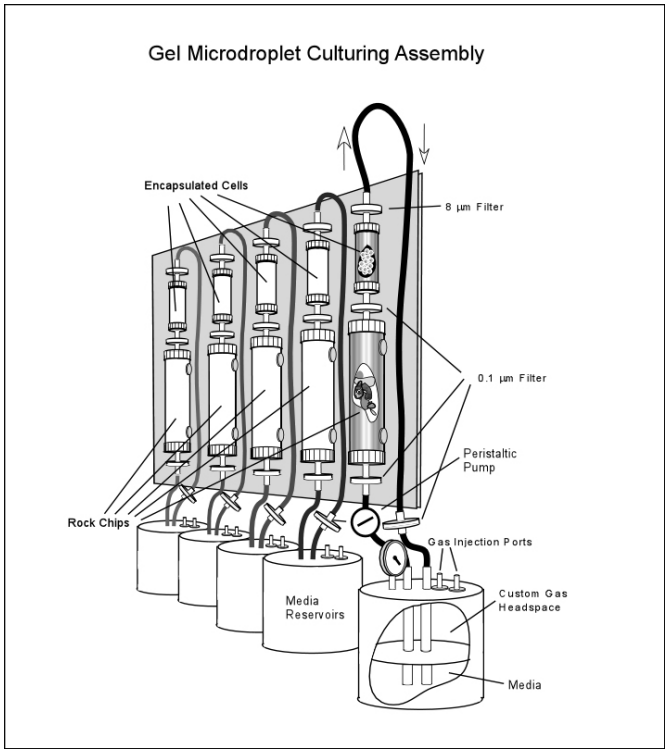


Figure 24. Cell cultivation assembly for GMD's.

Table 1. Supplements to be added to filtered fissure water in column experiments.

Target physiology	Gas mix	Organic electron donor	Electron acceptor
Multiple physiologies[a]	yes[b]	no	no
Sulfate reduction; acetoclastic methanogenesis	yes[b]	yes[c]	no[d]
Iron reduction [500 µM Fe(III)NTA]	yes[b]	yes[c]	yes[e]
Thiosulfate disproportionation (500 µM $S_2O_3^{-2}$)	yes[f]	no	no

[a] Includes hydrogenotrophic methanogenesis. [b] $N_2/CH_4/H_2/CO_2$/Propane. [c] Formate/acetate/lactate/methanol/benzoate, 20 µM each. Benzoate is a model for aromatic compounds detected in fissure water.

[d] Fissure waters typically contain natural sulfate. [e] Fe(III)NTA at 1 mM. [f] N_2/CO_2, 80:20

Sorting- The GMD's will be sorted after 4 to 8 weeks of growth. Anaerobic sorting of GMD's is performed by connecting the growth column (residing in the anaerobic grove bag) to a flow cytometer/cell sorter pressurized with N_2, and using degassed liquid containing O_2-scavenging chemicals.

The diversity and phylogeny of cultures from each growth column will be sequentially screened at the level of the 96-well plate. An aliquot of cells will be removed from each well, all 96 aliquots pooled, DNA extracted and amplified with universal 16S rDNA *Bacteria* and *Archaea* primers, and a clone library constructed from each PCR. Randomly-chosen clones (n=96) will be sequenced and analyzed by blast to determine their phylogeny. Because a GMD may have multiple cell types, rarefaction curves will be constructed to estimate diversity in the clone library from each plate. If diversity is high, additional clones may be sequenced. Plates with clones representing highly novel and/or previously uncultured

groups of microbes will be sub-divided into groups of 8 cultures, and screened again to determine the region of the plate containing the microbe(s) of interest. Single wells from that section of the plate will then be individually amplified, cloned, and sequenced to determine the wells containing the microbe(s) of interest. Alternatively, it may be possible to design a highly specific gene probe for the microbe(s) of interest and use fluorescently labeled nucleic acid probes to identify the wells containing the microbe(s) of interest. Fluorescent nucleic acid probes could also be mixed with GMDs before flow cytometry and sorting carried out to collect GMDs with hybridized microcolonies; however, this approach would be used to analyze only a portion of a growth column, as GMD microcolonies would be killed after exposure to the chemicals used for fluorescence in situ hybridization.

Sequencing of pathways from isolated microbes that appear unique or deeply rooted based upon their 16S rDNA sequences will be performed by Hazen's group at LBNL as part of their Genes-to-Life program. The ability to grow such microbes in GMD's will allow us to partially sequence their genome. Because the genome is a biomolecular record of ancient life (addresses goal 4 of NAI roadmap), these ancient genomes will provide insight into molecular, genetic, and biochemical mechanisms that control and limit evolution, metabolic diversity and acclimatization of life (goal 5 of NAI roadmap). The genomic content of GMD microcolonies can be studied using multiple-primed rolling circle amplification (MP-RCA). This DNA polymerase from bacteriophage phi-29 performs in vitro DNA amplification from linear and circular single- and double-stranded DNA templates (Dean et al., 2001). The method employs random hexamers for priming DNA synthesis and results in exponential amplification of the original target sequence. This method has been used by the Department of Energy sequencing center to directly amplify genomic DNA from as few as approximately 1000 cells of the microbe *Xylella fastidiosa* (Hawkins et al., 2002). Current research at DOE, Diversa, and other private companies is examining post-sequencing genome coverage when genomic DNA is amplified from low numbers of cells. Currently, genome coverage in shotgun libraries (3-4 kb inserts) produced from MP-RCA with an input of 100-1000 cells appears to be equivalent to that obtained by 2x to 3x sequencing of shotgun libraries produced from 10^{10} cells (unpublished data). This will no doubt improve significantly over the next few years. Thus, a whole genome shotgun library can be produced from the cells in a single (or few) GMDs using the MP-RCA technology, and sequencing of the library will capture a substantial portion of the genome. If ancient microbes are identified in the deep subsurface biosustainable environments, GMD's linked to MP-RCA and genome sequencing has the potential to make large contributions to the evolution of life on Earth.

In Situ Experiments

Introduction-A major challenge in determining experimentally in situ microbial respiration and incorporation rates is obtain meaningful information without disruption to the natural setting of the organisms. Traditionally determination of substrate utilization was done by bringing samples to surface laboratories and culturing them under a variety of potential substrates and growth conditions. While valuable, extrapolating this type of result back to the field is always uncertain. We propose instead to perform in situ tracer experiments to evaluate substrate utilization. Such experiments have not to our knowledge been attempted systematically in the challenging environment of the deep subsurface, the results will provide critical data for resolving substrate utilization in biosustainable environments and for identifying biogenic versus abiogenic metabolites.

Experimental Design-Based on these results of the previous investigations, we will piggyback on 1-3 exploration boreholes to be drilled at a Canadian mine site using a recirculating fluid spiked with rhodamine and fluorescent microspheres, two standards approaches for determining the extent of drilling fluid penetration of the fluids and fractures, and will be used for in situ field experiments. Immediately after borehole completion, the holes will be sealed off at the head to minimize any further contamination but allowed to flow slowly through a check valve. Geochemical, microbial and isotopic samples will be collected over time and analyzed until the borehole parameters stabilized and all traces of the drilling fluids have been flushed out of the system.

Compound specific stable isotope analysis and the use of stable isotope tracers (^{13}C, ^{15}N, ^{18}O and $^{34,36}S$ and ^{81}Br) will be used to address the issue of what substrates, provided by the fluids, gases and or rocks themselves, are being utilized by the communities in the deep subsurface.

To introduce these tracers we will utilize (in-situ) microcosms of solid phase "bio- traps" with BioSep® beads in flow-through Delran sampling tubes arranged along a supporting flexible spine. Each sampling tube is connected with gas impermeable tubing to the head of the borehole ((Mislowack et al., 2002)) and will also contain either crushed rock fragments of the country rock or specific rock or mineral substrates. A prototype was successfully deployed for 1 month in a South African mine. Each microcosm will contain a different labeled substrate and one microcosm without substrate is used as a control. The system has a modular design for autoclaving prior to assembly and insertion into the borehole and resealing of the borehole. The outlet of the gas impermeable tubing for each microcosm drips (~1 drop/minute) directly into an evacuated 140 mL serum vial sitting on dry ice. The serum vials are changed on a daily basis depending upon the drip rate.

1. In order to be able to detect the nanomolar levels of activity anticipated, in situ experiments will be conducted using ^{13}C-labelled substrates of a) DIC; b) CH_4 and C_2-C_4; and c) selected organic acids such as acetate, propanoate, butanoate, benzoate and formate identified in the *in situ* waters, ^{15}N-labelled substrates of NH_4^+ and NO_3^- and ^{36}S labelled SO_4^{2-}, $S_2O_3^{2}$ and sulfide. The substrates selected for the experiments will be based in part upon the 16SrDNA analyses of the borehole community and the results of GMD isolation.
2. Over a period of two months, samples will be taken to determine rate of conversion of various substrates and potential incorporation of ^{13}C-label into microbial biomass. Isotopic analyses of inorganic and organic C pools and biomass will be done to develop a C mass balance for these systems, and used to identify the substrate basis for the borehole microbial community.
3. After two months if the results on the effluent samples indicate microbial activity is occurring in some of the microcosms, the multi-level sampler is removed and the tubes disconnected, stored and returned to the lab. In the lab, the contents can be removed and the lipids and DNA of the microbial biomass adhering to the rock chips and the BioSep® beads can be extracted for analyses. Assessment of the extant in-situ microbial communities in subsurface environments will be most effectively accomplished if aliquots of solid phases in the samplers can be readily colonized by the resident microbiota and those systems be subsampled and analyzed over time.

BioSep® beads-BioSep® beads accurately capture viable biomass diversity and community composition and serve as excellent enrichment devices. We have recently employed Bio-Sep® beads (Sublette et al., 1996), 2-3 mm spherical beads consisting of 25% (w/w) aramid polymer (Nomex) and 75% (w/w) powdered activated carbon (PAC). The bulk density is about 0.16 g/cm^3 with a porosity of 74%, and an adsorptive surface is greater than 600 m^2/g. The beads are surrounded by an ultrafiltration-like membrane with a median pore diameter of 1.9 microns and with some large macropores > 20 microns. Beads can be purged of organic carbon by incubation at 350°C for at least 5 hours prior to deployment representing an organic-free inert adsorptive matrix ideal for microbial colonization in extreme environments. Bacteria are immobilized inside Bio-Sep® beads by culturing the bacteria in the presence of the beads in a natural environment. These beads were recently successfully deployed for several days at ~1800 mbls. in Evander Au mine in S. Africa. As in other installations, solid phase samplers with these beads have been shown to enhance the formation of biofilms in drinking water. For example, when deployed in groundwater Bio-Sep® beads generated a viable biomass (as measured by PLFA) that was more than 4-10 times greater than that formed on a plastic surface or glass wool (White et al, 2003, Peacock et al, 2003, Gouffon et al, 2002). Recovery of both lipid and DNA from these bio-traps proved much easier than from sediments and membrane filters. Enrichment of subsurface populations with solid-phase BioSep beads was demonstrated after infusion of 1-3 mM acetate induced a greater than

3-fold increase in viable biomass, with a 7 to10-fold increase in the specific PLFA characteristic of Geobacter, low-G+C Gram-positive Clostridium, and dissimilatory Fe(III) reducers in a metal-reducing groundwater system. Experiments performed in collaboration with the UFZ in Leipzig, Germany, used BioSep® beads containing ^{13}C-benzene in a polluted aquifer. GC/IRMS analysis of PLFA extracted from the beads showed ^{13}C incorporation into a small specific subset of the total PLFA indicating a specific set of organisms in the biofilm could utilize the ^{13}C-benzene. These experiments revealed the power of ^{13}C as a biomarker and allowing scientists to follow the metabolism of labeled compounds through extreme environments, including the synthesis of lipids in organisms.

