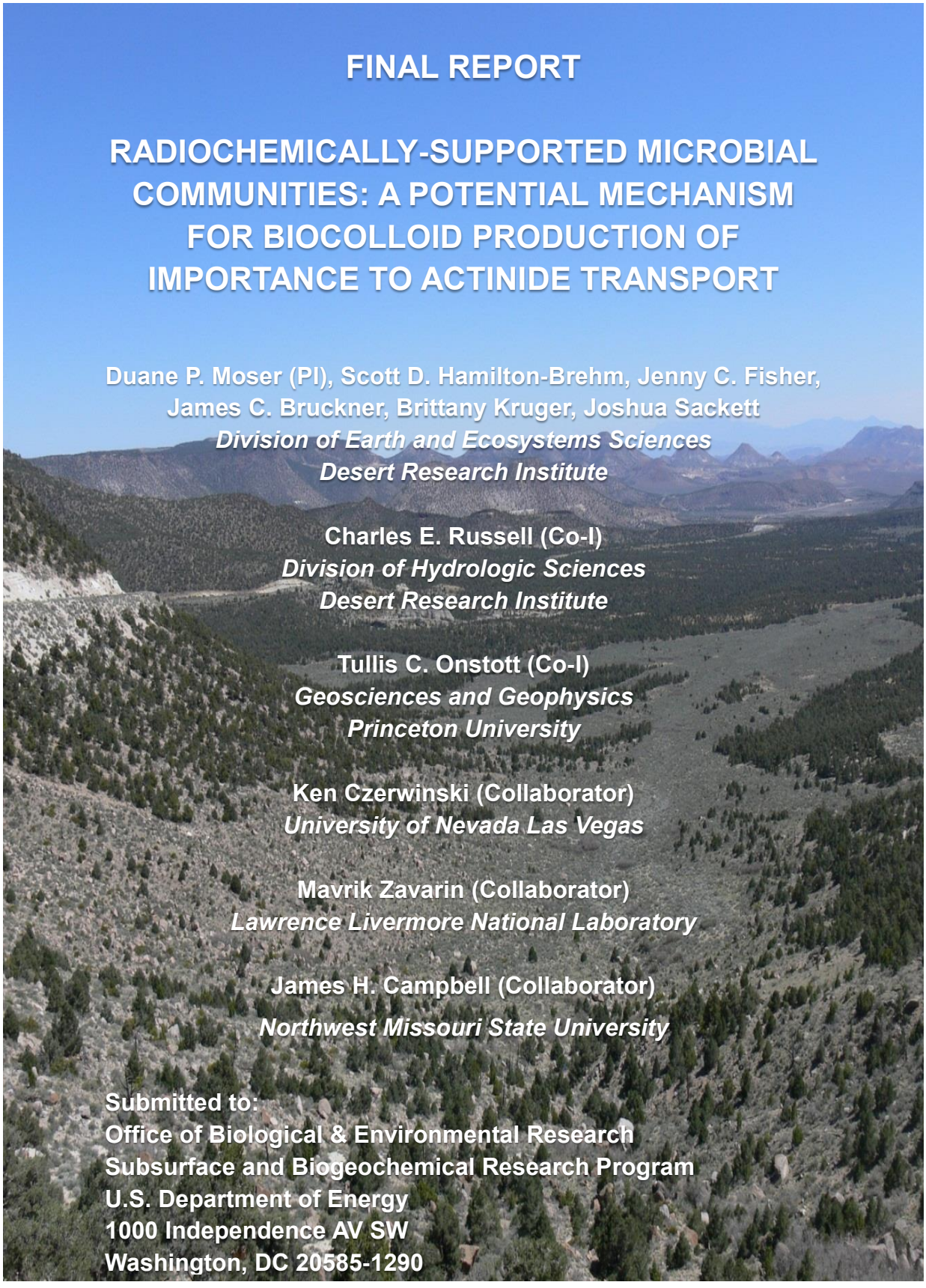

FINAL REPORT

RADIOCHEMICALLY-SUPPORTED MICROBIAL COMMUNITIES: A POTENTIAL MECHANISM FOR BIOCOLLOID PRODUCTION OF IMPORTANCE TO ACTINIDE TRANSPORT

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Submitted to:
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Subsurface and Biogeochemical Research Program
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LIST OF ACRONYMS

NNSA	National Nuclear Safety Administration
NNSS	Nevada National Security Site
NTTR	Nevada Test and Training Range
PCR	Polymerase Chain Reaction
ORF	Open Reading Frame
UGTA	Underground Test Area Activity
HRC	Harry Reid Center for Environmental Studies
LLNL	Lawrence Livermore National Laboratory
MEGA	Molecular Evolutionary Genetics Analysis
SBR	Subsurface Biogeochemical Research
SSU rRNA	Small Subunit of the Ribosomal RNA gene
UNLV	University of Nevada Las Vegas

03/20/2015

Cover Photo: View from West Pahute Mesa.

EXECUTIVE SUMMARY

Due to the legacy of Cold War nuclear weapons testing, the Nevada National Security Site (NNSS, formerly known as the Nevada Test Site (NTS)) contains millions of Curies of radioactive contamination. Presented here is a summary of the results of the first comprehensive study of subsurface microbial communities of radioactive and nonradioactive aquifers at this site. To achieve the objectives of this project, cooperative actions between the Desert Research Institute (DRI), the Nevada Field Office of the National Nuclear Security Administration (NNSA), the Underground Test Area Activity (UGTA), and contractors such as Navarro-Interra (NI), were required. Ultimately, fluids from 17 boreholes and two water-filled tunnels were sampled (sometimes on multiple occasions and from multiple depths) from the NNSS, the adjacent Nevada Test and Training Range (NTTR), and a reference hole in the Amargosa Valley near Death Valley. The sites sampled ranged from highly-radioactive nuclear device test cavities to uncontaminated perched and regional aquifers. Specific areas sampled included recharge, intermediate, and discharge zones of a 100,000-km^2 internally-draining province, known as the Death Valley Regional Flow System (DVRFS), which encompasses the entirety of the NNSS/NTTR and surrounding areas. Specific geological features sampled included: West Pahute and Ranier Mesas (recharge zone), Yucca and Frenchman Flats (transitional zone), and the Western edge of the Amargosa Valley near Death Valley (discharge zone).

The original overarching question underlying the proposal supporting this work was stated as: *Can radiochemically-produced substrates support indigenous microbial communities and subsequently stimulate biocolloid formation that can affect radionuclides in NNSS subsurface nuclear test/detonation sites?* Radioactive and non-radioactive groundwater samples were thus characterized for physical parameters, aqueous geochemistry, and microbial communities using both DNA- and cultivation-based tools in an effort to understand the drivers of microbial community structure (including radioactivity) and microbial interactions with select radionuclides and other factors across the range of habitats surveyed.

In response to greater than anticipated access to a large number of high-quality sites over the course of this project, a greater proportion of effort was ultimately placed on primary characterizations than was originally envisioned. As a result, DNA-based assessments of microbial community structure comprise the bulk of the work presented here. To complete these characterizations, two complementary 16S rRNA gene-based methodologies were used to capture both short-read (Illumina) and full-length-gene (PCR-amplified clone libraries) surveys of microbial community structure. Extracted DNA from subsurface fluids, using next generation sequencing on the MiSeq platform, produced 1.3 million reads of about 300 nucleotide in length. The same extractions were also used in polymerase chain reactions (PCR) to amplify bacterial and archaeal 16S rRNA genes for the construction of clone libraries from the 19 sites to generate 1,654 full-length and 886 partial sequences. These overlapping datasets enabled a

discrete cataloguing of major and minor phylogenic microbial groups present in a loose transect of wells across the entire length of the DVRFS. To the best of our knowledge, this is the first detailed survey of microbial diversity of a fractured rock flow system of this geographic extent. As detailed in our report, microbial communities were diverse and diverged from site to site. Overall however, bacterial phyla detected by both approaches across the aggregate dataset, revealed an average composition of 52% Proteobacteria, 10% Firmicutes, and 13% Bacteroidetes; and lesser proportions of other groups, including many to-date-undescribed phyla (e.g. so-called "microbial dark matter" lineages). In contrast, the archaeal libraries were much simpler in composition and dominated by recently-recognized phyla such as the Thaumarchaeota (average 55%) and Parvarchaea (average 10%).

Although most of the radionuclide inventory at NNSS is in the form of tritium (H_3), significant quantities of actinides, especially isotopes of Pu, also exist. The tendency of actinides to sorb to mineral surfaces and potential biocolloids was examined. These biocolloids span several orders of magnitude in size: microbial cells (~1 µm), cell exudates (from 0.1-1 µm), and exopolysaccharides (EPS; 0.001-0.1 µm). An initial set of experiments indicated that sorption of actinides to volcanic tuff may be enhanced by the presence of bacterial cells. These experiments also illustrated the propensity of living cells to sorb significant amounts of U-233 in the absence of tuff, indicating that particle-attached and free-living cells may also impact the transport of actinides in this environment. The type(s) of bacteria present, their growth phase, and attachment status all affected actinide mobility. The role of microbial exudates in this process was confirmed with dialysis experiments, which compared the diffusion of Pu-239(IV) across a 3-kDa membrane in the presence and absence of EPS preparations from an NNSS *Pseudomonas* spp. isolate.

For a number of sites, molecular ecological approaches were supplemented with traditional cultivation-based assessments of microbial diversity, targeting dominant physiotypes predicted by aqueous geochemistry. Specific media formulations were modeled after NNSS groundwater composition to culture and quantify a range of microbial physiotypes, and 40 pure isolates from the site are archived (-80°C in glycerol) and available for future study. Two of these isolates, currently designated as strains DRI-13 and DRI-14, have been extensively characterized. Both were cultured from the anoxic well U-3cn#5, which is a far-field well associated with post-shot monitoring of the "Bilby" nuclear test. This well penetrated the regional carbonate aquifer below the detonation cavity. These strains are distantly related to cultivated microorganisms. DRI-14 is most closely related (~91%) to *Candidatus Desulforudis audaxviator* and, to the best of our knowledge, represents its closest cultivated relative. Bacterial 16S rRNA gene libraries revealed the presence of closer relatives of *C. D. audaxviator* in five boreholes and two tunnels across our sample set. This microorganism is best known as an inhabitant of the very deep terrestrial and possibly marine subsurface. It is thought to subsist on H_2 and SO_4^{2-} produced by radiochemical

reactions independent of solar input. Its detection in preliminary data from several NNSS boreholes led to the hypothesis that radiochemically-produced substrates might stimulate the production of microbial biomass and was a justification for this study. The genomes of both DRI-13 and DRI-14 have been sequenced and, as of this writing, assembly/annotation are nearly completed. The metabolisms of these microorganisms differ, with DRI-13 being a fumarate fermenter, whereas DRI-14 is a chemolithoautotroph that appears to couple methane oxidation directly to sulfate reduction (the first bacterium known to mediate this process).

KEY FINDINGS

- Bacterial and archaeal populations were detected in 19 boreholes accessing portions of the Death Valley Regional Flow system. Patterns of microbial diversity corresponded to general position in the regional flow system (e.g. recharge zone vs discharge zone) and host lithology.

- Sorption of actinides to NNSS mineral surfaces (e.g. volcanic tuff) was enhanced by the presence of bacterial cells. Microorganisms and their exudates also significantly sorbed U and Pu in the absence of tuff, indicating that particle-attached and free-living cells may impact the mobility of actinides in this environment.

- Bacterial and archaeal populations demonstrated a multivariate response to ecological variables: primarily temperature for bacteria and potassium/pH/temperature for archaea.

- 16S rRNA gene library data revealed that bacterial and archaeal populations were especially responsive to dissolved oxygen (dO_2), with anoxic and high-temperature samples reflecting dominance by predicted thermophilic and/or methanogenic archaea and bacterial Firmicutes.

- Across the sample set, most sites revealed significant concentrations of dO_2, even at great depth, and in these locations, archaeal populations were dominated by Thaumachaeota ($\bar{x}$ = 64.4%).

- Radioactivity was not a major driver of microbial density or diversity. The bacterium *Candidatus Desulforudis audaxviator*, while present at multiple locations, was not predicted by radioactivity and its highest density was recorded at a non-radioactive control site.

- A collection of 40 pure microbial isolates of a variety of physiotypes (including copious exopolysaccharide production) was obtained.

- Two novel bacterial strains from the deep carbonate aquifer, DRI-13 and DRI-14, have been extensively characterized. DRI-13, a fumarate fermenter, likely represents a new bacterial genus. DRI-14 showed 91% 16S rRNA gene identity to *C. Desulforudis audaxviator* and appears to represent the first bacterium able to grow by anaerobic methane oxidation coupled to sulfate reduction and likely represents a new bacterial family.

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1. INTRODUCTION

A total of 2,036 nuclear tests have been conducted at various locations around the world since 1945; with 942 of these being carried out by the United States (1). Although US nuclear tests were conducted at a variety of locations and for a range of purposes, the vast majority were carried out at a single facility, the Nevada National Security Site (NNSS, formerly known as the Nevada Test Site (NTS)). The NNSS is located in Nye County in southern Nevada; the southernmost point of which is about 105 km northwest of Las Vegas. The site contains 3,500 km^2 of federally-administered land with restricted access and varies from 45 - 56 km in width (east-west) and 64 - 88 km in length (north-south). Although nuclear tests at the NNSS were initially conducted above-ground, following the implementation of the Limited Test Ban Treaty in 1963, which prohibited nuclear tests in the atmosphere, underwater, and in space; all testing at the NNSS has been underground. From 1951 to 1992, 828 underground nuclear detonations were conducted at the NNSS (2). The resulting radionuclide contamination, over a range of rock types, temperatures, redox chemistries, and hydrologic conditions represents a unique opportunity for the study of subsurface microbial responses to radioactivity and potentially their involvement of transport of legacy wastes. These factors informed the initial motivation this project and an earlier Environmental Remediation Sciences (ERSP) Exploratory project entitled: *Characterization of Microbial Communities in Subsurface Nuclear Blast Cavities of the Nevada Test Site* (FG02-07ER64406).

At the NNSS, the Underground Test Area (UGTA) Sub-Activity is an ongoing environmental management project tasked with addressing groundwater contamination associated with underground nuclear testing at the NNSS. The US Department of Energy (USDOE or DOE), National Nuclear Security Administration (NNSA), and Nevada Field Office (NFO) have oversight responsibility for this project. Despite the large radioactive inventory (calculated total of 1.321×10^8 Ci) for the six principle geographic test centers at NNSS (Frenchman Flat, Pahute Mesa, Ranier Mesa, Shoshone Mountain, and Yucca Flat), the majority of the underground environment on and around NNSS remains primarily uncontaminated (3). However, recently traces of radionuclide contamination were detected in groundwater off the NNSS proper (on the Nevada Test and Training Range, NTTR (3)). The majority of samples (e.g. 16 out of 24) collected for this work contained either no detectable tritium or were below the regulated threshold of 20,000 pCi/L (3). Thus, the groundwater samples upon which our project was developed cover the spectrum from pristine to severely-impacted (literally, 10s to 10s of millions of pCi/L tritium activity).

Radioactive sites, when available, remained a priority for our work. Whereas, numerous microbiological characterizations of nuclear material production and ore processing sites (e.g. Hanford in Washington State (4-6), Oak Ridge, TN (7-10), and Rifle, CO (11-14) have been conducted, very little work has been done on weapons testing sites, either in the US or in the former Soviet Union. NNSS is

unique in that, unlike at production sites, underground nuclear testing initially sterilizes the local subsurface environment by the extremely high temperatures and pressures (15). Although a large amount of research and monitoring effort has been dedicated to characterization of the hydrology and geochemistry of subsurface systems at the NNSS (16-21), prior to our study, microbiological investigations have not been a priority. It should be noted, however, that a number of pioneering studies of hard rock microbiology were conducted at the NNSS by the US DOE Subsurface Microbiology Program in the 1980s and 1990s (22-27), especially at Ranier Mesa. However, none of these systematically targeted radioactive samples nor could they incorporate the advanced DNA-based technologies of today. The challenges for microbial life in these environments range from high-fluxes of ionizing radiation, persistently high temperatures, and toxic (e.g. Pb from vaporized instrument rack shielding), and/or radioactive materials (e.g. Pu). Although, at the beginning of this project it was unknown whether or not microorganisms existed in the most radioactive of our study sites, results from the DOE Hanford Site (6), for example, indicating up to ~10^4 culturable microorganisms per g in highly radioactive sediments led us to predict that life would be present. Hence, one goal of this study was to determine if microbial community structure varied in response to the radioactive environment.

This project, like most that have examined life in the continental deep subsurface (28-30), "piggy backed" upon activities conducted for other purposes (e.g. mining and drilling). In this case, both were relevant. Two sampling opportunities, associated with the U12n tunnel complex, were collected from groundwater that had accumulated within a hydrologically-isolated mine. All of the others were from deep sentinel monitoring boreholes/wells. As anticipated during the preparation of our proposal, all of our relevant sampling opportunities were of groundwater. In no case were we able to obtain contaminated rock (e.g. nuclear melt glass) or fresh core material, and thus, solid samples were not pursued. Our practical focus on groundwater was and remains justified based upon the coincident focus on this mobile milieu by site managers. Although airborne transport of radioactive materials remains a possibility at NNSS, extensive monitoring for atmospheric contamination by the Community Environmental Monitoring Program (CEMP, http://www.cemp.dri.edu), has failed to detect any locally-sourced contamination in decades.

Although, the interactions between anthropogenic radioactive contamination and life were a theme throughout this work, the project also matured, in its own right, into one of the most comprehensive spatial assessments of microbial life in deep fractured rock environments ever undertaken. The exceptional suitability of this area for deep biosphere research was augmented by both the nature of the local geology and availability of numerous well-characterized windows into it (e.g. wells).

The NNSS and much of the NTTR are situated within an internally-draining sub-basin of the Basin and Range physiographic province known as the Death Valley Regional Flow System (DVRFS). The DVRFS is a deep, fracture-controlled aquifer that encompasses ~100,000 km^2 of mountain ranges (up to 3,600 m above sea level) and valleys, which can reach below sea level (e.g. Death Valley, at -86 m, the lowest point in North America (31)). Climate across the DVRFS is controlled by altitude: with the highest peaks receiving ~100 cm/y of precipitation and Death Valley, the hottest place on Earth (32) and driest place in North America, a mere ~4 cm/y (33). DVRFS geology is both active (Nevada is the US' 3rd most seismically-active state (34)) and complex; recording tectonic and sedimentary (marine and continental), metamorphic, and intrusive igneous histories from over 2 Ga. Geologically-significant events in the history of the area include: 1) formation of thick Paleozoic (~550 Ma) marine carbonates that underlie most of the DVRFS and much of the NNSS, 2) Tectonic contraction resulting in the development of multiple thrust plates, 3) extrusion of a broad Tertiary (23 – 2.6 Ma) volcanic plateau which comprises much of the NNSS/NTTR, and 4) crustal extension, from ~16 Ma to present, that created the Basin and Range and Death Valley (~2 – 3 Ma, (35)). This region has thin crust (e.g. ~17 – 25 km under Death Valley (36)) and is extensively faulted (e.g. typified by horst/graben structures). Valleys (grabens), such as Yucca and Frenchman Flat on the NNSS, are partially filled with sediment (37).

North-South trending fault networks of the DVRFS are thought to facilitate groundwater flow over long distances; from montane recharge areas (e.g. Pahute and Ranier Mesas) to large-discharge springs on the valley floors, some as far afield as Death- and Amargosa Valleys. This so-called "interbasin flow" concept enjoys a great deal of support (38-41), yet remains controversial (42-45). As a result of societal demand for water and DOE activities related to legacy waste management, the deep regional hydrology of the DVRFS is possibly the best understood in the world (Wayne Belcher, US Geological Survey (USGS), pers. comm.), and 3-D groundwater flow models of ever-increasing sophistication are being developed by the USGS and DOE. A comprehensive DVRFS hydrologic model, with 1,500 m grid cell spacing (194 rows, 160 columns and 16 layers, surface to 6 km below sea level), already exists and is being continuously updated by the USGS. This model incorporates several embedded models, including equally comprehensive 50-m-resolution Yucca Flat and Southern Amargosa eMbedded Models (SAMM).

Attempts to model the sorptive behavior of radionuclides at the NNSS are difficult because the geo-hydrologic setting of each nuclear test is unique; with a distinct chemical composition and microbial community. Groundwater samples collected from NNSS wells vary widely in pH, temperature, dissolved oxygen concentration, alkalinity, and radioactivity. The fact that bacteria may be present in fairly high numbers, possibly up to 10^7 cells/mL (this work), in NNSS subsurface fluids, suggests a possible role in controlling the mobility of redox-active radionuclides. Over the past several decades, our understanding of the

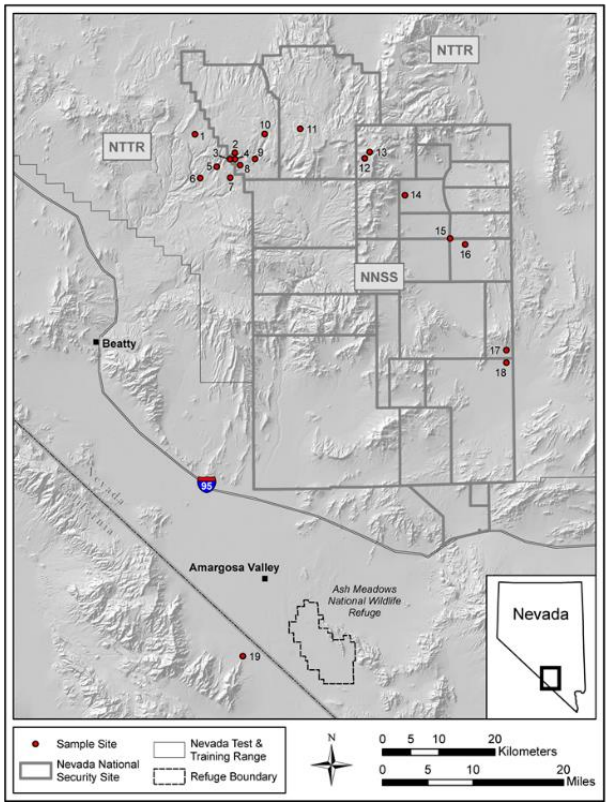
ability of microorganisms to mediate redox transformations of radionuclides has significantly expanded. Microorganisms are currently known to reduce or sequester U (46-50), Tc (51-53), Pu (54, 55), Am (56), and Np (57). Of these, U, Pu, and Tc are less mobile when reduced, and all can be reduced and/or oxidized by microorganisms (48, 58-63). Microorganisms are well known to mediate the biosorption and precipitation of transuranic elements (64-69). However, few studies have addressed the additive or subtractive effect of bacteria on the sorption of radionuclides to environmental substrates. The major published study to consider a component additivity approach to bacterial sorption (70, 71) examined the effects of a single species of Gram-positive bacteria on U sorption to kaolinite clays. In this study, we compared the sorption kinetics and distribution ratios (Kds) of uranium vs. tuff samples from the NNSS subsurface in the presence and absence of bacteria (both isolates and endemic bacteria present in NNSS water samples). The major objective of this portion of the work was to determine if, and to what extent, microbial communities might affect the sorption of U and PU, with the aim of enabling the incorporation of this information into future models.