Analyses

Lipid- Lipid analyses will be performed using previously reported important precautions to achieve quantitative results (White and Ringelberg, 1998) and published analytical procedures (Lytle et al, 2000a,b).

DNA analyses-Nucleic acids will be analyzed using the procedures discussed in section 3.

Stable Isotope Analyses-Stable isotopes (^{2}H, ^{13}C) combined with PLFA have recently been used to link degradation/metabolic activity with specific microbial populations (Boschker et all 1998; Nold et al, 1999; Hanson et al, 1999, Alexandrino et al, 2001; Pombo et al, 2002; Boschker and Middelburg, 2002). The problem with ^{2}H of lipids is that they reflect a combination of organic substrate and water and are fractionated and in that regard have yet to be proven diagnostic of a particular source substrate. Carbon isotope compositions of the FAME will be determined by GC-IRMS by Pratt research group in Indiana University. The measured isotopic ratios of methylated fatty acids are corrected for the methyl moiety (Abrajano et al. 1994) using:

$$\delta\ ^{13}C_{FA}\ (‰) = [(C_n + 1) \times \delta\ ^{13}C_{FAME} - \delta\ ^{13}C_{MeOH}]/C_n$$

where $\delta\ ^{13}C_{FA}$ is the $\delta\ ^{13}$C of the fatty acid, C_n is the number of C atoms in the fatty acid, $\delta\ ^{13}C_{FAME}$ is the $\delta\ ^{13}$C of the methylated fatty acid, and $\delta\ ^{13}C_{MeOH}$ is the $\delta\ ^{13}$C of the methanol used for the methylation reaction. Precision of an internal fatty acid standard (19:0) is ±1.01‰ (n = 5) for the FAME. Carbon isotope ratios of total biomass and CO_2 will also be determined and all C isotope values reported against the PeeDee belemnite standard.

The δ^{15}N of NO_3^-, NH_4^+ and the whole cells extracts from the BioSep® beads will be made in Sigman's lab at the Department of Geosciences at Princeton University. The δ^2H and δ^{13}C of the C_{1-4} compounds and the δ^{15}N of N_2 isotopic analyses will be performed in the Sherwood-Lollar's lab at the University of Toronto. The δ^{36}S and δ^{18}O of SO_4^{2-} and $S_2O_3^{2}$ and the δ^{36}S sulfide will be measured at Indiana University.

FISH-Using the results of 16SrDNA analyses of the borehole samples and serum vial samples, probes will be designed to target the 16S rDNA gene. These probes will be applied to biofilms that have colonized rock chips from the samplers for which two opposing faces have been previously polished. Microscopic observations will be performed at Princeton University using an Olympus BX60 microscope operating at 2000x with epifluorescence illumination. The microscope is equipped with a digital, color CCD camera, an image analysis software package, an internet connection and UV cubes and filter sets appropriate for DNA and protein stains.

Geochemical analyses-Cations and trace metals will be measured by ICP-MS and anions by IC-MS (Princeton University). Gas compositions will be measured at the University of Toronto.

Data analysis-It will be necessary for this multi-dimensional data to be processed so that microbial ecologists can achieve understanding of the relationships among bacterial presence, function, geochemical characteristics and other environmental factors. Non-linear analysis, i.e., artificial neural network analysis, will be used to analyze relationships between PLFA or gene frequency data and site geochemistry (Pfiffner et al, 1999, Almeida et al, 1998, Noble et al, 2000).

4. STRESS RESPONSE PATHWAYS IN DEEP SUBSURFACE BIOSUSTAINABLE ENVIRONMENTS

(Hazen, Phelps, Onstott, Brockman, Pfiffner, Moser)

In July 2002 the Department of Energy funded a major new Genomes to Life Program (GTL) project lead by Lawrence Berkeley National Laboratory. This 5 year, multi-institutional project is focused on the rapid deduction of stress response pathways in metal and radionuclide reducing bacteria. This project was used to start the Virtual Institute for Microbial Stress and Survival (http://vimss.lbl.gov) whose mission is research on stress and survival mechanisms in microorganisms in a variety of environments. This institute's principal research goal is to determine stress and survival pathways and networks from the molecular level to the ecosystem level. The stress response pathways being studied by this DOE funded project, e.g. O_2, pH, T, N, and P, are some of the same parameters that impact the survival and sustainability of microorganisms in deep subsurface and extraterrestrial environments.

The Focus of the VIMSS GTL Project.

The GTL Project addresses DOE cleanup and collateral research needs. Our focus is on experimentally elucidating and computationally modeling the stress response pathways of three target metal and radionuclide reducing organisms: *Desulfovibrio vulgaris, Shewanella oneidensis* MR-1, and *Geobacter metallireducens*. Microbial metal reduction plays an important role in biogeochemical cycling of C and N, as well as in the bioremediation of metals, radionuclides, and organic contaminants. A number of bacteria have demonstrated the capability to reduce radionuclides and other metals. Numerous microorganisms capable of coupling the oxidation of organic compounds to the reduction of metals have been isolated and studied from the standpoints of physiology, ecology, and phylogeny.

The overarching goal of our DOE research is to develop criteria for monitoring the integrity (health) and altering the trajectory of an environmental biological system (process control). To achieve this requires a more complete understanding of how the biological "units" comprising the system are organized, regulated, and linked in time and space (genes, genomes, cells, populations, communities, and ultimately, ecosystems). Key to these objectives is a more complete understanding of stress response systems and their environmental context.

These studies will directly support the proposed NASA research by enabling a comparison of stress response pathways in deep subsurface microbial environments. Indeed, a critical factor that enables biosustainability in deep subsurface and even extraterrestrial environments are stress response pathways. By comparing the stress regulatory pathways that we are currently studying to those we will examine in deep subsurface microbial communities, we will be able to discriminate between those stress regulatory pathways and conserved components that are shared across species and habitat boundaries from those that maybe unique and critical to the survival of life in deep subsurface environments.

Overview of the VIMSS GTL Approach.

The goals for our DOE project fall into two categories: applied and pure. The pure goals, which are particularly relevant to the proposed NASA NAI project, are to understand, from a physical-chemical, control-theoretical, and evolutionary point of view, the structure, function, and dynamics of the pathways involved in the biogeochemistry of soil microbes under a wide variety of conditions.

First, we measure in physical detail the time-dependent activity of as many pathway components as possible under a variety of conditions, stresses, and other perturbations. From both perturbation-response data and direct measurements of molecular interaction, we will then deduce pathways involved in the stress response of target organisms during the task of metal reduction. The same perturbation-response data are also a necessary precursor for understanding how changing soil conditions and the applications of external stimulatory agents to these organisms will change and control their behaviors.

In the case of this proposal, our team will analyze time-dependent activity for 1) experiments performed in the Deep Subsurface- Process Simulator using deep mine fissure water and crushed rock and 2) for the in situ borehole experiments.

Why Many Pathways from Multiple Organisms in Great Detail?

By creating detailed causal/physical models of the stress response pathways, we will learn what the principles of control in these pathways are at a molecular level. In order to do this we must have extensive measurements of the time-dependent changes in activity for all molecular players and their interactions including for the base line, pre-stimulation or pre-perturbation state. It does not suffice to have only the cis-regulatory structure that comprises the immediate control of gene expression, nor only the protein-protein interactions. Ideally, all the state-dependent interactions among the cellular components should be traced. This is because bacterial regulatory networks are less stratified than it might seem at first. For example, the sporulation initiation pathway in *Bacillus subtilis* (a cryptobiotic pathway; Fig. 25) demonstrates that all types of interactions can play a major role in cellular decision-making. That is, control of gene expression, signal transduction, secretion of small peptides and proteins, and metabolite consumption are all coupled to arrive at the final decision to sporulate. The network in Figure 25 was experimentally discovered over a 25-year period and contains a large amount of genetics and biochemistry. Even so, the exact control of sporulation has still not been solved. To match and exceed this level of detail in our target organisms will require a concerted effort to measure as many cellular species as possible, as well as their interactions and their behaviors, under a wide variety of external conditions and mutant states. The functional genomics bioanalytic pipeline described below is designed to address this need.

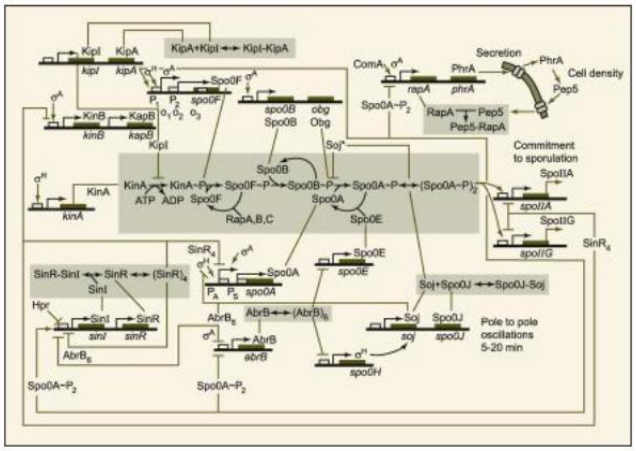


Fig. 25. The sporulation initiation pathway in *Bacillus subtilis.*

The sporulation initiation pathway diagram in Figure 25 is artificially truncated. Many other molecular species are integrated into the function of this pathway. In addition, many of these other species, for example ComA, are involved in other pathways. Figure 26 shows how the seemingly innocuous notation for ComA in Figure 25 (middle, upper right of that figure) can lead into more complex networks. One of the central challenges of network biology will be development of methods that discover modular structure in these pathways, if such exists. Certainly the pathways can at least be conceptually broken up by overall function. For example, the stress response pathways in *Bacillus subtilis* interact with each other in such a way that a perturbed population of cells splinters into a number of different cellular behaviors, each cell implementing one or more stress responses (Figure 27). These stress responses modulate each other so that incompatible behaviors such as sporulation and chemotaxis are not expressed simultaneously (or contemporaneously). This sort of interaction among pathways is more than simple cross-talk. Cross-talk, at least in engineering, implies parasitic signals that move

uncontrolled among closely spaced systems. Yet in this discipline, cross-talk has strong biological implications. This strong coupling and the resultant population heterogeneity necessitate the study of more than one stress response and the development of experimental methods to follow which cells choose which responses. Indeed, a central goal of the theoretical work in our group is to derive formal methods for determining modularity in these networks so that subsystems can be tested and modeled without complete information about the "cross-talked" systems.