Our sorption/desorption studies showed that more U bound to bacteria than to tuff based on weight in artificial carbonate free groundwater (Section 3.5). The presence of viable bacteria also slightly enhanced sorption to tuff, but was reversible upon cell death. Experiments with filtered and unfiltered water collected from the NNSS subsurface and water with added bicarbonate showed significantly-reduced sorption of U for [HCO_3] ≥ 1 mM, likely due to the formation of highly soluble uranyl-carbonate complexes.

2. MATERIALS and METHODS

2.1. FIELD SITES

The UGTA Sub-Activity is an ongoing environmental management activity tasked with addressing groundwater contamination associated with underground nuclear testing at the NNSS. The DOE, NNSA, and NFO have oversight responsibility for this project. As a result, project health and safety requirements (e.g. Field Activity Work Packages (FAWP), Radiological Work Permits, (RWP) and chain of custody documentation were integrated into ongoing operations of preexisting NNSS activities. In total, 17 wells, sampled from FY2008 to FY2014, are considered in this report (Fig. 1). With the exception of two samplings of accumulated mine water conducted via mountaintop ventilation holes (U12n.Vent#2 and U12n.10), all samples were borehole fluids. Specific circumstances of individual well sampling events varied (Table 1). Primarily, samples were collected from flowing sample lines at the end of multi-day hydrologic pumping tests or were obtained from static water columns down hole



Sample Name	Key Code
PM 3-1 & 2	1
ER 20-7	2
ER 20-11	3
ER-EC-11	4
ER-EC-15 (Up/Mid/Lo)	5
ER-EC-13 (Up/Lo)	6
ER-EC-12	7
ER 20-8 (Up/Lo)	8
ER 20-4	9
UE-20N#1	10
U19V PS Almendro	11
U12n.Vent#2	12
U12n.10	13
UE-2ce Nash	14
UE-3E#4 Aleman	15
U-3cn#5 Bilby	16
ER 11-2	17
ER 5-5	18
BLM-1	19

Fig. 1. Locations of sampled boreholes and tunnels employed for this project. Corresponding key code numbering shown here is used throughout document. (Map image by Cheryl Collins, DRI).

via discrete sampler deployments (e.g. "bailers"). The sampling of sites on the NTTR was limited to West Pahute Mesa; whereas, the NNSS work spanned Pahute Mesa, Ranier Mesa, Yucca Flat, and Frenchman Flat. The down-gradient reference site, BLM-1, located on BLM land on the Eastern flank of the Funeral Mountains in the Armargosa Valley (Fig. 1), was drilled as part of the Yucca Mountain Project by an outside contractor (The Hydrodynamics Group, Ltd., Redmond, WA) and was sampled at the end of a 48-hour hydrologic pumping test (200 gallons (757 L) per minute) conducted by the Nye County Nuclear Waste Repository Program Office (NWRPO). Coordinates for each of the locations can be found in Table 1.

2.2. SAMPLE COLLECTION and PROCESSING

Physical Parameters and Sample Collection. Field operations at NNSS were photographically recorded under an UGTA camera permit issued to D.P.M.; whereas, NTTR sampling operations were not photographed. Field measurements of temperature, dissolved O_2 (dO_2), conductivity, and pH were made onsite, generally with a YSI sonde fitted with a flow cell (Yellow Springs Instruments, Yellow Spring, OH). In cases were a sonde was not available, onsite

measurements were conducted by the lead contractor, generally Navarro-Interra (NI), either via their own sonde or in-line sensors applied to water being discharged during pumping tests. In some cases, CHEMets ampuoles (CHEMetrics, Midland, VA) were used to obtain dO_2, iron, ammonia, nitrate, nitrite, and sulfide measurements onsite.

Depending on radiochemical properties from the well environment, microbial samples for DNA extraction from planktonic cells were collected, either by filtration at the wellhead (>100 L, Sterivex housings, EMD Millipore, Bedford MA) or later in the laboratory. Between three (early in the project and for radioactive samples) and eight individual filters were collected at each site. For pumped samples, a custom, valved copper manifold was secured to a GHT-threaded hose bib provided by the site contractor for sampling at the wellhead. For DNA-based analyses, pumped well fluids at about 55 kPa (8 psi) were directed through 0.22 µm Sterivex filters throughout the roughly eight-hour onsite sampling operation. Filters were covered with a tarp for protection from sunlight and flow was recorded. For radioactive or bailer-sampled wells, sterile 20-L Nalgene polypropylene carboys were filled onsite and filtered in the LLNL laboratory in Mercury, NV (typically within 4 hours of sampling) using 0.22 µm Sterivex filters under an air-pressurized headspace (72-74). After removal of residual water with filtered air, filters were stored on dry ice for transport to DRI, followed by storage at -80^{o}C. Many of these filters remain archived and available for additional study.

Samples for gas analysis were obtained using 500-mL, gas-tight Magnum Syringes (VICI, Baton Rouge, LA), preloaded with 100 mL of 99.99% pure argon (Air Liquide, Los Angeles, CA). Syringes were attached to a stainless steel manifold, constructed with Swage fittings, at the wellhead and 400 mL of sample was drawn into the syringe. Dissolved gases were then allowed to equilibrate with the argon (~16 hours, heating if necessary to match sample environmental conditions). The argon with entrained sample gas was then transferred from the Magnum syringe using a 22-gauge needle and gastight syringe into pre-evacuated 15-mL vials (over-pressurized to ~2 atm), and stored at room temp (~25°C). Sample vials were sent to Princeton University for gas analysis in the laboratory of T.C.O.

Biologically-viable water samples were collected by filling a 95% v/v ethanol-sterilized 500 mL Magnum Syringe with sample, followed by transportation on wet ice or, in the early days of the project, directly by filling of N-flushed, evacuated 160 mL serum vials off flowing sample lines as in (72). Once at DRI, the syringe samples were dispensed into sterile evacuated 60 mL serum bottles and stored refrigerated. Planktonic cells from water samples were collected in 50 mL polypropylene centrifuge tubes and counted by flow cytometry (MicroPro, Becton Dickinson) according to manufacturer's protocols within 48 h of collection. For this sample set, two separate assays were conducted: total cells (so-called "Biomass Assay"), and viable cells ("Total Viable Organisms = TVO"). Prior to

injection onto the flow cytometer, water samples were pre-filtered with 40-micron nylon cell strainers (Part no. 352340, BD, Becton Dickinson).

Samples for ions, nutrients, total organic carbin (TOC), TIC, tritium, conductivity, and turbidity were generally taken by UGTA or LLNL, subject to their QA/QC protocols and chain of custody systems, and analyzed by them or via third party vendors. The results of these measurements have generally been made available to us, some of which are reported in Table 1. After the passage of at least 1 L of sample, filtrate from the Sterivex filters was collected into new 60 mL VOA vials for organic C determinations and new 60 mL polypropylene Nalgene bottles, soaked overnight in nanopure water, for nutrient chemistry. Unfiltered water was collected for TOC. Samples were stored at 4^{o}C until needed.

2.3. ANALYTICS

Supplemental chemistry analysis for samples not processed as part of the UGTA pipeline were performed at the DRI Water Analysis Laboratory (Reno, NV) or at Princeton University (T.C.O.). At DRI, for nitrite, nitrate and ammonium, Alpkem RFA 300 and Technicon Automated Colorimetric Analyzers were used. Dionex Model ICS 2000 Ion Chromatograph was used for the measurement of Cl^-, Br^-, and SO_4^{2-}. A Brinkmann Metrohm Titrando Automated Titrator, capable of potentiometric titrations to fix inflection end points, was used in the determination of CO_3^{2-}, HCO_3^-, pH, EC, and F^-. An OI Analytical 1030W Carbon Analyzer was used for determination of dissolved organic carbon (DOC) and TOC in water samples. A Thermo Elemental SOLAAR M5 Atomic Absorption Spectrometer with air-acetylene flame and vapor generation capabilities was used for major cation and many metal analyses. A Thermo IRIS Intrepid Inductively Coupled Plasma Emission Spectrometer (ICP-AES) complements the capabilities of the atomic absorption spectrometer and permits lower detection limits for many trace metals.

As noted above, a subset of samples were analyzed in duplicate for anions (F^-, Cl^-, NO^{2-}, SO_4^{2-}, Br^-, NO_3^-, PO_4^{2-}) and for short chain fatty acids (acetate, lactate, formate, and propanoate) at Princeton University using a Dionex IC25 ion chromatograph (Thermo Scientific, MA) coupled to an MSQ Plus ESI-quadrupole mass spectrometer (Thermo Scientific, MA) (75).

2.4. MOLECULAR MICROBIOLOGICAL DIVERSITY ASSESSMENT

Molecular Methods. Nucleic acids were extracted from the filters at DRI or the Harry Reid Center (HRC) Radiochemistry Laboratory at UNLV according to manufacturer's protocol using the UltraClean™ Soil DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA), amended with three freeze/thaw cycles (-80°C/65°C; 20 minutes each) at the beginning of the extraction procedure. Filters from radioactive sites were processed at the HRC before being screened for radioactivity by scintillation (all of the samples were below detection limits post-

processing) and then transferred to the DRI for subsequent molecular characterization.