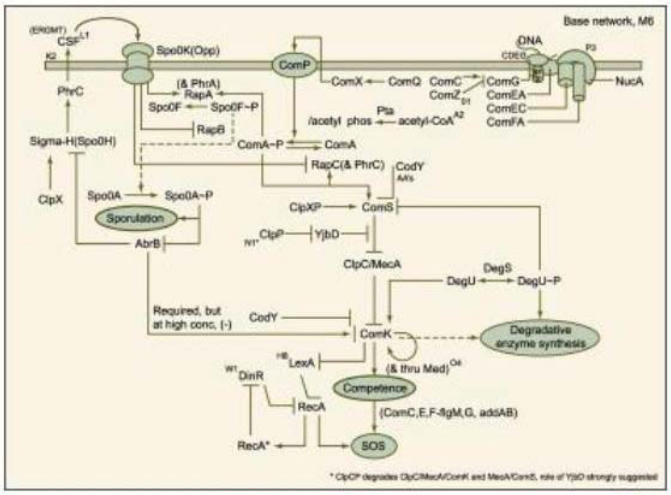


Fig. 26. Coupling of sporulation to other pathways.

Figure 27 shows the interaction among stress response pathways within a particular organism (in this case *B. subtilis*). Although other organisms may have homologous pathways to *B. subtilis*, the regulation among and within these pathways is likely to be different. As an example, in a recent comparative study of the chemotactic pathways in *Escherichia coli* and *B. subtilis*, it was found that, although the major complement of proteins was the same between the two organisms, there were both component differences and important differences in regulation. The study concludes that the systems respond very differently to perturbation in their machinery. Two classes of question arise in the face of this information. First, how do the differences in perturbation response behavior lead to differences in the endogenous ability to track chemical signals, and what do these dissimilarities imply about the differences in control that may be achieved by external (unnatural) signals? Second, are these differences traceable to the particular environments in which these two organisms live? *E. coli*, a gram-negative microbe, is an enteric organism that needs to live in animal hosts as well as in external environments, whereas *B. subtilis,* a gram-positive, spends most of its life in soil. Are there evolutionary roots to the disparate regulatory structures, or are they merely artifacts of evolutionary drift?

Although the three key organisms studied in our DOE will provide core models of the stress response pathways, obtaining information on the regulatory strategies of other organisms and their relative overall responses to different conditions will provide further confidence in the estimation of regulatory structure differences across and between niches and of how these regulatory differences lead to alteration of a variety of biogeochemical processes. For the NASA NAI proposal we will examine the diversity of regulatory strategies, both by genomically characterizing the microbial community in the deep subsurface, biosustainable environments and by comparing the response of this community to stress or stimulus with that of our three key organisms.

PNNL-Diversa team, homologous to the pathways identified in our key microbes, will lead to identification of both conserved and divergent sets of proteins and DNA cis-regulatory elements. Deducing which pieces of a given pathway are central to a given function can be done by observing which factors are conserved across niches. Within-niche variation of regulation should give insight both into the plausible flexibility of the regulatory network in achieving similar goals and the differential

regulation of organisms competing for resources within the same niche. Conserved differences between niches should yield insight into niche-specific regulatory strategies. Combining this information with experimental measurements on the population dynamics of the community under different stress conditions will yield a mapping between regulatory strategy and behavioral phenotype. Models of the different strategies, modified from the highly tested models of the target organisms, will yield hypotheses for the role of each regulatory difference in the survival of the microbe and the fabric of microbial community interaction with the environment that controls biogeochemical processes.

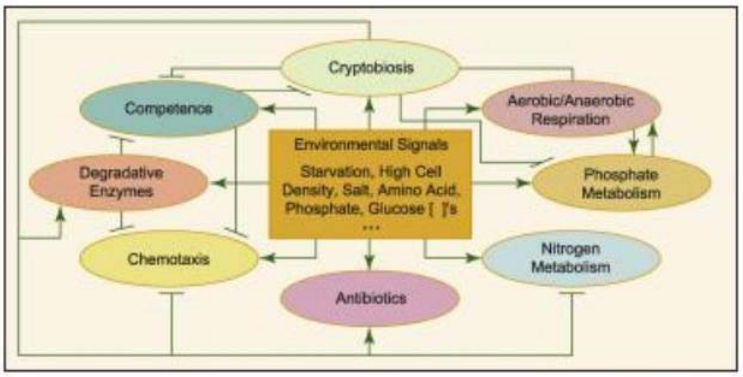


Fig. 27. Different stress responses caused by splintering of perturbed population of cells after interaction of stress response pathways in *Bacillus subtilis.*

Environmental Considerations: An Ecological Framework for Defining Stress

Extensive application of direct molecular measures of microbial community structure in different environments/habitats (primarily based on comparative 16S rDNA sequencing) has revealed some general themes that are directly relevant to the goals of the proposed research. First, population diversity is present in the deep-seated brines and the greater part of environmental diversity has yet to be recovered in pure culture.

Second, a natural organization to microbial diversity within this habitat appears to exist with heterotrophic sulfate reducers being the most obvious dominant respirer and aceticlastic methanogens dominating less saline and more carbonate rich fissure water. Generally, populations (as identified by the 16S rRNA sequence type) are affiliated with well-resolved phylogenetic groups (clades or "phylotypes") (Britschgi and Giovannoni, 1991; Liu and Stahl, 2001). This is also true of the Columbia River Basalt aquifer in which metal or sulfate reduction is a major process have revealed a high diversity of related populations (e.g., members of Geobacteraceae and Desulfovibrionaceae) (Fry et al., 1997). Thus, at higher taxonomic ranks there is a less complex natural order—high species diversity is captured by a limited number of major phylotypes. This suggests that the natural assemblages share a similar ecological function (e.g., metal reduction or sulfate reduction).

Little understanding of the ecological factors contributing to, or sustaining, high diversity within a phylotype (e.g., within the families Geobacteraceae or Desulfovibrionaceae) exists for the deep subsurface. We suggest that high species diversity among populations comprising a single phylotype contribute to functional stability within an environmental system. Since environments are not static but are constantly changing with respect to key physical-chemical variables (e.g., substrate concentration, temperature, pH, salinity, osmolarity, light, redox potential, etc.), it follows that not all populations in a system are simultaneously experiencing optimal growth. Those populations growing under suboptimal conditions, or entering resistant (e.g., spores) or moribund states, are experiencing stress even under the slowly evolving conditions of deep subsurface brine systems. Thus, we suggest that it is not sufficient to monitor stress. It is essential to understand the environmental context of stress response for different

populations. We hypothesize that within any well-adapted and dynamic system, some fraction of the community will be experiencing stress. The key question is, what levels of stress response signal a system that is in distress or that is not optimally adapted or sustainable over geological time intervals?

Experimental Methods for Determining Stress Response Pathways in Biosustainable Deep Subsurface Environments.

The two major experimental approaches to examine the stress response in deep subsurface environments were outlined above. Using the deep subsurface stimulator, the inoculate microbial population and constituent water and crushed rock will be exposed to good stress, nutrients amendments, and bad stress, CO_2 injection and dehydration with water ice and clathrates formation. Using a brine emanating borehole and down hole multi-level sampler array the microbial community, hypothetically in a overall state of stress, will be exposed to growth or energy supplying substrates. Both experiments will be performed over a time scale of months. Both experiments will be preceded with microcosm experiments using single species isolated from the environments by either conventional or GDM techniques.

Identification and Recovery of Stress-Responsive Populations from Complex Communities

Deep subsurface environments change at a much slower rate that the shallow environments being studied by DOE, yet at the same time, microbial turnover rates are also orders of magnitude slower in deep subsurface environments than shallow aquifers. Consequently, it still follows that all populations in a natural system are not simultaneously growing optimally. Those populations growing under suboptimal conditions, or entering resistant (e.g., spores) or moribund states, are experiencing stress and this component of the community may be greater in deep subsurface environments than shallow aquifer environments. Thus, we suggest that it is not sufficient to monitor an extrinsic parameter known to be associated with stress—it is essential to understand the environmental context of stress for individual populations. We hypothesize that within any well-adapted and dynamic system, some fraction of the community will be experiencing stress. This component of the community will be identified using a general activity measure (rRNA synthesis) to identify populations that respond to changing chemical or physical environments by entering a cryptic or low growth state. Cells comprising specific stress-responsive populations will then be physically recovered using a combination of a high-speed cell sorter and fluorescent in situ hybridization (FISH), using fluorescent probes designed to target specific rRNA sequence types. In this way, we will achieve full integration of system elements, linking specific stress-regulated genes to specific populations shown to respond differentially to specific stresses.

These studies will be directly integrated and in support of the expression analysis and modeling studies of our DOE GTL project. For example, these systems will later be used to monitor whole-system stress response using microarrays developed from the collection of stress responsive genes and related to model predictions, etc. The same microarrays will also be used to measure the stress responsive genes in the biosustainable deep subsurface environments being studied in the current NASA proposal.

Radiomicroarray Analysis.

Change in growth status will be evaluated using a radiomicroarray format to measure changes in the rRNA synthesis of individual populations resident in the DSPS and the in situ borehole experiments described above. The microarray will build upon our research program developing a platform for comprehensive monitoring of microbial populations in complex communities. Unlike the more standard glass-slide array, the gel-based format immobilizes DNA probes in small acrylamide pads ($100 \times 100 \times 20\mu m$) arrayed on a glass surface. This provides for much higher probe density (and target capture capacity) than possible by direct immobilization on the glass surface. Probes immobilized on individual pads are designed to complement specific rRNA sequence types, either unique to individual species or encompassing larger phylogenetic groups. Our studies have demonstrated the utility of this format to directly detect environmental rRNAs using an optimized fragmentation and fluorescent dye labeling

protocol (Guschin et al., 1997). This microarray format has been well characterized with respect to specificity and reproducibility.

We have recently combined the gel-based array with radiolabeling. In this format the microbial community is labeled by adding $^{33}PO_4$ (or ^{3}H uridine) to the growth medium immediately following perturbation/stress (e.g., altered pH, metal addition). The $^{33}PO_4$ is incorporated only into the nucleic acids of actively growing populations. Following recovery of total rRNA and hybridization to the microarray, the array is coated with a Ag halide emulsion. The emulsion is developed following an appropriate period of incubation, and radioactivity of the rRNA bound to each individual gel pad is quantified by observing precipitated silver grains with a diameter of approximately 0.2 μm using microscopy. Thus, nongrowing populations are labeled only with fluorescent dye, whereas active populations are identified by generating both fluorescent dye and Ag grain deposition signals. We recognize that not all cells of a specific population will be of comparable physiological status, for example, if cells are attached to rock chips versus being planktonic. Unfortunately we have no feasible approach for examining changes in attached cells during either the DSPS or in situ borehole experiment. Microcosms studies on the other hand that are examined with nondestructive SR-FTIR approaches may provide some insights into changes occurring on the rock surfaces during environmental changes.

Recovery of Stress Responsive Populations via Flow Cytometry

Flow cytometry is a well-established method of measuring physical characteristics of individual cells, including light scatter (size and shape) and fluorescence emission at wavelengths of interest. This technique was initially used in biological oceanography to quantify and sort naturally fluorescent marine picoplankton. Our earlier work documented the feasibility of using flow cytometry in combination with FISH to quantify specific microbial populations using probe-conferred fluorescence (Amann et al., 1990) and this method has now received general use in environmental microbiology (Fuchs et al., 1998).