For PCR-amplified clone libraries, universal bacterial primers 27F-YM/1492R (AGAGTTTGATYMTGGCTCAG/TACCTTGTTACGACTT) and universal archeal primers were used 21F/1492R (TTCCGGTTGATCCYGCCGGA/ TACCTTGTTACGACTT) to generate full-length 16S rRNA gene amplicons (76). PCR was performed using LATaq (Clontech, Mountain View, CA) using thermocycler settings of 95^{o}C, 30 s; 53^{o}C, 30 s; 72^{o}C, 60 s; for 30 cycles. Amplicons were purified with an UltraClean GelSpin DNA Purification Kit (Mo Bio Laboratories Inc., CA). PCR amplicons were cloned using a TOPO-TA kit (Invitrogen, CA). Clones were selected to fill a 96-well plate and fully- or partially sequenced by Functional BioSciences (Madison, WI) using vector primers. Contigs were generated using Sequencher™ 4.9 (Gene Codes, Ann Arbor, MI), aligned, matched with nearest neighbors and checked for chimeras using Greengenes (77) or DECIPHER (78). Alignments were further refined and phylogenetic relationships determined using MEGA (79). Quantitative Insights Into Microbial Ecology (QIIME) version 1.8.0 software was used to form operational taxonomic units (OTUs) from clone library sequences and assign putative taxonomic identification to each OTU (80). OTUs were picked using the 'pick_otus.py' command using default parameters. One representative sequence per OTU was chosen using the 'pick_rep_set.py' command, following default parameters. This enabled the removal of duplicate sequences matching to the same OTU, drastically decreasing the computational time and effort required to process the data while accounting for the number of sequences captured when the representative set was chosen. Taxonomy was assigned to each representative sequence using the 'assign_taxonomy.py' command, using default parameters. This command compared each representative sequence against the August 2013 GreenGenes (77) database to determine sequence identity. Taxonomic assignments were then used for downstream analysis.

Extracted DNA from the filters was also prepared for high-throughput sequencing using the Illumina MiSeq platform. Amplification of the V4 region of the 16S rRNA genes of Bacteria and Archaea was performed as previously described (84), with the following modification to increase our coverage of the Archaea from 53.6% of known near-full-length Archaeal 16S rRNA genes to greater than 89% (81, 82): the cytosine at position 4 of the forward primer 515F (5'-GTGCCAGCMGCCGCGGTAA-3') was changed to Y (either pyrimidine [C or T]). A corresponding modification was made to the primer used for the first sequencing primer. HotMasterMix DNA polymerase (5 Prime Inc., Gaithersburg, MD) was used in a 33-cycle PCR to produce amplicon libraries, and sequencing was performed by MicroSeq Enterprises (Las Vegas, NV).

Illumina sequence data were processed in mothur v1.33.3 (83) using a modification of the pipeline presented by Kozich et al. (84) at the "classify.seqs" command. At this step, we used the Greengenes (P. Schloss, personal

communication) database (77) and a 50% cutoff (reference=gg_13_5_99.fasta, taxonomy=gg_13_5_99.pds.tax, cutoff=50) for improved taxonomic assignment of sequences belonging to unclassified prokaryotic groups. OTUs were defined at a genetic distance of 0.03, and any OTUs that could not be taxonomically classified were curated manually. Finally, singleton OTUs were removed from downstream analyses (85). All mothur commands were run in batch mode on the Stampede supercomputer at the University of Texas Austin (https://www.tacc.utexas.edu/stampede/) under an allocation from XSEDE (https://www.xsede.org/).

Sample-to-sample comparison of whole communities sequenced in Illumina libraries was performed in the PRIMER-E environment (86) using the same statistical pipeline for OTUs of both bacteria and archaea. Counts of sequences within each OTU were normalized to percentages within each sample to nullify effects of unequal sequence numbers within sample. Percentages were square-root transformed to down-weight the effects of exceptionally abundant OTUs. Bray-Curtis resemblance matrices were then calculated from square-root transformed percentages. Bray-Curtis matrices were visualized using Nonmetric Multidimensional Scaling (NMDS; two-dimensional) and hierarchical clustering (average-neighbor algorithm).

Bacterial or archaeal communities detected in each sample were compared with selected water chemistry parameters using the PERMANOVA+ add-on package (87) of PRIMER-E. The Distance-Based Linear Model (DistLM) algorithm was used as a statistical test of effect of each water-chemistry measurement on microbial assemblages. Multi-collinearity of variables was assessed (87) using Draftsman plots, but was not detected. The statistical significance (p-value) of effects of each water-chemistry variable was tested under a randomly permuted (n=9999 permutations) null model. The proportion of variation explained by individual variables (prop. values) was calculated using DistLM. Models were selected based upon combinations of measured variables producing the "Best" selection procedure for fit of community data as evaluated using an adjusted R-squared value (R_2^a). Lastly, ordinations of bacterial or archaeal communities constrained by water chemistry and were visualized using a Distance-Based Redundancy Analysis (dbRDA).

Primers were designed for the 16S rRNA gene from bacterium, *Candidatus D. audaxviator* using the online website, Primer3 (88). The forward primer was designated F484 and the reverse R876 (GGGTTGACGGTACCTCAGGAATAAG/ GGTTACCTACGGCACTGAAGGGTAT). The expected amplicon resulting from these newly designed primers is 392 bp.

2.5. CULTIVATION EXPERIMENTS

Cultivation. Unfiltered well or tunnel water samples in evacuated crimp-sealed 60 mL serum vials were used for enrichment cultivations. Enrichment cultures

were decimally diluted (1 mL sample/9 mL diluent x 5 – 6 rounds each) into pre-made, sealed Balch tubes containing non-radioactive microbiological growth media. Each sample was diluted into a minimum of five different microbial growth media prepared to resemble NNSS groundwater in terms of major ions and pH (89). Media used included formulations for aerobic heterotrophy and anaerobic iron/sulfate/nitrate reduction and fermentation. Isolates were generated by plating enrichments onto the equivalent substrate in 1.5% agar plates. Colonies were picked and transferred back to liquid media. When visible turbidity was achieved, DNA was extracted using an UltraClean Microbial DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA) and amplified with universal bacterial primers and LATaq (Clontech, Mountain View, CA) using thermocycler settings of 95°C, 30 s; 53°C, 30 s; 72°C, 60 s; for 35 cycles. Amplicons were sent to Functional Biosciences (Madison, WI) for Sanger sequencing of the 16S rRNA gene using bacterial primer sets. Isolates were stored in a medium buffered with 20% glycerol solution at -80°C.

Enrichments for obligate anaerobes were performed in 160-mL serum bottles with 25 mL of "Subsurface Microbe Cultivation" (SMC) medium: 3.6 g/L 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 284 mg/L Na_2SO_4, 174.2 mg/L K_2PO_4, 0.3/L resazurin, 400 mg/L $MgCl_2$•6 H_2O, 500 mg/L KCl, 268 mg/L NH_4Cl, 250 mg/L $NaHCO_3$, 1 mL/L ATCC Minimal Vitamins (ATCC, VA), and 1 mL/L ATCC Minimal Minerals. Media were prepared anaerobically using the Hungate technique modified with a lower concentration (600 mg/L) of Na_2S•$9H_20$ as a reducing agent (90). Well water sample enrichments were incubated at their environmentally-relevant temperatures based on observed data at the time of sampling (e.g. temperatures from 25-60°C, in 5°C increments). Carbon/energy source combinations included: glucose (10 mM), maltose (10 mM), tryptone (0.1% w/v), casamino acids (0.1% w/v), cellulose (0.8 g/L), methane (99%), yeast extract (0.05% w/v), acetate (10 mM), formate (10 mM), lactate (10 mM), propionate (10 mM), H_2/CO_2 (80:20%), pyruvate (10 mM), fumarate (10 mM), and methanol (1 mM). Respiratory electron acceptors included: sulfate (10 mM), inorganic sulfur (1 g/L), sulfite (10 mM), thiosulfate (10 mM), nitrite (5 mM), nitrate (5 mM), and lignin (0.5 g/L).

Cell densities in liquid culture were monitored using a Petroff-Hausser counting chamber (Hausser Scientific Co., Horsham, PA) and an Axioskop2 Plus microscope (Zeiss, Thornwood, NY, USA) under phase contrast. To complete purification, liquid enrichment cultures were transferred at least three times into the same medium and conditions (e.g. initial cell densities $>10^6$ cells/mL). The acquisition of pure isolates from enrichments was further enhanced by the application of multiple adaptations, such as heat shock/thermal selective pressure, increased salt concentration, substrate limitation/introduction of inhibitors plating/slants, and serial dilutions. Upon the third transfer, DNA was extracted from putative isolates and universal bacterial or archaeal primers were used to PCR amplify the full-length SSU rRNA gene. The PCR products were then sequenced to identify

the isolate. Unique isolates were stored in either anaerobic 20% glycerol or 100% medium vials at -80°C.

Strain DRI-13 was enriched on 0.1% w/v peptone and isolated following heat shock treatments. During logarithmic growth, the culture was subjected to heat shock by shifting the culture bottle from 45°C to 60°C for 1 hour and then back to 45°C. This was repeated for several transfers until a single morphology persisted. Growth experiments were conducted in 160 mL serum bottles, containing 25 mL volume of SMC medium and 10 mM fumarate with a headspace of 100% N_2. Cultivation experiments, to define temperature optima, were incubated at 37, 45, 50, 55, 60, and 65°C in the dark without shaking. Cultivation experiments to determine optimal pH had alternative buffers added at a final concentration of 10 mM to generate the desired pH values. These included: 1,4-Piperazinediethanesulfonic acid (PIPES) for pH 6.5-7.5, HEPES for pH 8-8.5, and 3-([1,1-Dimethyl-2-hydroxyethyl]amino)-2-hydroxypropanesulfonic acid (AMPSO) for pH 9. All culturing optimization tests were done in quadruplicate.

Strain DRI-14 was isolated in 25 mL SMC medium in a 160 mL serum bottle with the headspace filled to 2 bar with 99% methane. Carbon utilization was determined by culturing DRI-14 under the same conditions with the exception of pressurizing under different headspace gases (e.g. 80/20% hydrogen/carbon dioxide, and 100% nitrogen).

2.6. GENOMIC DNA EXTRACTION and SEQUENCING

High-molecular-weight DNA was extracted from isolate DRI-13 by cultivating the organism anaerobically in SMC medium with five, 1-L batch cultures at 50°C. Cells were harvested by centrifugation at 15,000 ×*g* and DNA was purified by phenol/chloroform extraction (91). DNA was RNase treated, visually checked for quality on a 1% agarose gel, and shipped to the DOE Joint Genome Institute (JGI) for draft sequencing using Illumina technology. The sequences were assembled according to JGI protocols using Velvet, annotated using a suite of gene characterization tools in the IMG pipeline, and made available as a part of the JGI-IMG data warehouse (92). The genome of DRI-13 has been released to the public according to JGI protocol and is available both through the DOE IMG platform and via NCBI.

High-molecular-weight DNA was extracted from isolate DRI-14 from two batches of eight, 160-mL serum bottles with 25-mL medium volumes (400 mL total). Two batches were incubated at 45°C and 50°C for 60 and 40 days, respectively. Genomic DNA was extracted using a Promega Wizard Genomic DNA Purification kit (Madison, WI) and sent to Josephine Bay Paul Center at the Marine Biology Laboratory (Woods Hole, MA) for Illumina Miseq sequencing. To create a shotgun library for sequencing, the DNA was sheared to an average fragment length of 500 bp using a Covaris S220 Ultrasonicator. A Nugen ovation ultralow library construction kit was used for end repair, adaptor ligation and amplification.