Our more recent studies have shown that high-speed sorting can be used to physically recover specific populations, using FISH as the sorting parameter. Figure 29 shows the results of a successful sort in which a specific population of cells was recovered based on probe-conferred fluorescence. The pooled cells then serve for general and specific genetic characterization. For example, a few thousand sorted cells were sufficient to recover 16S rRNA genes using PCR amplification.

Previous attempts to view cells from South African fissure water using a flow cytometer and FISH were unsuccessful due to the low signal (small number of ribosomes). In the in situ borehole experiment, however, where isotopically labeled nutrients are being added, if growth does occur, then sufficient numbers of cells will likely be recovered for use in other sequence-based analyses, including hybridization and large-fragment cloning for the directed recovery of homologous stress response genes. This will require the use of evacuated serum vials partially filled with ethanol in order to preserve the cellular integrity and rDNA for FISH hybridization.

The proposed combination of methods provides a technical framework to link a physiological response of a specific population within a community setting (radiomicroarray) and to associate that population with a specific genetic system of stress response.

Flow Cytometric Sorting of FISH-labeled Populations

Flow cytometry has been productively used to provide general information of single-cell abundance and certain aspects of their activities (e.g., cell size, DNA and RNA content, membrane potential). Most past applications have been of relatively coarse resolution because analyses were restricted to populations having unique intrinsic properties such as autofluorescence (Button and Robertson, 2001).

The use of fluorescent dye-labeled probes to label single cells via fluorescence in situ hybridization (FISH) now provides the basis to selectively confer fluorescence on any population using genetic criteria. Our group contributed to both the early development of the basic format and the early documentation of the combined use of FISH and flow cytometry (Amann et al., 1990). A key goal of our proposed research

will be to use fluorescent probes complementary to the 16S rDNA's to selectively recover specific environmental, DSPS and in situ borehole experimental populations via flow cytometric sorting, providing a mechanism to associate genes recovered from the bulk community with specific members of the community. Probes will be developed to target specific populations highlighted by the radiomicroarray studies. The success of sorting a population from field-site samples will in part depend on our ability to isolate a relatively clean cellular fraction from the environmental substratum and correlate its forward and

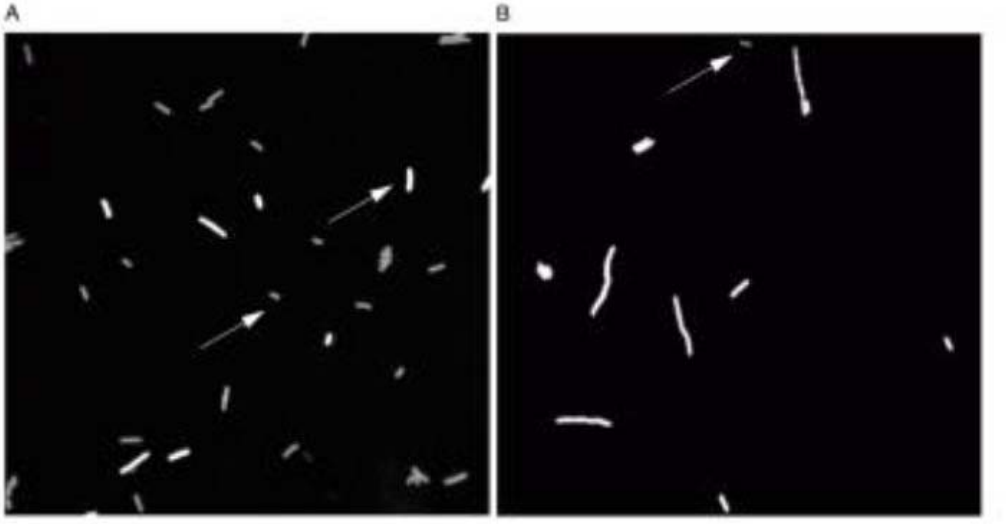


Fig. 29. Fluorescence photomicrograph of bacterial cells fixed with paraformaldehyde and labeled by hybridization with fluorescently labeled, group-specific 16S rRNA oligonucleotide probes. Panel A shows a mixture of *Escherichia coli* and *Pseudomonas aeruginosa* (noted with arrows) cells before flow cytometric sorting. Panel B shows the same population of cells after sorting. The arrow denotes a single cell not properly excluded during the sort. Courtesy of A. Schramm.

side scatter properties with a specific 16S rDNA sequence. We will also evaluate the use of enrichment cultures developed from environmental samples, eliminating or reducing the need for fractionation of cells from a solid substratum. Sorted populations will be further characterized using PCR and hybridization to link them with specific genes implicated in stress response pathways.

Direct Stress and Community Comparison with PLFA and Metabolite Analyses.

As discussed above PLFA has been used extensively for characterizing and monitoring microorganisms in all types of environments. The normal detection limit is about 10,000 cells; however, recent studies have shown that much lower densities can be detected using microassay techniques (Hazen, 1997). The extraction process for the lipids has also been used to simultaneously extract nucleic acids, providing a method to relate sequence information (e.g., as determined by DNA probing) and lipid signature compounds from the same environmental sample.

We will use this approach to relate sequence-specific information (e.g., T-RFLP mapping, DNA probing) to lipid profiles in the mine borehole samples, the DSDP samples and the in situ borehole experiment. The combined analyses will provide a way to associate populations identified by sequence with molecular signatures associated with physiological status.

The metabolites, dissolved organic and inorganic compounds and complexes, will be measured by ICP-MS and IC-MS and the dissolved gas species by GC and Kappa 5 (low H_2 and CO concentrations).

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PLAN FOR STRENGTHENING THE ASTROBIOLOGY COMMUNITY

INTRODUCTION

The Indiana-Princeton-Tennessee Astrobiology Institute (IPTAI) entitled "Detection of Biosustainable Energy and Nutrient Cycles in the Deep Subsurface of Earth and Mars" focuses on subsurface microbial communities that have been sequestered from the surface photosphere for tens to hundreds of millions of years. Using a combination of field and laboratory experiments, we proposed to characterize environments supporting deep-subsurface microbes on Earth. Observational and experimental data will be used to design innovative instruments, datalogging systems, and algorithms for differentiation of non-biotic (sterile) from biotic biogeochemical cycles on Earth and, potentially, on Mars. We seek specifically to analyze microbe/mineral and microbe/ion interactions; to determine isotopic signatures of organic and inorganic species present in gaseous, aqueous, and solid phases; to document genomic diversity and metabolic strategies; and to study protein expression and origin of metabolites in deep microbial ecosystems. This information is crucial for the development of life-detection approaches that can be tested on Earth and can be deployed as flight-capable instruments on future Mars drilling missions.

Education and Public Outreach (EPO) activities in IPTAI are designed around three areas of emphasis. First: educational workshops for undergraduates and high school teachers where participants actively collect and interpret data from laboratory and field experiments. Second: public outreach through a web site with premiere-quality digital media including animations and video that illustrate how and why scientists conduct research in deep mines. Third: mentoring undergraduate and graduate research at Indiana, Princeton, and Tennessee universities. These astrobiology students will work with faculty to design a series of web-based quantitative and investigative activities for all pre-college students but highlighting the diverse careers of leading women on the IPTAI team.

Inclusion of collaborators from the School of Fine Arts, IU Instructional Support Services, and University Information Technology Services at Indiana University is an unusual aspect of this proposal. High-resolution digital video/audio materials will be collected during field experiments and will be use in both research and educational components of the IPTAI. Videos produced by scientists will document research methods in a substantially different way from conventional commercial films. We hope to capture examples of both set-backs and advances in research resulting from unanticipated and challenging conditions in deep mines. Given severe time and access constraints in deep mines, digital documentation of the physical conditions and the configuration of instruments is essential for interpretation of experimental results (Fig. 1).

EDUCATION AND PUBLIC OUTREACH

The science and engineering fields within the United States are lacking in minority participation especially African-American and American Indian students. In the Science and Engineering Indicators 2000 submitted by the National Science Board to NSF, representation of Black, Hispanic and American Indian individuals among scientists and engineers is substantially lower than their respective proportions of the total U.S. population. Demographics on terminal degree also show disparities. In 1999, 61% of black engineers and scientists had bachelor's degrees as their highest degree compared to 56% of all engineers and scientists. Furthermore, the fields of social sciences, computer and mathematic science rather than life or physical sciences are the more prominent fields for black scientists and engineers. These differences are attributed to diverse factors including lack of funding, guidance/mentorship, and opportunity. Given that the U.S. faces a dire shortage of engineers and scientists for educational,

industrial, and governmental programs, it is important to foster participation by currently underrepresented groups. Aggressive recruitment may be necessary for development of new intellectual

Figure 1. Pratt checking flow on a massive filtration apparatus connected to a borehole valve located at a depth of 3.3 km below the surface in Kloof 4-41 Mine, South Africa.

capital in critical areas such as economy, environment, health and security. Developing underutilized human resources can lead to a competitive advantage for the U.S. in a global economy.

The established scientific community must find better ways to motivate minority students towards graduate-school education. Many explanations are posited for the relatively small numbers of students of color entering PhD programs: today's sluggish market for college instructors, the high costs of graduate education, limited financial support, and more lucrative opportunities in other professional fields (Davidson and Foster-Johnson, 2001). Projections by the Bureau of the Census, however, show that the population of elementary school children in the United States in 2030 will be equally divided between non-Hispanic whites and all other racial/ethnic groups combined (NASA, 1996). If more minorities are encouraged to attend graduate school and pursue academic careers, they will serve as tomorrow's role models and mentors for our increasingly diverse population.

Purpose and Goals

Educational and public outreach in the IPTAI proposal is targeted on recruiting, encouraging, and facilitating retention of underrepresented or minority undergraduates and teachers. Our efforts will be mainly within the U.S. but likely will include participants from Canada and South Africa. We will specifically design materials for pre-college girls and for workshop participation by undergraduates and high-school teachers. The annually hosted workshops will be the most visible and costly component of our EPO effort. Our past experience indicates, however, that a one-on-one approach is the most effective way to foster genuine engagement with future scientists and science educators. Workshops will target U.S. minority undergraduate students and will be supervised by an international group of mentors. Internships and workshops will be conducted primarily at laboratories in the U.S. but also will be

conducted in tandem with field experiments at mines in the U.S., Canada, and South Africa. Our over-arching EPO goals are:

- To engage students and teachers in multidisciplinary team research using internship and workshop activities. Particular effort will be devoted to minority and female students.
- To involve undergraduate students, graduate students, and post-doctoral researchers in field experiments at deep mines. Field research will be supervised by faculty members with extensive underground experience in order to ensure attention to issues of training and safety.
- To increase student awareness of career opportunities in astrobiology and in related growth industries such as geomicrobiology, biotechnology, bioremediation and environmental engineering.
- To recruit undergraduate majors and graduate students in astrobiology and geomicrobiology.
- To foster transfer of innovative technologies between academic, government and industrial research groups through collaborative geomicrobiological research.

The proposed educational experiences will explore the unusual geochemical and microbial environments that exist within and around the world's deepest mines. Astrobiology internships and workshops will encompass environmental field techniques, microbial and geochemical laboratory exercises, and tutorials on a broad range of topics including controversial aspects of environmental remediation, origins of life, and genetic engineering.

Graduate Education and Post-Doctoral Training

The single largest component of funding in the proposed NAI is advanced educational training in the form of graduate assistantships and post-doctoral associateships. A total of six graduate assistantships and four post-doctoral associateships are included in our budget under a combination of NASA-requested and institutional-matching funds). External funding for student positions is a critical step in getting academic administrations to consider new degree and certificate programs in astrobiology. Proposals for astrobiology and/or geomicrobiology degree programs are currently under consideration at Princeton University and Indiana University. Regardless of the degree title, a substantial number of new graduate degrees will result from the proposed investigation, and these young scientists will identify themselves as members of the astrobiology community. Pratt and Onstott have successfully advised a total of 23 graduate degrees of which four are female PhD's and three are female MS's. It is notable from the perspective of role models that the proposed NAI includes three women scientists (Pratt, Pfiffner, and Sherwood-Lollar) in leading roles.

Pratt, Onstott, Pfiffner, and Sherwood-Lollar have all demonstrated their ability to supervise students under demanding and difficult conditions in deep mines. Post-doctorate and graduate students training was essential to the NSF LExEn research project "Collaborative Research: South African Ultradeep Mines - Long-Term Sites for Interdisciplinary Studies (LSLIS) into the Extreme Environment of the Deep Subsurface@ (EAR-99788267; Ben-Ari, 2002). Students involved with the South African project came from university and DOE laboratories in the U.S. and from academic institutions in Canada, Japan, and Germany. More than twenty undergraduate students, graduate students, and post-doctoral associates participated in joint field exercises and co-authored publications. These students and post-doctorate researchers were trained in geochemical, isotopic, microbiological and molecular field methods. We believe that the proposed NAI field experiments offer a similar extraordinary opportunity for participation on a multidisciplinary research team. With incorporation of EPO as an integral part of the NAI proposal, we will be able to effectively link programs in pre-college outreach, undergraduate research mentoring, graduate research advising, and post-doctoral supervision. We believe that public outreach should go hand-in hand with student mentoring and scientific research if we want to successfully recruit under-represent groups to careers in science and engineering.

Workshops for Undergraduates and Teachers

We propose to model our internships and workshops on previously successful efforts in South Africa. The University of Tennessee, Knoxville was funded by NSF to hold two consecutive workshops in 2001 and 2002 (http://geomicro.utk.edu/) entitled, "The South African Field Laboratory Workshop: Experience for Minority Undergraduates (Christen, 2002) and Life in Extreme Environments and Biotechnological Applications." In these workshops, thirteen to sixteen minority U.S. and previously disadvantaged South Africa (S.A.) students participated in a series of hands-on, field exercises and laboratory tutorials over a five day period under the guidance of U.S. and S.A. mentors in S. Africa (Fig. 2). Over 80% of attendees would recommend this experience to someone else and over 75% of the attendees have enrolled, are applying, or plan to apply when they complete their bachelors degree, to advanced degree programs or internships in research laboratory (NIH, CDC). The overwhelming success of these workshops and the experience we gained from conducting these educational outreach efforts has encouraged us to formulate a 7-week long program under the National Science Foundation's (NSF) Research Experience for Undergraduates (REU) program (proposal pending) with realistic goals.

Figure 2. Undergraduate Science Majors sampling biofilms in a deep South African Gold Mine. Photo taken during 2001 University of Tennessee Workshop.

Students training to be engineers and scientists need a sophisticated understanding of the situations in which they will be working. As either practitioners or researchers, most individuals holding a science degree do not work exclusively within technical and scientific spheres. It is important for students to realize that they will work in complex social, political and international contexts. Research and study abroad provides an effective way to add ethical and humanistic dimension to the scientific enterprise. Research shows how overseas educational experiences lead to increased awareness about ethical concerns that face society as a whole, increased interest in international affairs, world-mindedness, and cross-cultural empathy as a result of overseas educational experiences. Also, students returning from overseas generally demonstrate a change in behaviors, including increased involvement with organizations that focus on international issues and participation in activities with the goal of increasing international understanding. An abroad opportunity to collaborate with mentors and students in inquiry-based learning allows students to broaden their understanding of their career goals during transition from formal education to profession. The deep-mine workshops proposed in the Indiana-Princeton-Tennessee NAI

will give students a social and cultural context for scientific research. Issues of intellectual property, patents, biodiversity, and national and/or planetary resources will be considered in the context of scientific research. Sociological research indicates that vocational exploratory behavior may occur in studies abroad, often leading to an increase in career options. (Hannigan, 2001; De Winter, 1997; Handelsman, 2002).

In the U.S. and many other countries, earth science education in high school is minimal. Subsequently, students are unprepared to pursue geology, earth science, and environmental sciences at the university level. To address this need, we plan to hold a weekend workshop for secondary teachers during years 2 through 5. These training workshops would assist in curriculum ideas related to astrobiology and geomicrobiology. We think these workshops will help teachers capitalize on the excitement of exploring for life on other planetary bodies. We will use existing materials on NASA web sites and we will develop new materials based on our research activities in deep mines.

Relationships with Minority Institutions

The Center for Environmental Biotechnology at Tennessee has a long history of research collaborations with Florida A&M University (FAMU) which is a historically black college or university (HBCU). We have successfully involved FAMU students in our previous South African workshops. Dr. Pfiffner and Ms. Davis have an open seminar invitation from the Environmental Sciences Institute at FAMU. We will use that opportunity to actively recruit college students in astrobiology, geomicrobiology, and educational outreach in the proposed astrobiology institute. In addition to FAMU, we have expanded our HBCU interactions to include Hampton University, Xavier University of Louisiana, and the University of Puerto Rico. Word of mouth through student participants at workshops has been, by far, our most effective form of advertising. We plan to continue emailing flyers to numerous universities and posting workshop notices in journal outlets like Geology, EOS, and the University Faculty Voice.

Outreach Science for Pre-College Girls

Pratt and Pfiffner have considerable experience developing hands-on and quantitative science exercises for pre-college girls. For five years in the mid-1990's, Pratt coordinated the geological and environmental components of Brownie Math and Science Day at Indiana University. More than 300 girls in first and second grade attend this event each year. Pfiffner has worked extensively with the "SHaring ADventures in Engineering and Science" (SHADES) colloquium is for 6th and 7th grade females and their math and science teachers in East Tennessee counties. The SHADES program consists of highly interactive, demonstration-oriented presentations and exhibits on science and engineering topics. The goal of the program is to show students that mathematics, physical sciences, life sciences, chemistry, and the engineering disciplines are "fun" and interesting and that career options are very diverse. The students attend a presentation (25 minutes) with hands-on activities for each the five disciplines and then participate in a team design competition. Presenters are engineers and scientists from the local professional societies, academia, and corporations who are interested in motivating young people to pursue careers in science and engineering. Career planning information is included in the registration packet. The program is offered one Saturday every Spring and Fall with the location moving through local schools and schools in neighboring counties.

Both the Brownie Math and Science Day and the SHADES program are designed for any girl who has an interest in and aptitude for science and math. These programs do not target the best students but serve a broad spectrum of young girls who might be motivated to take the high school math and science courses allowing them to major in technical fields in college. Sessions for teachers are planned as a part of the SHADES colloquium, and future plans include in-service credit for participating teachers. Students and teachers who attended the previous conferences have been very enthusiastic about it. SHADES is sponsored by the Greater Knoxville Math/Science Coalition. Coalition member organizations include the American Association of University Women (AAUW), Association for Women

in Science (AWIS), Society of Women Engineers (SWE), American Nuclear Society (ANS), and the Tennessee Society of Professional Engineers (TSPE). A similar program is called Broaden Your Horizons.

As part of the EPO activities in the proposed NAI, we will develop specific presentations to be used in Brownie Math and Science Day at Indiana and in the SHADES colloquium at Tennessee. The exercises developed and tested at local science events will be refined and loaded on the IPTAI web site for use by other elementary and junior high-level schools. Astrobiology graduate students will work with faculty on the development and refinement of these materials. Specific attention will be given to illustrating these exercises with photographs showing women scientists in diverse roles. We think it is important for young girls to see photos of actual women scientists and to have links to career biographies for these women. The proposed IPTAI has several women in leading roles who would be appropriate subjects for career biographies.

Programmatic Evaluation

Evaluation of the EPO program would be conducted as one part of the overall science program assessment. At the end of the workshop abroad or in the U.S., the students, teachers, and mentors will fill out an exit survey form that will solicit a written evaluation of the program and suggestions for improving this program. A follow-up questionnaire will be included as suggested by NSF's "Looking Beyond the Borders" handbook and the "2002 User-Friendly Handbook for Project Evaluation" (Frechtling, 2002; Loretz, 2002). Additionally, we plan to track the students' career development for four years by communication with the student and their advisors. Students who have participated in the South African workshops have routinely asked the mentors for advice on graduate schools or career advancement programs, and for letters of recommendation to the aforementioned. To date, we have had maintained good communication with undergraduates that participated in the NSF 2001 and 2002 workshops.

Annual reporting will include information on the overall efforts from student recruiting and selection through the abroad and stateside research and educational training internships / workshops as part of the EPO program for the proposed NAI. This information will include the undergraduate students' demographic information, year of schooling completed, their home institution and its highest degree granted in the student's field of study. Additional information about the students will include their research activities and findings with respect to specific research results and the training and development they received during the program. The training and development may include special skills learned, opportunities to present the research work within the NASA Astrobiology Initiative and in professional meetings, and efforts made with respect to graduate school and career advancement. Students will be encouraged to present their research at their home institutions, regional and national meetings, and to publish their research findings. One of the U.S. undergraduate participants, Kristi Coleman, of the South African workshops has presented a poster at the 2002 American Geophysical Union International Fall Meeting in San Francisco, CA (Coleman, et al. 2002). Another student, Jonesta Nolan will participate in a poster presentation at the American Society for Microbiology International Annual Meeting in Washington, DC, May 2003 (Nolan, et al. 2003). These types of presentation and publications will be documented and reported. Outreach activities to the international universities and mining companies will be described, as well as the impact of these international collaborations toward enhancing a diverse, competitive, and internationally networked workforce.

Summative program evaluation will be used to measure the success of the program. Measures to consider in the evaluation are student completion of their undergraduate programs, matriculation to a graduate program in the sciences or engineering, and recruitment in science and engineering careers as compared to a control group. Additional assessment will be garnered from the student and mentor surveys, questionnaires and evaluations of the EPO program for this NASA Astrobiology Initiative program. The program evaluation will include a cost assessment element. The UT Center for International Education will assist with program evaluation, data analysis, and assessment of this proposed EPO.

The technical/educational advisory committee whose members will be comprised of outside educators and scientists along with some NAI team investigators. This committee will review and evaluate the program progress, provide feedback for refinements for future activities.