Eighteen cycles of amplification were necessary to produce the amount of material required for sequencing. A Pippin Prep (Sage Science) 2% cassette was used to target library molecules of 620 bp. The resulting oligonucleotides were sequenced with an Illumina MiSeq using a 2 x 250 bp PE kit, resulting in 10.6 million pairs (21 million individual 250 bp reads) from 60% of a lane. Reads were filtered for quality using the analyze-illumina-quality-minoche quality-filtering pipeline (https://github.com/meren/illumina-utils), resulting in the removal of 1,587,851 reads (15%). A single round of digital normalization (github.com/ged-lab/khmer/tree/2012-paper-diginorm) was also applied to the quality-filtered data to simplify the dataset for *de novo* assembly. The CLCgenomics workbench (Boston, MA) was used for *de novo* assembly, resulting in 45 contigs with an average length of 55,335 bp. Raw reads were mapped to the contigs if they matched 100% of length and 97% identity. Under these parameters, 47% of reads mapped to the reference and indicate 1000× coverage of the genome. Contigs were uploaded to RAST for annotation. Assembly of the quality-filtered data is pending and the digitally normalized data produced an assembly comparable to the raw data.

2.7. SCANNING ELECTRON MICROSCOPY and SAMPLE PREPARATION

DRI-13 and DRI-14 cultures were harvested during logarithmic phase and fixed in 10% formalin in phosphate buffered saline (PBS). Cells were prepared for scanning electron microscopy (SEM) as previously described (93). Briefly, cells were washed twice in cold 1× PBS, washed once in ultra-pure water, and re-suspended in 25 µL of molecular grade ultrapure water. This cell concentrate was dripped onto a 0.2-µm, 13 mm Whatman Nuclepore polycarbonate filter (GE Healthcare Bio-Science Corp., Piscatamy, NJ) and allowed to air dry. The cells affixed to the filter were sequentially dehydrated in 10, 25, 50, 75, 95, 100% ethanol for 15 minutes each. Cells were critical-point-dried and visualized on a JEOL JSM-7001F-LV scanning electron microscope (Tokyo, Japan) located at the Center for Electron Microscopy and Microanalysis, University of Southern California.

2.8. SORPTION/DESORPTION OF RADIONUCLIDES

Exopolysaccharide (EPS) extractions were carried out, using a a previously-published method (94, 95). Briefly, cells of NNSS isolate, *Pseudomonas* str. U12n.10 were grown as a biofilm on 150 mm agar plates for 96 hours, washed from the plate with a saline buffer, and pelleted by centrifugation. Cell washing with both saline buffer and an EDTA-based buffer removed loosely- and tightly-bound EPS from the cells, respectively. EPS was filtered (0.22 µm) to remove residual cells, concentrated via 3-kD centrifugal filters (Amicon ultra-4, EMD Millipore), and then precipitated with ethanol overnight. Precipitated EPS was resuspended with distilled deionized water, then dialyzed at 2 kD (Slide-A-Lyzer MINI Dialysis Devices, Pierce, Rockford, IL) for 96 hours to remove remaining salts and ethanol. Cell suspensions were plated and grown overnight, after removal of

EPS, to demonstrate viability post-extraction. Flow cytometry (MicroPro, Becton-Dickenson) confirmed that the number of viable cells was statistically equivalent to the total number of cells, indicating that the extracts consisted of only EPS material and little or no cell lysate.

Bacterial cultures used in sorption experiments were grown overnight in Luria-Bertani (LB) liquid medium to stationary phase at a density of ~10^9 cells mL^{-1}. Cells were concentrated by centrifugation at 3500 ×*g* for 10 minutes. Cells were washed twice and resuspended to a final density of 10^9 cells mL^{-1} in SMC medium (minus bicarbonate), which contained no organic carbon, phosphorus, or nitrogen.

Desorption of U-233 from tuff, obtained from the NNSS, was measured in column experiments using a solution spiked with bacterial cells as the eluent. For Pu-293, sorption experiments were conducted with cells only (at I=0.01M, pH=7), cells + tuff, or EPS only. Cultures included *Shewanella* CN-32 grown in liquid culture, *Shewanella* CN-32 grown on plates (to increase the relative amount of EPS associated with cells), *Shewanella* CN-32 DM2 (an EPS^- mutant; Michael Leonardo, Coe College, unpublished data), U12n.10-R (an EPS-producing *Pseudomonas* sp. isolated from the NNSS in our laboratory), and lysed *Shewanella* CN-32 cells.

2.9. SUBMISSION OF CLONE LIBRARY SEQUENCES

A total of 76 BLM-1 clone library sequences, representing unique OTUs, were submitted and accepted by National Center for Biotechnology Information (NCBI, Accession numbers KF939333-KF939408). Others are in progress as of this writing.

3. RESULTS and DISCUSSION

3.1. PHYSICAL PARAMETERS and AQUEOUS CHEMISTRY

Seventeen borehole wells and two tunnels were sampled for this project, with geographic locations ranging from Pahute and Ranier Mesa volcanics to dolomite-hosted fluids associated with Frenchman Flat or the Amargosa Valley, near Death Valley. Figure 1 shows the location of the wells/tunnels and a key code numbering system used throughout this report (Note: some wells were sampled at multiple depths and are decimally enumerated; i.e. ER-EC-15 Upper is 5.1, ER-EC-15 Middle will be 5.2, and so-forth). Photographic depictions of well sites and field activities from the NNSS and the Amargosa Valley control site are shown in Figures 2 and 3.

A summary of the physical and chemical datasets developed over the course of this work can be found in Tables 1a and 1b. The wells sampled encompassed a

range of radiological, physical and geohydrological conditions. The zones sampled varied from 300 – 1,116 m below land surface. Rock types were primarily volcanic, with three sites (UE-3E #4 [Aleman], U-3cn5 [Bilby], and BLM-1) either sampling dolomite directly or producing groundwater that was sourced via fractures to the underlying regional carbonate aquifer.

Temperatures, as sampled, ranged from a low of 11.3°C in the U12n.Vent#2 sample to a high of 58.5°C in ER-EC-15-Lower. The cool temperature in U12n.Vent#2, however, almost certainly reflects the nature of the sample collection method (e.g. via bailer through a ~700 m air-filled vent hole in the winter), rather than the actual conditions in the tunnel below. Overall, temperatures generally tracked depth (Table 1), with the deeper samples reflecting higher temperatures.

Fig. 2. Examples of sampling sites. A) Yucca Flat from the air (NTS Photo Library, http://www.nv.doe.gov/library/photos/nts.aspx); B) ER-EC-13 being drilled, from Bill Wilborn, publicly-released 2011 Devils Hole Workshop Talk; C) ER-EC-13 during hydrologic pumping test; D) water discharge at end of pumping test; and E) Amargosa Valley reference well, BLM-1, during pumping test, Jamie Walker (NWRPO) shown. All images from published sources (upper) or cleared for this work (lower).

Table 1(a). Wells Sampled with Corresponding Metadata and Aqueous Chemistry.

Assigned #	1.1	1.2	2	3	4	5.1	5.2	5.3	6.1	6.2	7	8.1
Name of Well	**PM 3-1**	**PM 3-2**	**ER 20-7**	**ER 20-11**	**ER-EC-11**	**ER-EC-15 (upper)**	**ER-EC-15 (middle)**	**ER-EC-15 (lower)**	**ER-EC-13 Upper**	**ER-EC-13 Lower**	**ER-EC-12**	**ER 20-8 Upper**
Date Sampled	8/12/2013	8/23/2013	9/23/2010	8/5/2013	5/18/2010	11/6/2013	1/10/2014	2/17/2014	7/13/2012	3/28/2013	11/28/2011	6/27/2011
Latitude	37.24	37.24	37.21	37.2	37.2	37.11	37.11	37.11	37.17	37.17	37.17	37.19
Longitude	-116.56	-116.56	-116.48	-116.49	-116.48	-116.31	-116.31	-116.31	-116.55	-116.55	-116.49	-116.47
Sampling Depth (m)	610	475	616	800	1116	475	700	920	610	900	1113	822
Total Well Depth (m)	668	668	895	896	1264	992	992	992	914	914	1240	1049
Sampling method	JP[1]	JP[1]	P[2]	P[2]	P[2]	P[2]	P[2]	P[2]	P[2]	P[2]	P[2]	P[2]
T (°C)	26.7	27.1	34.1	39.0	44.0	47.7	48.5	58.5	41.0	45.0	46.0	41.8
pH	7.18	7.73	8.30	8.30	8.70	8.01	8.19	8.17	7.90	8.20	8.70	8.35
Rock type	T[4]	T[4]	T[4]	RL[8]	T[4]	RL[8]	T[4]	T[4]	RL[8]	T[4]	T[4]	T[4]
ODO (mg/L)	2.4	2.3	6.0	4.5	3.4	3.5	2.3	0.6	3.7	4.8	1.9	4.7
ODO (%Sat.)	31.3	30.1	87.4	70.0	55.0	59.6	38.7	11.1	58.3	78.8	32.5	76.0
^{3}H (pCi/L)	300	340	1.77E+07	1.87E+05	6,805	677	320	906	651	2,318	370	2,813
TOC (mg C/L)	0.3	0.4	0.6	0.4	0.4	1	7.4		5.4	0.8	1.3	0.2
TIC (mg C/L)	29.3	30.2	37.9	28.0	28.7				35.0	30.3	25.4	26.0
Viable Cells	32,345	68,092		3,399	1,849,817	10,120	369,309	71,001	15,356	4,042	124,274	25,600
Total Cells	67,711	140,881		5,525	4,078,069	11,396	277,905	89,799	22,612	5,050	210,240	25,831
Conductivity (µS/cm)	783	749	510	450	517	778	732	705	692	659	450	462
Turbidity (NTU)	13.8	12.9	8.2	20	1.1	2.1	15.4	9.9	15.7		40.5	3.4
Acetate (mg/L)	0.008	0.017		0.01	0.1	0.005	0.013	<d.l.	0.02	0.011	<d.l.	5
Lactate (mg/L)	0.076	0.078		0.065	0.09	0.032	<d.l.	0.08	0.26	<d.l.	1.1	0.91