DEVELOPMENT OF COURSES AND PROGRAMS RELATED TO ASTROBIOLOGY

At Princeton University, Onstott will develop a sophomore level course on Astrobiology supported by the University's Sophomore Initiative. This course will draw upon the expertise of faculty from Geosciences, Chemistry, Engineering and Astrophysics departments. This course will provide the basis for the developing a curriculum in Astrobiology. Many courses are already offered that are relevant to Astrobiology, including Freshman Seminar 160w-Terraforming Mars (Onstott-Geosciences), FRS-Origin of Life (Dismukes), FRS153 "Elements of Life" (Stiefel), CHM544/ENV544 "Metals in Biology" (Stiefel). A new Geoscience course-A tour of the Solar System is being developed by Duffy. Ge201 "Earth and the Terrestrial Planets" taught by Suppe. The ultimate goal is to have an undergraduate certificate in Astrobiology at Princeton University by 2008.

At Indiana University, Pratt is developing a new graduate course in geocmicrobiology. This course will be offered for the first time in Spring 2004 and will draw on faculty from the Geological Sciences Department, the School of Public and Environmental Affairs, and the Biology Department. For the past eight years, Pratt and associate instructors have taught a freshman-level course entitled "Earth: Our Habitable Planet." This course is taken by more than 200 students each the fall semester and more than 100 students each the Spring semester. Habitable Planet is designed to introduce students to the complex interactions among atmosphere, biosphere, hydrosphere and geosphere. A major component of Habitable Planet is current hypotheses concerning the origin of life and presence of life on other planetary bodies. Planets and Meteorites, taught by A. Basu, is another fully enrolled introductory science course taught in the Department of Geological Sciences. Pratt gives guest lectures based on her deep-subsurface research in an introductory Physics course entitled "Physics of Extraterrestrial Life." which is taught by Charles Horowitz. Pratt and Horowitz are working collaboratively with Chemist Donald Burke on the development of an introductory astrobiology course. We are designing this course for high enrollment (+200 students per semester) and for rotating faculty instructors from Geological Sciences, Biology, Physics, and Chemistry. If successful, this new course will be the first step toward a certificate or minor in astrobiology at Indiana University.

SUPPORT FOR ELECTRONIC COMMUNICATION AT INDIANA UNIVERSITY

Recognizing the importance of emerging communication and collaboration tools to teaching, research, and administrative activities, specific action items regarding videoconferencing, media streaming and collaboration were included in the Indiana University Information Technology Strategic Plan (ITSP) 1998-2003. The ITSP provided funding for service establishment and enhancement of existing centrally-funded management and operations. Through strategic plan initiatives, the University provides services and support for videoconferencing, media streaming and collaboration, without fee to academic, administrative and research users.

For the proposed IPTAI, Indiana University is able to provide institutional commitment to a wide range of communication and collaboration technologies through an extensive support organization. Indiana University Digital Media Network Services operates one of the largest University videoconferencing networks, provides media streaming services, and is in process of establishing an institutional data collaboration service. The vision is to make these three services function seamlessly - any event involving participants in distributed locations can take advantage of any or all of the services on a reserved or ad-hoc basis depending on their particular need.

Videoconferencing

Videoconferencing is used extensively throughout Indiana University's eight-campus system in support of teaching, research, and administration. NASA-deployed videoconferencing systems are identical to the systems currently supported at Indiana University. The technology currently used on campus is standards-compliant H.323, Internet-based. Approximately 145 group systems (conference rooms and classrooms) and 250 desktop systems are represented. Group systems are Polycom ViewStation and desktops are Polycom ViaVideo models. Two carrier-grade Polycom MGC100 videoconferencing bridges, provide for multipoint conferences (more than two parties in a single conference call), and can support approximately 200 simultaneous 384kbps endpoints in conference. Gateway bridging to ISDN H.320 videoconferencing is provided. The Radvision ECS200 gatekeeper supports 1000 registrations and 200 simultaneous calls in routed mode. Indiana University participates in the Internet2 Commons, ViDeNet and Global Dialing Scheme (GDS), allowing for easy dialing via E.164 "telephone-like" numbers to participating sites throughout the globe. Within the University, dialing is supported through a Global Address Book service. Key infrastructure components, such as the conferencing bridge and gatekeeper, are duplicated to provide fail-safe operation. Funds are requested in the IPTAI budget for two additional Polycoms that can be deployed in offices of the Institutional PI's at Princeton and Tennessee.

Media streaming

Media streaming services at Indiana University will support the encoding, storing and delivering of a variety of digital formats, including Real, Windows Media, QuickTime, MP3, MPEG-1, and MPEG-2. MPEG-4 support is in development. Current infrastructure for storage and delivery includes a Real Helix server with 1 TB of storage and capacity for 400 concurrent streams. The Helix server supports Real, QuickTime, Windows Media and MP3 formats. A VideoCharger server provides storage and delivery of MPEG-1 and MPEG-2 formats. Content-provider access to the streaming storage and server is provided via accounts on the institutional web publishing server. This method makes the process to publish streaming content intuitive, and familiar to anyone already publishing web content on the institutional web environment.

Implementation of a re-architected system is in progress that will significantly enhance the media streaming infrastructure. The new architecture is based on a 4TB network-addressable storage (NAS) server that provides a common storage service for a diverse collection delivery severs, supporting the range of delivery formats. The storage will be mirrored on identical systems on our Indianapolis and Bloomington campuses, interconnected by a University-owned fiber intranet between the two campuses. A third snapshot server will take periodic snapshot backups.

In 2003, an activity will be completed to establish a digital asset management system. Currently, all assets are managed as file objects with unique user spaces. The digital asset management system will provide methods to preserve and manage resources, through the use of standards-based video metadata and catalog, and will provided expanded access through search and retrieval systems. Commercial systems will be evaluated, as well as implementation of open-standards profiles such as the ViDe Application Profile for Digital Video. Also in 2003, in conjunction with the deployment of a digital asset management system, a digital rights management system will be implemented. Currently, assets can be quasi-secured through obfuscation. A comprehensive, secured method for providing rights management will be implemented.

Videoconference-to-streaming gateway

Videoconference-to-streaming gateway service to record and stream videoconferences was brought into production in the fall of 2002. The service, referred to as IStream, is built around Polycom Viewstations whose audio/video output feeds directly into streaming encoders; plus a comprehensive software system built to provide scheduling, operations, and user access to the content. The service allows any H.323 videoconference, occurring anywhere in the world, to be easily recorded and streamed, live or

on-demand. Real Networks and MP3 formats are supported. The service currently has capacity to capture eight concurrent videoconferences, and is scaled according to demand.

The University has installed videoconference endpoints in auditoriums at the IUB and IUPUI campuses specifically to take advantage of the IStream service. Lectures are recorded and optionally streamed live. The recorded format can be audio and video using Real Networks, or audio-only using MP3. The MP3 option has proven extremely popular - students can download lectures directly to desktop PC's or portable MP3 players. During the fall 2002 term, 30 classes utilized the I-Stream service. In addition to classes, guest lectures and one-time events are streamed using IStream, and administrative meeting have made use of IStream to establish meeting records.

Data collaboration

Data collaboration taxonomy can describe categories of remote presentation, interactive collaboration, and persistent shared virtual workspace. Remote presentation, typically used in conjunction with audio or video conferencing, provides for a member of a conference group to share, in real-time, the display of a computer desktop or application, with other conferees - and to share the information at native computer resolution, rather than say, at reduced resolution though in-stream display in a videoconference. Interactive collaboration provides for the live sharing of a computer desktop or application among a group - with any member of the group able to take control of the application. Interactive data collaboration tools can be specialized, such as web co-browsing or whiteboard, or may be generalized to share any application or desktop. Persistent shared virtual workspaces (PSVW) have the feature that information and work are captured and persistent over time in the shared space. PSVW tools range from simple threaded discussion lists, to complex environments that support tool sets such as threaded discussion, file sharing, project planning, outliners, meeting management, document review, calendar and others.

Remote display and interactive data collaboration are considered to the critical next step in establishment of electronic collaboration services at Indiana University. Several products are in pilot test at the University. A production implementation is planned for early summer 2003. Remote display and interactive data collaboration services will be established using easy-to-use web-based tools, hosted on University servers. Shared virtual workspaces are being explored in specific projects rather than enterprise service context. Videoconference classrooms and conference rooms are being equipped to permit concurrent display of videoconference and remote data display images.

Service Support and Cost

The Indiana University Digital Media Network Service unit is charged to provide infrastructure services and comprehensive user support for videoconferencing, media streaming and collaboration services. In addition to the infrastructure services described, DMNS provides support for acquisition, training, use, facility and resource scheduling, management, operations and help desk - staffed to provide instantaneous response to user service problems and requests. The DMNS operations center utilizes advanced commercial and custom tools to monitor and operate the network, including the Polycom Global Management system. DMNS is staffed with three videoconferencing engineers, two media streaming engineers, a data collaboration engineer, a systems administrator, a scheduler, a help desk coordinator, hourly help desk staff and a unit manager.

Members of the Digital Media Network Services unit are deeply involved in national and international digital video activities and collaboration technology groups and activities. The DMNS manager is currently co-chair of the Internet2 Digital Video Initiative; chair of the Video Development Initiative (ViDe), and is past chair of the Committee on Institutional Cooperation (CIC) Video Working Group.

The videoconferencing, media streaming and data collaboration services and support organization are provided as centrally-funded IT services. The services and support are provided without fee to academic, research and administrative users. Users are obliged to purchase their own end-systems, such

as Polycom videoconferencing terminals, but the support services, and central systems services such as multipoint conferencing bridging, streaming storage and servers, and data collaboration servers are provided without fee.

PRODUCTION OF PREMIERE-QUALITY DIGITAL MEDIA FOR WEB AND VIDEO DISTRIBUTION

Scientists and students on the IPTAI team will benefit substantially from collaboration with a professional staff of artists and digital media consultants. This production collaboration will enable IPTAI to develop premiere-quality materials for use on the web site and at conferences and workshops. We believe this collaboration will result in educational videos that illustrate how and why scientists conduct research under difficult and dangerous conditions such as in deep-mine locations. Video material of this type is unlikely to result from commercial television programs or magazines where the emphasis is on scenery and drama rather than scientific goals.

Digital Media Services (DMS) is a unit within University Information Technology Services (UITS) at Indiana University. DMS provides premiere-quality digital media solutions including Macromedia Flash animations, World Wide Web site design, production and maintenance, Digital Video Production including acquisition and post-production using the next generation of video standards including High Definition Television (HDTV), high-quality 3-dimensional computer animation, interactive Digital Versatile Disk (DVD) production as well as duplication services for the various media.

DMS is especially well-positioned for producing materials for the educational outreach and visualization components of the IPTAI proposal. Producing both short-form and long-form video programming for documentary-style materials can be applied to a variety of purposes including broadcast documentaries, short video packages, provision of material for public information entities, use in museum and other various educational venues, interactive DVD and streaming media via the Internet.

Acquisition and Animation

DMS maintains the position that capturing the visual and aural experiences of any situation in the highest quality format allows for the material to be skillfully manipulated to fit a wide variety of specific informational, educational and display purposes. To accomplish this, DMS acquires motion content using advanced digital imaging cameras. Typically, content would be shot using 720p or 1080i High Definition Television equipment. DMS is currently evaluating a number of products for purchase including the Sony CineAlta and the Panasonic AJ-HDC27F. For shooting conditions which could be potentially hazardous, DMS will rely on cameras such as the AG-DVX100.

Digital Media Services utilizes several 3-dimensional computer animation packages including Alias/Wavefront Maya, Discreet 3D Studio Max and Softimage XSI. Besides utilizing professional university staff to produce 3D content, DMS also capitalizes on skilled students from such schools and departments as the IU School of Informatics and the IU Department of Telecommunications.

Compositing, Postproduction and Distribution

Digital Media Services employs several talented in-house digital artists to acquire still images and to manipulate those images along with motion content and graphics with a variety of tools including Adobe Photoshop, Adobe Illustrator and Pinnacle Commotion.

The Avid DS|HD High Definition Nonlinear Edit System is the postproduction tool of choice for Digital Media Services. The Avid DS|HD allows for total image and motion control, extensive layering and compositing and advanced audio mixing. Materials edited on the DS|HD allow the editor to create productions in full HDTV but also allows for the finished project to be exported in a variety of frame rates and resolution sizes including the current NTSC standard. In effect, productions are “future-

proofed" allowing for use with today's display and transmission standards as well as the standards which will be in place within the next few years.

Selected Production Tools
1) Rimage Autostar II 2)Sonic Scenarist 3)Pioneer PRV-9000 4)Pinnacle StreamGenie 5)Helix Producer 6)Discreet Cleaner 7) Nikon Super Coolscan 4000ED 8) Epson 1640XLGA 9) Olympus Camedia E-20 10) Panasonic DVC Pro Camera 11) Media 100 NLE 12) Avid DS|HD NLE 13) Avid Xpress DV NLE 14) HDTV Cameras (Product Review Under Way – Purchase by May 2003)

Digital Media Services distributes content using a wide variety of methods. Distribution modes include Real Media and Windows Media streaming via dedicated streaming servers maintained by Indiana University Digital Media Network Services, digital video tape, Betacam SP, CD-R and DVD duplication using the Rimage Autostar II with Everest printing. Productions can be made available on most professional broadcast media for over-the-air transmission.

World Wide Web and Interactive DVD

In addition to the video capabilities mentioned above, whether it is acquired in the field, studio or by purely electronic methods, content produced by Digital Media Services is ideally poised to export material to the World Wide Web using Macromedia Dreamweaver and Indiana University's robust web hosting environment and/or to interactive DVD using Sonic Scenarist.

Summary

By acquiring, compositing, editing and distributing content completely within the digital realm, content produced by Digital Media Services maintains the highest possible technical quality while ensuring compatibility with current and future technologies. This allows the content to be shareable, scalable and adaptable thus protecting the time and financial resources spent.

We anticipate writing media grants for supplemental funding to cover costs for production of a broadcast documentary in Years 4 and 5 of IPTAI. We estimate that production of a one-hour video including audio collection, audio mixing, video editing, and 3-D animations will cost about $120,000. Jasiak and Droppo (see CV's for non-funded collaborators) will take the lead on writing grants for video production if the IPTAI is funded.

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FACILITIES AND EQUIPMENT

Indiana University: Indiana University supports the IU Stable Isotope Research Facility (SIRF) by providing salary and benefits for Mr. Steve Studley (professional-rank technician). Additional technical expertise is provided by Mr. Jon Fong (lab technician), and Ms. Terry Stigall (electronics expert). Currently, SIRF maintains five isotope ratio mass spectrometers:

1. Finnigan 252 GC-C-IRMS (gas chromatograph-combustion-isotope ratio mass spectrometer)
2. Finnigan 252 dedicated for sulfur isotopic measurements, with dual-inlet, multiport inlet, continuous flow elemental analyzer, and laser fluorination system.
3. Nuclide 6-60 IRMS (soon to be retired)
4. Finnigan Delta E Mass Spectrometer
5. A new Finnigan Delta Plus with gas chromatography-combustion-elemental analyzer interfaces has recently been ordered as a result of the funding of an NSF equipment proposal. The new instrument will be dedicated to H and O isotopic analyses.
6. Finnigan MAT TSQ 700 (gas chromatograph-molecular mass spectrometer)
7. Perkin Elmer 5100 Atomic Adsorption Spectrophotometer with flame and graphite furnace capabilities as well as hydride generation
8. Jobin Yvon ICP JY-38

Indiana SIRF is one of the few laboratories in the U.S. with a modern isotope mass spectrometer (Finnigan MAT 252) dedicated to running sulfur samples. This instrument has an elemental analyzer (EA) inlet that is well calibrated for samples containing between 0.1 and 1 micromole of sulfur in the form of $BaSO_4$ or AgS. Our reputation for high accuracy and precision of sulfur isotope measurements using the EA is validated by numerous requests for access to the SIRF mass spectrometers. The institutional affiliation of our collaborations and contracts for $*^{34}S$ data over the past two years is as follows: Max Planck Institute for Marine Microbiology (Germany), Odense University (Denmark), University of Cincinnati, University of Missouri at Columbia, American Museum of Natural History, Harvard University, NASA Ames, Carnegie Geophysical Laboratory, Northern Illinois University, Kanazawa University (Japan), Iowa State University, University of Missouri, and Pennsylvania State University. In addition to routine $*^{34}S$ measurements, Ripley and Pratt are collaborating on the design and calibration of a laser-assisted fluorination chamber for determination of $*^{33}S$ and $*^{36}S$ on samples of AgS converted to SF_6. We currently are able to produce pure F_2 in our reaction line and we are able to detect all four sulfur isotopes in the form of SF_6. We anticipate having the fluorination chamber connected directly to the mass spectrometer via a cryogenic trap within two months. We have a waiting list of more than twenty researchers who have requested rare sulfur isotope measurements when the fluorination apparatus is completely installed. Many of our outside requests for rare isotope measurements are from members of currently funded Astrobiology Institutes.

Princeton University:

Laboratories**:** The Geomicrobiology lab resides in the basement of the department and houses fume hoods, crushing, grinding, powdering, and separation equipment. The lab also includes chemical stock room, two HEPA filtered hoods, UV sterilization hood, -80^{o}C freezer, -20^{o}C freezers, refrigerators, anaerobic glove bag, incubators, water baths, high temperature ovens, centrifuges, UV spectrometer, Robocycler for DNA amplification and electrophoretic gels. UV thermal printer is available in Ward lab for printing DNA gel runs. The lab's analytical facilities include Perkin Elmer ICP-AES, LICOR CO_2 analyzer and Dionex ICMS. The lab's optical microscopy and fluid inclusion section houses two microthermometry stages, cathodaluminocsope and two high quality optical microscopes. A digital, color CCD and analog B/W CCD camera and image analyzer is connected through the local computer network for uploading digital photomicrographs. The BMX60 microscope is equipped with epifluorescence imaging with UV cubes and filter sets appropriate for DNA and protein stains and FISH and phase contrast objectives. The department's machine shop resides on the same floor several lathes and drill presses included a fully-programmable lathe, which is used to manufacture our field sampling equipment. DNA sequencing facilities, FACScan flow cytometer, Molecular Dynamics Imager for mapping

radioactive samples and the laser confocal facility are readily available on the same floor in the Molecular Biology Department. The Geosciences X-ray diffraction lab resides on the same floor and houses two Scintag PAD spectrometers, one of which possesses a high temperature stage. The lab also makes use of CEBIT's ICP-MS also resides on same floor. The electron beam facility at the Princeton Materials Institute and houses a Phillips XL30 FEG SEM, a CAMECA SX50 EPMA, a Phillips CM200 FEG TEM, a Zeiss 910 TEM. GC-MS, HPMS and MALDI- TOF facilities for detailed organic characterization are available in the Mass Spectrometry Facility of the Chemistry Department.

University of Tennessee: The Center for Environmental Biotechnology at The University of Tennessee, Knoxville occupies 26,000 sq. ft. in the newly constructed Science and Engineering Research Facility on the main campus of the University of Tennessee, Knoxville and 8,000 sq. ft. at the Pellissippi Facility. CEB has skilled technical personnel and extensive capabilities for aerobic and anaerobic microbial cultivation, analytical and radiological methods used in microbial ecology, biodegradation, and molecular biology. This laboratory contains all the necessary basic equipment needed for microbial experiments including shakers, incubators, waterbaths, autoclaves, laminar flow hoods, laboratory and field anaerobic glove bags, liquid scintillation counters, centrifuges and spectrophotometers. The laboratory contains 8 capillary or packed column gas chromatographs equipped with detectors (TCD, FID, ECD, PID and GPC) and autosamplers, and a Hewlett Packard bench-top 6890 GC/MS and a VG Platform II HPLC electrospray, atmospheric pressure chemical ionization, GC/MS. The HP system is also configured with the Entech autosampler and serial preconcentrators for VOC's. Analytical computer systems are used for data management. Additional equipment available to the project include a Dionex ASE-200 Accelerated Solvent extractor with a 24 station autosampler, HPLC with fluorescence, UV-Vis, electrometric, conductivity, and radiochemical detectors, supercritical fluid capillary chromatograph, and Fowler cells. Microscopes include the Laser Precision Analytical triple-beam ATR/FTIR with an Analect IR Microscope, Zeiss Axioplan microscope, and a confocal microscope and Hamamatsu C4200-77 photon-detecting camera with the Argus-10 imaging system. In the molecular biology laboratory, a Perkin-Elmer-Cetus thermal cycler, bioimaging analyzer for gels (Alpha Innotech Image analyzer / densitometer the data from is Window 95/97 compatible), conventional gel electrophoresis units, "Fast-prep" bead beater (Bio 101) and a DGGE unit with a TGGE converter (BioRad), Beckman Oligo 1000 DNA Synthesizer, GSI Lumonics ScanArray 5000, Engineering Services, Inc. DNA Micro-Arrayer (SDDC), BioRad iCycler QPCR. The Department of Microbiology has a flow cytometer and the Department of Geological Science has an isotope ratio mass spectrometer. In addition, the University of Tennessee has a dedicated Molecular Biology Resource Facility (MBRF) with an Applied Biosystems 373A Automated Sequencer. The University also maintains the Biology Services Facility with machine and electronics fabrication/repair services.

Oak Ridge National Laboratory: *ORNL Environmental Sciences Division* has an extensive array of instrumentation.

Seafloor Process Simulator and Deep Subsurface Process Simulator. The high pressure biogeochemical laboratories contain tow major high pressure devices, a 72 liter 3000 psi seafloor process simulator and a <1 liter 5000 psi subsurface sediment analysis system. Both systems are computer instrumented and controlled and are in explosion proof vented facilities. All safety and health consideration are fully implemented and highly trained scientific personnel are operators. Facilities are used for geochemistry, biogeochemistry, isotopes studies, methane and carbon dioxide hydrates and supercritical carbon dioxide studies in complex native sedimentary materials.