Formate (mg/L)	<d.l.	<d.l.		<d.l.	0.47	<d.l.	0.015	<d.l.	<d.l.	0.022	<d.l.	0.81
Propanoate (mg/L)	<d.l.	<d.l.		<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	20	<0.01
SO_4^{2-} (mg/L)	129.5	92.8	53	35.6	68	47.4	97	59.1	96	91	35	49
NO_3^- (mg/L)	2.21	1.3	2.27	0.99	2.27	0.64	1.07	0.2	1.79	1.73	1.24	2.22
NO_2^- (mg/L)	<0.005	<0.005	0.14	0.02	0.04	<0.005	0.022	0.053	0.01	<0.016	0.02	0.14
NH_4^+ (mg/L)	0.01	0.007		<0.005		<0.005	0.012	0.042		<0.005		
PO_4^{3-} (mg/L)	0.0038	0.02		0.022	0.0092	0.25	0.23	0.016	0.028	0.02	1.05	0.004
HCO_3 (mg/L)			171	120	146	160	195	195	150	150	207	122
Cl (mg/L)	110	95	30	21.9	44.5	64	51.3	25.4	62	55.1	11.2	28.3
Na (mg/L)	138	130	118	109	110	130	150	150	120	130	85	88
K (mg/L)	11	14	4.8	2.2	0.71	3.2	3.3	4	4	2.9	0.471	2.17
Ca (mg/L)	32	28	6.62	3.03	5.8	6.8	4.2		10	12.1	3.65	2.31
B (mg/L)			0.209		0.018				239	236	0.029	0.123
Mg (mg/L)	2.5	1.7	0.181	0.022	0.0089	1	1	0.013	0.072	1	22	0.024
Br (mg/L)	1.7	0.29	0.181	0.832	0.464	0.093	0.14	0.093	0.2	0.737	0.072	0.09
Fe (mg/L)	4.9	3.8	0.007	0.005	15	0.1	2.3	2.8	0.1	19.9	7.3	<0.015
Mn (µg/L)	0.16	0.12	4.32	0.73	2.02	7.6	45	0.5	10	2.39	14.4	3.78
Mo (µg/L)	5.8	7.44	15.6	1.88	3.9	11.72			11.1	9.9	6.84	4.9
Zn (µg/L)	4	1.844	4.8	1.73	0.79	6.3			50	20	4.3	28
Cu (µg/L)	0.78	0.588	0.78	0.82	2.5	2.67			3	2	1.19	0.3
Cr (µg/L)	1.6	<0.15	4.5	1.33	0.84	0.818	5.3	3.3	0.87	0.89	0.34	0.41
Pu (pg/L)			0.703		0				0	0		0
U (µg/L)	5.4	1.3	8.2	3.9	1.64	4.63			6.8	7.7	2.27	2.7
Pb (µg/L)	<0.024	0.029	0.51	0.153	1.02	0.388			0.95	0.4	1.52	<0.03
Sr (µg/L)	111	53.3	5.9	4.12	34	24.4			11.8	28.1	10.6	1.85
Cs (µg/L)	0.36	0.379	1.12	1.81	3.9	6.44			2.27	1.7	2.37	1.32
W (µg/L)	0.66	1.19	1.33	1.35	2	2.78			1.61	1.08	0.86	1.67
He (% vol/vol)				0		0	0	0	0.004	0	0.000	0.000
H_2 (% vol/vol)				0.00174		0.00083	0.00004	0.00073	0.004	0.00247	0.002	0.001862
O_2 (% vol/vol)				5.57		9.02	3.00000	11.41	23.730	7.08	14.434	20.26

N_2 (% vol/vol)	18.00	34.22	19.18000	43.94	76.681	24.66	81.339	78.63
CO_2 (% vol/vol)	0.16126	0.17353	0.02930	0.04624	0.963	0.04551	0.302	0.0060
CH_4 (% vol/vol)	0.00019	0.02779	0.00013	0.00044	0.005	0.00016	0.001	0.00001
CO (% vol/vol)	0.00317	0.00164	0.00189	0.00172	0.004	0.00228	0.002	0.000618
C_2H_6 (% vol/vol)	0.00343	0.00040	0.00004	0.00140		0.00152		

[1]Jackpump, [2]pump, [3]bailer, [4]Tuff, [5]Carbonate, [6]Dolomite, [7]Alluvium, or [8]Rhyolitic Lava. <d.l. below detection limit.

Table 1(b). Wells Sampled with Corresponding Metadata and Aqueous Chemistry.

Assigned #	8.2	9	10	11	12	13	14	15	16	17	18	19
Name of Well	**ER 20-8 Lower**	**ER 20-4**	**UE-20N#1**	**U19V PS#1ds (Almendro)**	**U12n.Vent#2**	**U12n.10**	**UE-2ce WW (Nash)**	**UE-3E #4 (ALEMAN)**	**U-3cn5 (Bilby)**	**ER 11-2**	**ER 5-5**	**BLM-1**
Date Sampled	8/8/2011	9/21/2011	5/24/2012	9/15/2009	10/5/2011	8/20/2008	7/2/2008	6/10/2009	3/29/2011	7/18/2013	5/12/2013	6/21/2011
Latitude	37.19	37.2	37.24	37.25	37.20	37.21	37.14	37.07	37.06	36.89	36.87	36.40
Longitude	-116.47	-116.44	-116.42	-116.35	-116.22	-116.21	-116.14	-116.05	-116.02	-115.94	-115.94	-116.47
Sampling Depth (m)	982	850	700	1097	362	366	474	441	793	375	300	884
Total Well Depth (m)	1046	1066	1006	1170	Tunnel	Tunnel	503	563	924	400	317	884
Sampling method	P[2]	B[3]	P[2]	B[3]	B[3]	B[3]	P[2]	B[3]	P[2]	B[3]	P[2]	P[2]
T (°C)	49.8	40.5	36.0	28.0	11.3	23.0	34.0	25.6	44.7	28.9	25.0	61.0
pH	8.50	8.40	7.50	9.30	7.79	9.40	6.80	7.05	7.69	8.60	8.40	7.30
Rock type	T[4]	RL[8]	RL[8]	T[4]	T[4]	T[4]	C[5]	D[6]	T[4]	T[4]	A[7]	T[4]
ODO (mg/L)	4.9	7.4	4.0	2	9.2	3.4	2.3	3.5	0.2	4.3	6.9	0
ODO (%Sat.)	85.3	116.8	59.9	26.7	88.8	42.2	33.5	44.9	3.2	58.2	87.8	0
^{3}H (pCi/L)	2,800	142	5.55E+07	8.49E+07	1.03E+06	6.26E+06	2.67E+05	1.56E+06	6.5	1,680	1.0	1.0
TOC (mg C/L)	0.1	0.8	2.1	22.0	14.9			15.2	1.5	4.0	1.2	
TIC (mg C/L)	27.3	16.6	20.1	40.9	55.5	25.6	84.2	12.7	50.3	74.8	36.2	
TVO	6,172	11,488			4,694				4,245	2,820	12,574	7,005
Biomass	8,729	14,514			5,742				10,887	33,546	13,611	15,582
Conductivity (µS/cm)	360	240	433	784	355	1108	736	1,858	845	1,100	410	830
Turbidity (NTU)	4.3	12.3							3.675	779	<1	
Acetate (mg/L)	0.08	0.04							0.4	0.02	<d.l.	<d.l.
Lactate (mg/L)	0.08	1.14							0.4	0.11	0.008	<d.l.

Formate (mg/L)	0.36	3.48							0.65	<d.l.	0.009	<d.l.
Propanoate (mg/L)	<d.l.	76.0							<0.01	<d.l.	<d.l.	0.782
SO_4^{2-} (mg/L)	0.14	0.024	<0.024	3.9	8.75	289	18.1	9.15	36	100	41	167
NO_3^- (mg/L)				0.519	0.759	1.9	5.7	4.95	0.26	3.6	3.76	0.097
NO_2^- (mg/L)	0.032	1.88		0.2	<0.024				<0.14	0.048	0.004	0.029
NH_4^+ (mg/L)	134	114	93	3.54					0.05	0.024	<0.005	
PO_4^{3-} (mg/L)	23.8	4.7	11.2	4	1.5				0.033	0.24	0.012	0.0086
HCO_3 (mg/L)	93	62	64.1				428		229		150	
Cl (mg/L)	1.55	1.32	2.4	73.1	10.7	35.5	14	503.6	27.4	50	8.0	41.4
Na (mg/L)	3.55	4.45	6.7	162		221	38	99	53.5	200	87	221
K (mg/L)	0.125	0.087	0.123	13.2	6.64	13.6	20.6	61.2	8.5	5.3	7.4	14.6
Ca (mg/L)	0.0324	0.046	0.162	0.48	5.51	14	71.8	202	37.2	4.8	7	32.2
B (mg/L)	0.081	0.052	0.664	1.1				0.39	0.213		0.303	
Mg (mg/L)	0.0049	0.0038	1.7	0.0089		0.19	30.8	0.185	16.5	2.3	3.2	
Br (mg/L)	3.37	2.6	97		0.865	0.65	<0.12	9.58	0.09	2.1	0.04	0.097
Fe (mg/L)	6.5	9.6	17.8	0.146	0.96	0.102	0.801	0.59	1.57	8.8	5.7	
Mn (µg/L)	30	0.48	37.6	3.7	61	11.4	101	24	101	36.7	97	
Mo (µg/L)	0.82	0.94	4.39	1400	15.9	24.1	2.47	22.6	4.8	48.6	7.4	
Zn (µg/L)	0.6	0.02	0.54	28.4	24.2	46	57	62	10.9	25	50	
Cu (µg/L)	0	0	0.005	6.1	6.17	2.4	2.3	1.68	<0.09	1.07	7.5	
Cr (µg/L)	2.8	1.6	2.87	9.1	6.2	1.16	<0.27	0.84	<0.18	27	3.2	
Pu (pg/L)	<0.06	0.3	<0.012	0.06	16.4	11.3	<0.13	<0.002	0	-	-	n.d.
U (µg/L)	6.1	5.4	11.1	0.127	1.67	0.2	4.95	0.045	0.83	12.5	8.5	
Pb (µg/L)	1.75	1.03	1.42	18	12.6	5.01	32.3	1.8	0.072	0.44	0.084	
Sr (µg/L)	1.92	1.19	2.65	16.7	7.9	25.7	259	518	248	6.82	24.9	
Cs (µg/L)	<0.01		0.00	1.5	0.092	0.167	0.269	8.7	2.11	0.22	0.1	
W (µg/L)	<0.01		0.00	101	9.3	34.8	0.199	0.93	0.49	38.5	2.47	
He (% vol/vol)	19.86		20.27		0.004				0.000		0	0.008
H_2 (% vol/vol)	78.89		78.69		0.015				0.002		0.00086	0.008
O_2 (% vol/vol)	0.36		0.01		6.847				2.121		7.68	0.496

N_2 (% vol/vol)	0.02		0.00	89.474	97.670	21.99	51.190
CO_2 (% vol/vol)			0.00	0.627	0.892	0.22980	1.149
CH_4 (% vol/vol)				0.005	0.164	0.00099	0.056
CO (% vol/vol)	0.14	0.024	<0.024	0.019	0.001	0.00209	0.001
C_2H_6 (% vol/vol)						0.00199	

[1]Jackpump, [2]pump, [3]bailer, [4]Tuff, [5]Carbonate, [6]Dolomite, [7]Alluvium, or [8]Rhyolitic Lava. <d.l. below detection limit.

Fig. 3. Microbiological sampling. A) Non-radioactive Pahute Mesa well being sampled; B) PI Moser sampling U19ad PS#1A (Chancellor), a well both high in radioactivity and temperature; C) U12n.Vent.10 being sampled through mountain-top vent hole via improvised bailer; D) Sterivex filter array during pumped sampling of a well on Pahute Mesa, used for DNA-based analyses. Note: dissolved gases accumulating as bubbles (All photos cleared).

Geothermal gradients at specific locations, however, appear to vary. Holes where more than one depth zone was sampled are especially useful for assessing geothermal gradients. ER-EC-13, is most typical of this portion of the DVRFS. This well had temperatures of 41°C and 45°C at 610 m and 900 m, respectively; values which would roughly correlate to a geothermal gradient of ~14°C/km. Low values, such as this, were common across Pahute Mesa (unpublished), suggesting the recent downward movement of meteoric water. Other wells of similar depth had similar or lower measured water temperatures (e.g. 34.1°C and 37°C for ER 20-7 and UE-20N#1, for depths of 616 and 700 mbls, respectively). It should be noted, however, that the very low temperature of PM 3-1 (610 m and 26.7°C) is probably an artifact related to its low pumping rate (11.3 L/min Jeff Wurtz, UGTA, pers. comm.). A second hole, sampled at multiple depths (three) was ER-EC-15. Temperatures in this well were measured at 47.7°C, 48.7°C and 58.7°C at 475, 700, and 920 mbls; indicating much more complex patterns of fluid flow. For example, if one considers the 8°C difference between the 475 and 700 m samples, a gradient of only ~4°C/km would be indicated. Conversely, the 10°C difference in temperature between the 700 and 920 m samples reflects a gradient

of ~45°C/km. Such widely divergent gradients in the same hole are most readily explained by the incursion of a deeper, hotter end member into the lower portions of ER-EC-15, an observation with may be further supported by shifts in microbial community structure noted between the different depths in this hole may reflect this (Figs. 4, 5, and 6).

As might be expected, the geothermal gradient in the discharge zone of the DVRFS (e.g. BLM-1) was generally higher. Since this well was only sampled at a single depth, this determination is less accurate. However, if one considers the average annual air temperature of nearby Amargosa, NV (19.6°C (96)), the downhole temperature of 61°C at 884 mbls would indicate a geothermal gradient of ~47°C. This value is even higher than the accepted Death Valley regional average of about 33°C/km (97, 98) and suggestive of significant upwelling in the vicinity of this borehole. Since the depth to water in BLM-1 is only about 33 m and land surface at the well site is ~300 m above the surrounding valley floor, the well can be considered artesian, a factor again consistent with discharge from the regional flow system.

Across the sample set, pH ranged from a low of 6.8 in UE-2ce (Nash) to a high of 9.3 in U19V PS#1 (Almendro). pH values from Pahute and Ranier Mesa volcanic substrates were generally highest (e.g. >~8.0), which is typical of volcanic aquifers (99, 100). Conversely, samples from the three dolomite-associated samples (UE-3E #4 [Aleman], U-3cn#5 [Bilby], and BLM-1) were 7.05, 7.69, and 7.30, probably reflective of carbonate buffering, as would be expected.

In groundwater, dissolved oxygen is a primary controller of redox potential (101, 102) and microbial biogeochemistry (e.g. terminal electron accepting processes (103, 104). Oxygen dynamics of groundwater flow over long distances have been studied in a number of regional/fractured rock flow systems (105-109) including the DVRFS (110). This deep transport of dissolved O_2 (dO_2) in groundwater is especially prominent in the recharge area (39, 42-44) of the regional flow system (e.g. the Pahute and Ranier Mesa portions of this study). This aspect is supported in general by the high concentrations of dO_2 that occur to sometimes great depths in the volcanic uplands of the NNSS/NTTR. Although the dO_2 concentrations do vary from location to location, in several cases, these values begin to approach saturation (e.g. ER 20-7, ER-EC-13-Lower, ER 20-8, ER 5-5 at 88%, 79%, 85%, and 88%, respectively). In one case, the measured dO_2 exceeded saturation (e.g. ER 20-4 at 116%, Table 1).

In the absence of any obvious source of oxygen in this system, the best explanation for the high concentrations of oxygen probably relate to very low biological and geochemical oxygen demand, especially in the volcanic uplands. TOC was measured for most of the samples and ranged from relatively low values, for example, 0.11 mg C/L in ER 20-8, to values exceeding 10 mg C/L in many of the detonation cavity-associated samples (Table 1). The reason for this trend, however, is not clear. A second factor which may impact organic C

measurement may be the presence of residual drilling amendments (e.g. polymers). Thus, collectively, these possible compounding factors render the utility of TOC/DOC suspect for the purpose of estimating biological oxygen demand in this sample set.

Also consistent with the presence of dO_2 across most of this subsurface system is the consistently high concentration of nitrate in the groundwater (e.g. average of 1.9 mg across the dataset and as high as 5 mg/L in UE-3E #4). Since, upon the depletion of oxygen, nitrate reduction is a preferred environmental electron accepting process (103, 111-114), the near universal detection of nitrate across this system indicates that oxygen was indeed present and suppressing this process. Correspondingly, all samples in this dataset with >1 mg/L nitrate were also low in dO_2 (e.g. ER-EC-15 Lower, U12n.Vent#2, and BLM-1). It should be noted, however, that although substantial dO_2 was detected in U12n.Vent#2 (Table 1), this sample was collected in an open bailer through an air-filled vertical ventilation shaft and thus had likely equilibrated with the atmosphere. Given that high concentrations of methane had accumulated in this hole prior to sampling (Jeff Wurtz, pers. comm.), it is probable that the flooded tunnel fluids were anoxic *in situ* and thus the relative depletion of nitrate in this sample (0.759 mg/L) should not be unexpected.

A common proxy for microbiological activity level in the subsurface is standing stock cell density (115-118). Planktonic cell concentrations in this dataset, as measured by flow cytometry, ranged from a low value of 5.1×10^3 per mL in ER-EC-13-Lower to as many as 4.1×10^7 in ER-EC-11 (Table 1). Given the geohydrological similarity of these sites, one to another, we suspect that the high values are most likely in error or reflect some drilling-associated artifact (e.g. cells growing on added polymers). When these data are taken in the context of related projects, it may be possible to assess the confidence that should be assigned to the high cell numbers in this dataset. For example, in a detailed study of nine high-flow springs and wells associated with the Ash Meadows National Wildlife Refuge in Amargosa Valley near site BLM-1, total cell counts were consistently between 1×10^3 and 1.4×10^4 per mL (119). It seems unlikely that pristine samples from the deep subsurface in this region would host cell densities three orders of magnitude higher than those of a surface-impacted environment such as a spring outlet. Thus, we consider the relatively low values (e.g. those of 10^5 and lower: ER-20-11, ER-EC-15, ER-EC-13, ER-20-8, ER-20-4, U12n.Vent#2, U-3cn5, ER-11-2, ER-5-5 and BLM-1) from this dataset as more likely to be accurate representations of this system. Moreover, if one considers two of the three wells where multiple zones were sampled, two of these (ER-EC-13 and ER-20-8) showed convincing declines in cell concentrations between upper and lower zones (22,612 vs. 5,050 and 25,831 vs. 8,729, respectively), possibly consistent with trends that might be expected in a down-welling recharge zone, such as West Pahute Mesa. Conversely, the trend of increasing cell concentration with depth in ER-EC-15 (e.g. cell counts of 11,396, 27,905, 89,799 in Upper, Middle and Lower zones, respectively) may reflect an injection of hydrothermal fluid from

depth, as suggested by the geothermal gradient and microbial community structure of this site.

Regardless, the generally low cell counts in most of these wells would be consistent with the low heterotrophic oxygen demand inferred by the presence of dO_2 to across most of this system and may explain the presence of dO_2 at relatively great depths.

3.2. MICROBIAL COMMUNITY STRUCTURE

Our first major project objective was to determine if life exists and could be detected in a remotely-sampled, deep-subsurface environment. Prior work by the PI (30, 118, 120), and others focused on deep subsurface microbial communities (121), indicated that this task may not be completely straight forward. In particular, the likelihood of drilling- or sampling-associated sources of microbial contamination were of concern throughout this project. All of the drill holes or other site access portals sampled were created years prior to our project. Thus, the unavailability of contamination reference materials, such as drilling fluid, negated the proven approach (118) of subtractive identification of indigenous microorganisms. Given that most of the wells from this study were extensively developed (e.g. millions of liters pumped) prior to sampling, we have confidence that well artifacts were minimized.

For this study, bacterial and archaeal lineages were compared by two complementary DNA-based approaches: next-generation Illumina and PCR-amplified clone libraries of the 16S rRNA gene. The Illumina dataset in particular provided very deep coverage of microbial communities (642,260 reads after quality control processing). Coincident with the Illumina assessments, amplified libraries were also constructed to provide near-full-length sequences for more phylogenetically-informative assessments of major microbial lineages (e.g. 96 well format). Summaries of both data types are provided below.

3.3. MICROBIAL COMMUNITY STRUCTURE: ILLUMINA

3.3.1. BACTERIA

After computational processing, the MiSeq Illumina sequencing produced 612,944 bacterial DNA reads from across the dataset, resulting in 4,998 OTUs, defined at 97% or greater percent identity. OTUs, representing >1% of the total and grouped at the phylum level, are summarized in Figure 4. Given the diversity of habitats sampled, the variation in profiles across them is probably not unexpected. Commonly detected phyla present included Proteobacteria, Bacteroidetes, Firmicutes, Cyanobacteria, Verrucomicrobia, Acidobacteria, and Planctomycetes. Proteobacteria were present in all samples, representing from 10.1% in ER-EC-15-Lower to 88.9% in PM 3-1. This group was the most abundant phylum in all but three samples (ER-EC-15 Lower, U19V-PS Almendro, and BLM-

1). In all, five sites had a strong Firmicute presence: ER-EC-15-Lower, 42.7%; U19V-PS (Almendro), 64.9%; U12n.10, 20.7%; U-3cn#5, 25.0%; and BLM-1, 70.2%. Correspondingly, Firmicute-rich sites were of higher temperature and anoxic. The apparent dominance by Firmicutes in deep, hot continental subsurface locations is an emerging theme in the search for microbial life associated with fractured hardrock environments (30, 122-128), and is also confirmed by this work (129).