Microbiology and Molecular Biology. The Microbiology Laboratory has extensive facilities for work with aerobic and anaerobic microorganisms. A variety of controlled temperature incubators and chambers, autoclaves, freeze-drying facilities, storage facilities, refrigeration, and a wide range of state-of-the-art analytical equipment are available. The laboratory contains sterile transfer hoods, autoclaves, spectrophotometers, gas chromatographs, automated plating equipment, automated bacterial identification systems, laser colony counting equipment, fume hoods for work with volatile contaminants, gassing stations for the preparation of anaerobic media, two anaerobic glovebags (Coy Laboratory, Inc.), electrolytic respirometers, flow cytometry, an automated GeneTACÔ colony picker (Genomic Solutions),

a robotic liquid handler (RoboSeqÒ 4204 SE), HiGro System (GeneMachines), and Bioflo 110 modular benchtop chemostats (New Brunswick). Trained laboratory technicians keep this equipment operational. The Molecular Laboratory also includes 24 PCR thermocyclers, an iCycler Thermal Cycler (Bio-Rad) for real-time quantitative PCR, an AKTA FPLC (Amersham Pharmacia Biotech) for protein purification, a denatured gradient gel electrophoresis system, controlled temperature water baths and incubators, several high-speed centrifuges, and numerous microcentrifuges, an electroporator, and a UV crosslinker for DNA hybridization studies. A variety of electrophoresis equipment, power supplies, Oriel photomultiplier with collimating beam probe and fluorescence spectrometer are available. UVP digital photograph systems equipped with CCD camera for image capture are also available.

Geochemistry Laboratory. The Mineral Characterization Laboratory contains a Scintag XDS 2000 X-ray diffractometer (XRD), a FTIR spectroscopy (Nicolet Magna-IR 760 spectrometer), an Atomic Force Microscope (Digital Instruments) and a JEOL JSM-35CF Scanning Electron Microscopy (SEM), utilizing both secondary electron imaging and backscattered electron imaging in conjunction with energy-dispersive and wavelength-dispersive x-ray spectroscopy. The ESD has laboratory facilities designed for the detection and quantification of the elements relevant to FRC research that are available for use by this project. There are dedicated gas chromatographs with TCD, FID, ECD and MS detectors and high-pressure liquid chromotographs (HPLC) for the analysis of organic compounds and dissolved gases. There are ion chromatographs (IC) equipped with conductivity and spectral array detectors for anion analysis and the detection of chelated metals; capillary electrophoresis equipment with Ultraviolet/Visible detectors for anion, cation, and chelated metal detection; atomic absorption spectrophotometers with graphite furnace for the analysis of metals and an inductively coupled argon plasma spectrophotometer for determination of metals and some nonmetals (*e.g.* S, P

University of Toronto: In the past decade, compound specific isotope analysis (CSIA) has revolutionized the application of stable isotopes to understanding biogeochemical cycling. Continuous flow analysis provides an increased sensitivity of analysis of 4-5 orders of magnitude and is the key to routine application of isotopic analysis to compounds present at the kinds of low levels common in the natural environment. The Stable Isotope Laboratory at the University of Toronto played a leading international role in the application of this new area of technology to dissolved organic contaminants in hydrogeological studies and to naturally occurring gases in deep groundwater systems. and scientific research. The laboratory is equipped with a high sensitivity Finnigan MAT 252 continuous flow gas source mass spectrometer for compound specific carbon isotope analysis and dual inlet CHON analysis. In addition in 1999 the laboratory installed one of the first Delta+-XL Finnigan MAT continuous flow mass spectrometers for compound specific hydrogen isotope analysis. The laboratory has extensive experience and well developed sample preparation systems (vaccum lines, carbonate prep systems, CHN sealed quartz combustion lines, Toepler gas transfer systems) for both gaseous and dissolved organic contaminants. A full time research associate (Dr. G. Lacrampe-Couloume) and part-time technician ensure the highest level of sample throughput, standardization and QAQC. In addition the laboratory hosts 5 gas chromatographs equipped with FID, TCD and micro-TCD detectors and cryogenic capabilities, laminar flow hoods and an anaerobic chamber. In addition to the facilities of stable isotope analysis, in cognant departments the University of Toronto houses abiotic and biotic microcosm facilities, microbiological growth chambers, GC and LC, and equipment for isolation and characterization of micro-organisms and microbial DNA and. Through Dr. Sherwood Lollar's status as Adjunct Professor in the Dept of Chemistry we also have access to the Environmental Analytical Chemistry "Analest" Facility incorporating a wide range of sophisticated chromatographic, spectroscopic and GC-mass spectrometry instrumentation.

Pacific Northwest National Laboratory: Since 1970, Pacific Northwest National Laboratory has been a leader in studies of subsurface microbiology. While, most of this work has been directed toward the resolution of environmental problems, a great deal of fundamental investigation has been conducted both on-site and off- by PNNL staff members. Bench to field-scale research activities have formed the basis for understanding the subsurface fate and transport of radionuclides, metals, anions and organic contaminants, as well as microbial community structure and the ecological energetics of the subsurface

biosphere. These activities have been sponsored by DOE, DOD, EPA, NSF and private industry.

Environmental Microbiology Laboratories. Environmental Microbiology Group maintains research facilities at PNNL and has extensive capabilities for studying aerobic and anaerobic microorganisms and the processes they catalyze. This equipment and instrumentation includes anaerobic glove bags, custom-mix gassing stations for the preparing anaerobic media and culturing fastidious microbes, incubators, bioreactors of various sizes and configurations, confocal laser scanning and epiflourescent microscopes, flow activated cell sorter, kinetic phosphoresence analyzer (for U), liquid scintillation counter, HPLC, GC, FPLC, and reduction gas analyzer. The group maintains fully-equipped molecular biological facilities including thermocyclers for PCR (including real-time Taqman PCR), pulse-field gel electrophoresis equipment, a Perkin Elmer model 373 automated DNA sequencer, and GeneScan software package for Terminal-RFLP analysis.

Diversa Corporation. Diversa possesses the technology to encapsulate cells in gel microdroplets, run the growth columns, and has four MoFlo Cytomation flow cytometers for analysis and sorting of GMDs. Diversa is a medium-sized company (ca. 400 employees) with cutting-edge molecular biology capabilities and technologies. Diversa has invested tens of millions of dollars in their high-throughput cultivation system, which is now being used in a high-throughput manner for small molecule discovery.

Lunar and Planetary Institute, Universities Space Research Institute:

The Lunar and Planetary Institute is a focus for academic participation in studies of the current state, evolution, and formation of the solar system. The LPI maintains a resident research staff of 20 scientists whose main tasks are to provide the planetary expertise necessary for the Institute to achieve its goals, and to maintain their scientific proficiency through peer-reviewed research activities. The Computing Center at LPI is built around a local area network that connects SUN Ultra SPARCstations, an 8 node Beowulf Linux Cluster and over 100 Pentium IV PCs and G4 Macintoshes with a combined online disk storage of over 1 terabyte. The network is connected to the Internet via a 3 Mbps ATM link.

The LPI's Center for Information and Research Services (CIRS) collection includes over 50,000 monographs, journals, slide sets, maps, videos, and CD-Roms. The subject emphasis of the collection is astronomy and geology, with limited collection development extending into secondary support fields of computer sciences and remote sensing.

As a NASA Regional Planetary Image Facility (RPIF), CIRS curates data and provides a facility for researchers to examine and interact with data from planetary missions. The RPIF data collections contain images, maps and CD-Roms that have been obtained from the Surveyor, Ranger, Lunar Orbiter, Apollo, Magellan, Galileo, Voyager, Viking, Clementine, Mars Pathfinder, and Mars Global Surveyor missions. CIRS is also a member of AMIGOS, a network of over 700 regional libraries that provides continuing education classes, support services, resource sharing, and access to the Online Computer Library Center (OCLC), an international network of over 40,000 libraries sharing resources, cataloging, and cooperative development work.

Lawrence Berkeley National Laboratory: **LBNL Center for Environmental Biotechnology Core Facility:** The core microbiology facility of LBNL is in the Center for Environmental Biotechnology, located in building 70 and 70A. The seven-laboratory unit occupies a total area of 2,950 square feet. The laboratories are set up for class II, type A/B3 molecular- and microbiology work. Level 1 quality and safety assurance procedures are in place. The following work-specific equipment and instruments are available in the laboratories:

- Lietz Laser Con Focal Microscope with digital imaging;
- Affymetriz Gene Microarray Processor 3000
- SterilGARD II 6-foot vertical laminar-flow, biological safety cabinet (Baker);
- 2 Avanti J-25 high performance centrifuge (Beckman);
- 2 Coy Anaerobic Chambers one double wide with microscope and incubator
- DU 640 UV/VIS scanning spectrophotometer (Beckman);
- Ultra-low temperature freezer (Revco);
- 2 Axioskop RLF for DIC, phase contrast, and epifluorescence with microphotography (Zeiss

- Integrated SpeedVac (Savant);
- GeneAmp PCR system 9600 (Perkin-Elmer);
- Expedite 8909 DNA synthesizer (PerSeptive Biosystems);
- Model 377 ABI Prism automated DNA sequencer (Perkin Elmer);
- CHEF DRII pulsed field electrophoresis equipment (Bio-Rad)
- MIDI identification system (Hewlett Packard);
- High sensitivity MSD mainframe for the HP 6890 GC (Hewlett Packard).
- BIOLOG microbial identification system (BIOLOG);
- Environmental shakers with photosynthetic light banks (New Brunswick);
- Alliance HPLC system with a 996 photodiodphotodiode array detector and a 474 scanning fluorescence detector (Waters).

The core microbiology facility preserves and maintains laboratory strains and wild-type strains isolated from environmental samples, microbial genomic DNA, plasmids and cloned genetic material. We also have 3 glove boxes and incubators that allow us to work with anaerobic microorganisms and microaerophiles.

VIMSS-GTL Experimental Core Facilities: The Environmental Molecular Microbiology Facility (ORNL, LBNL, U. Washington, Diversa Inc.), Environmental Simulation and Culture Facility (LBNL, U. Washington, Diversa Inc.). Technologies and facility resources are under development at LBNL and Diversa for culturing hard to grow organisms in controlled, reproducible environments. LBNL is also developing defensible environmental model chambers at different scales for better mimicking the natural environment of the microorganisms under study. These facilities will be fully integrated with the Advanced Light Source Microscope Beam Lines (1.4.3, 4.0.1-2, 6.1.2, 7.0.1, 10.3.1, 10.3.2) to take advantage of its unique analytical capabilities for environmental and biological samples, (infra red and x-rays); the Center for Isotope Geochemistry and its ability to analyze environmental samples for stable isotopes, (isoprobe); and the Center for Environmental Biotechnology with it's facilities for PLFA and nucleic acid analyses from environmental samples, soil columns, bioreactors, SLCM imaging, and biological safety level 2 laboratory. The LBNL National Center for Electron Microscopy will also enable other studies of environmental and biological specimens using state-of-the-science electron microscopes, (scanning transmission electron microscopy). This integration of unique instrumentation and facilities at LBNL with the VIMSS facilities at University of Washington for flow cytometry, bioreactors, functional microarray construction, and the Diversa environmental culture, isolation, archiving facilities, make this facility a unique and valuable resource for DOE. These facilities are also key to obtaining the highest quality and quantity of biological material for the other experimental facilities and research cores in VIMSS.