Another prominent lineage detected in most of our Illumina libraries (commonly about 20% of the total, (Fig. 4) was that of the Bacteroidetes. Bacteroidetes is a diverse group of Gram-negative bacteria that do not form spores, is typically anaerobic, and is found a wide variety of environments. In this dataset they are present in large numbers by Illumina sequencing in all sampled sites, except for those with the highest temperatures or above average levels of tritium (Fig. 4). This phylum is also prominent in samples from some of the deepest sites ever

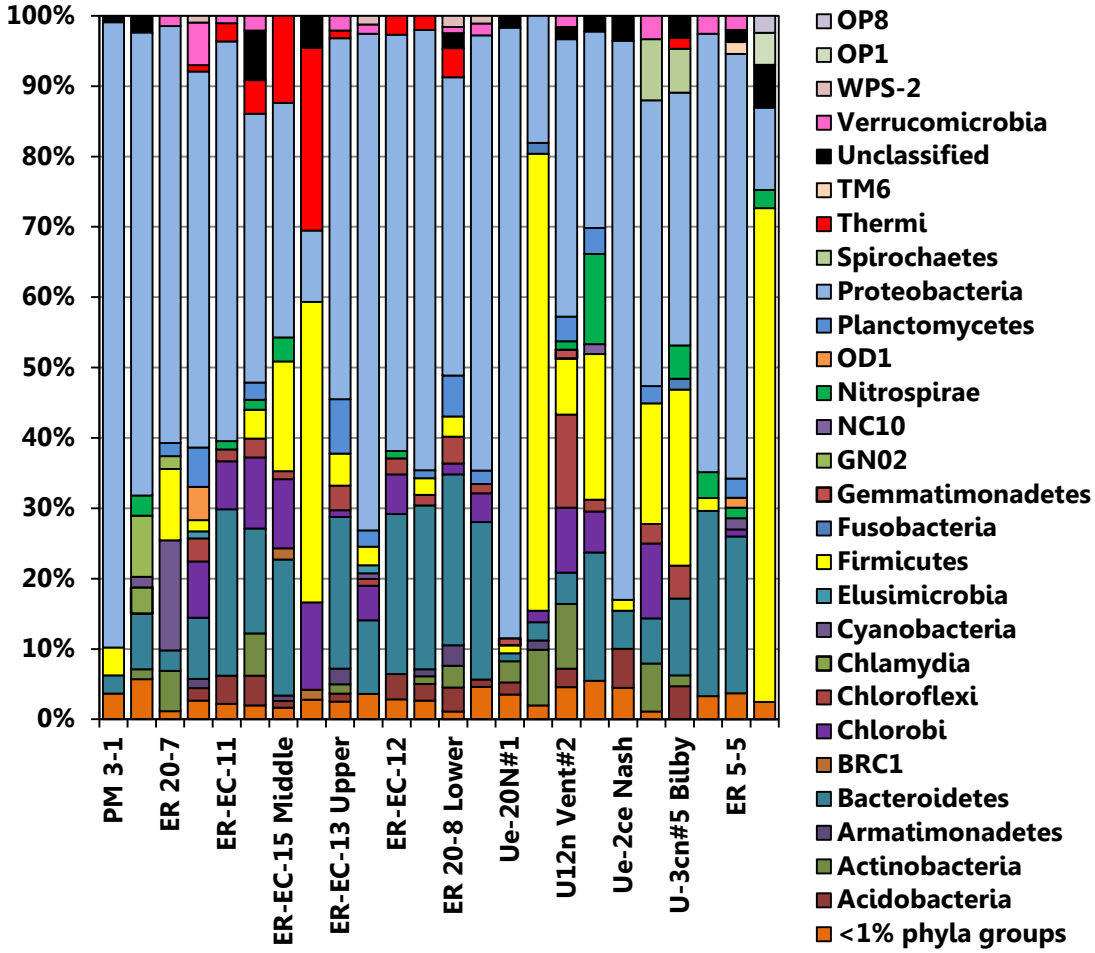


Fig. 4. Stacked bar graphs, normalized to percent, showing bacterial phylum-level distribution of 16S rRNA genes obtained by Illumina sequencing from 20 wells and 2 tunnels. A total of 612,944 DNA sequences are represented here, which are grouped into phyla based on Greengenes classification.

sampled in the South African deep subsurface (130) and fault-associated boreholes and springs in and around Death Valley (129, 131). Possibly supportive of a deep groundwater influence, the Thermi were also present in high proportions in several samples (up to about 28% in ER-EC-15-Lower). This candidate bacterial phylum is proposed for inclusion within a superphylym known as the Terrabacteria (132). Novel OTUs, not classified by Greengenes, are not present in all samples, but of the sample sites where they are found, the range varies from 0.9% at PM 3-1 to 7.1% at ER-EC-15-Upper.

One of our original hypotheses was that the microbial communities of these unusual habitats would be dominated by Gram-positive bacteria. This was in partial deference to our expectation that, as was noted in radioactive sediments from the Hanford SX-108 tank farm (6), relatives of the well-known radioresistant, Gram-positive bacterium, *Deinococcus radiodurans* (133, 134) would be present. It was, thus, somewhat of a surprise that in this dataset, despite the fact that hundreds of OTUs were analyzed, no close relatives of *D. radiodurans* were detected in our amplified gene libraries. In fact, Gram-negative bacteria dominated in most communities sampled.

3.3.2. ARCHAEA

With a C:Y substitution (see methods), the universal primer set that targets the v4 region of the Bacterial 16S rRNA gene also matches 89.9% of the near-full-length archaeal 16S rRNA genes in RDP (81). Even though archaeal coverage is incomplete, the amplifiable subset is useful for revealing broad patterns of diversity and group representation. In this dataset, 29,316 archaeal reads were generated, resulting in 153 OTUs. As part of our data processing pipeline, archaeal MiSeq analyses were restricted to libraries with >100 sequences. After singletons were removed, sites U19V-PS and U12n.10 had no reads remaining, Ue-3e#4 Aleman had 10 reads, ER-EC-15-Middle had 7 reads, U-3cn#5 (Bilby) had 16 reads, and ER-20-7 had 34. These sites were subsequently removed from the data set. Class groups of official and unofficial taxonomic archaeal clades that made up less than 1% of the total were grouped together into a "<1% class groups" cluster.

At the class level, members of the Thaumarchaeota constituted an average of 64.4% archaeal sequences detected across the dataset (Fig. 5) and were present in all samples with the exception of BLM-1. The abundance of Thaumarcheota ranged from 98.8% in ER-EC-11 to 18.1% in U12n.Vent#2. In the subset of samples with the highest temperatures, three classes appeared to supplant domination by the Thaumarcheota: the Thermoprotei, Methanobacteria and miscellaneous Crenarchaeotal group (MCG). Thermoprotei were present in ER-EC-15-Lower at 49.4%. The autotrophic methanogens, Methanobacteria, were present in eight samples, ranging from a low of 1.3% in ER-EC-13 to 99.9% in BLM-1. Unclassified classes were not present in all samples, but when present, ranged from as low as 1.0% in ER 20-4 to as high as 6.4% in ER 20-8 Lower.

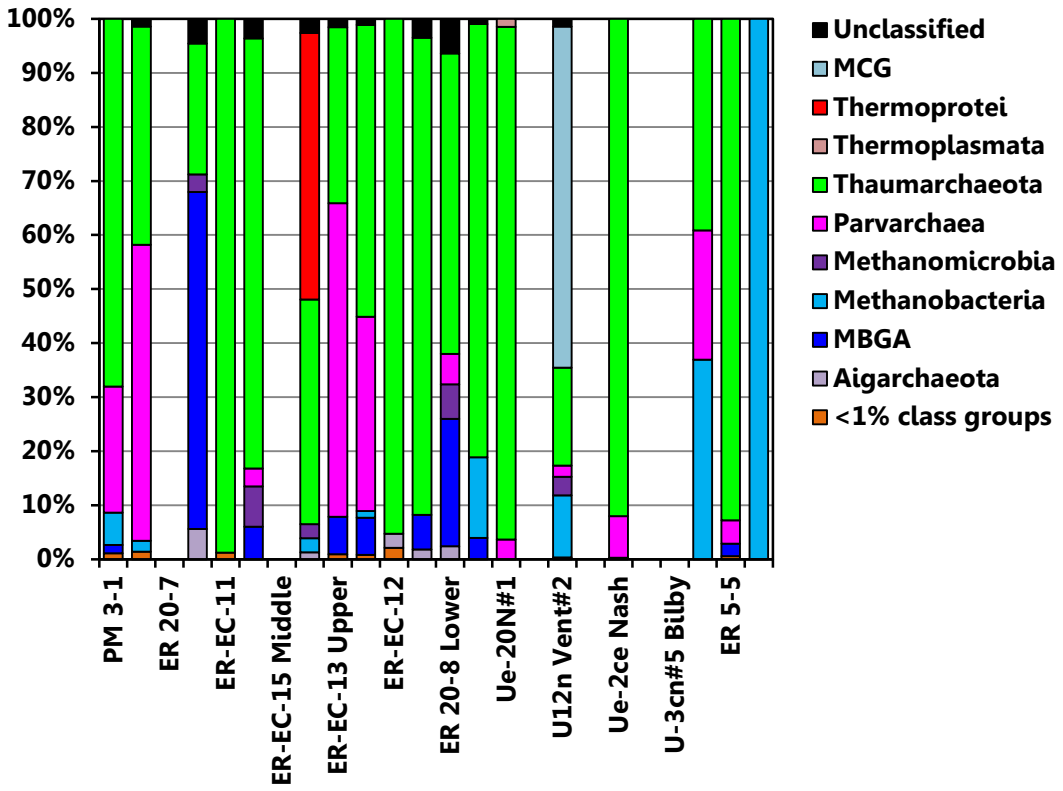


Fig. 5. Stacked bar graphs, normalized to percent, showing archaeal class-level distribution of 16S rRNA gene sequences obtained by Illumina sequencing from 19 wells and 2 tunnels sampled for this project. A total of 29,316 DNA sequences are represented here, which are grouped based on Greengenes. After processing and removal of singletons several samples (e.g. U19V-PS and U12n.10) lacked any OTU groups and were thus removed.

The dominance of the archaeal phylum, Thaumarchaeota (derived from the Greek word *Thaumas* meaning 'wonder') in the NNSS/NTTR sample sites is likely of importance in understanding microbial biogeochemistry of this environment and possibly hints as to its role in the subsurface may be obtained from other habitats. This group comprises 12-100% of the archaea in marine sediments and water columns (135, 136). In fact, Thaumarchaeota are thought to comprise as much as 20% of all marine prokaryotes (137, 138). Perhaps of greater relevance to this study, they are also found in terrestrial geothermal springs (139, 140). In the oceans, Thaumarchaeota are recognized as a major global moderator of the rate-limiting step in the nitrogen cycle (e.g. nitrification, the oxidation of ammonia to nitrite (141-146)). Both nitrite and ammonium concentrations are relatively low in our dataset and, although the relevant measurements were not made in all cases, no trends were apparent between samples with a high proportion of Thaumarchaeaota and any chemical parameter, including nitrogen compounds. The role this group of archaea plays in the NNSS/NTTR subsurface, thus, remains to be established.

3.3.3. CASE HISTORY: BOREHOLE ER-EC-15

We anticipate the development of a stand-alone paper based on our results from the well ER-EC-15, which was sampled over three different depth intervals. Select physical measurements and aqueous chemistry variables for this well are illustrated in Figure 6. This well is located in the overall recharge area of the system, but its relatively high geothermal gradient (Section 3.1) suggests the input of a deeper, hotter and more anoxic end member, especially in the deepest zone sampled (992 m). In ER-EC-15, as shown in Figure 6, the upper zone (475 m, 47.7°C, dO_2 3.5 mg/L) is dominated by Proteobacteria and Thaumarchaeota. These two groups are also present in the deeper zones, but at lower relative proportions. Conversely, the deepest zone (992 m, 58.8°C, dO_2 0.6 mg/L) was dominated by Firmicutes (Bacteria) and Thermoprotei (Archaea). In this well; whereas, the Firmicutes were present at all depths and in intermediate concentrations between shallow and deep in the intermediate zone, the Thermoprotei were only found in the deepest sample. This result is consistent

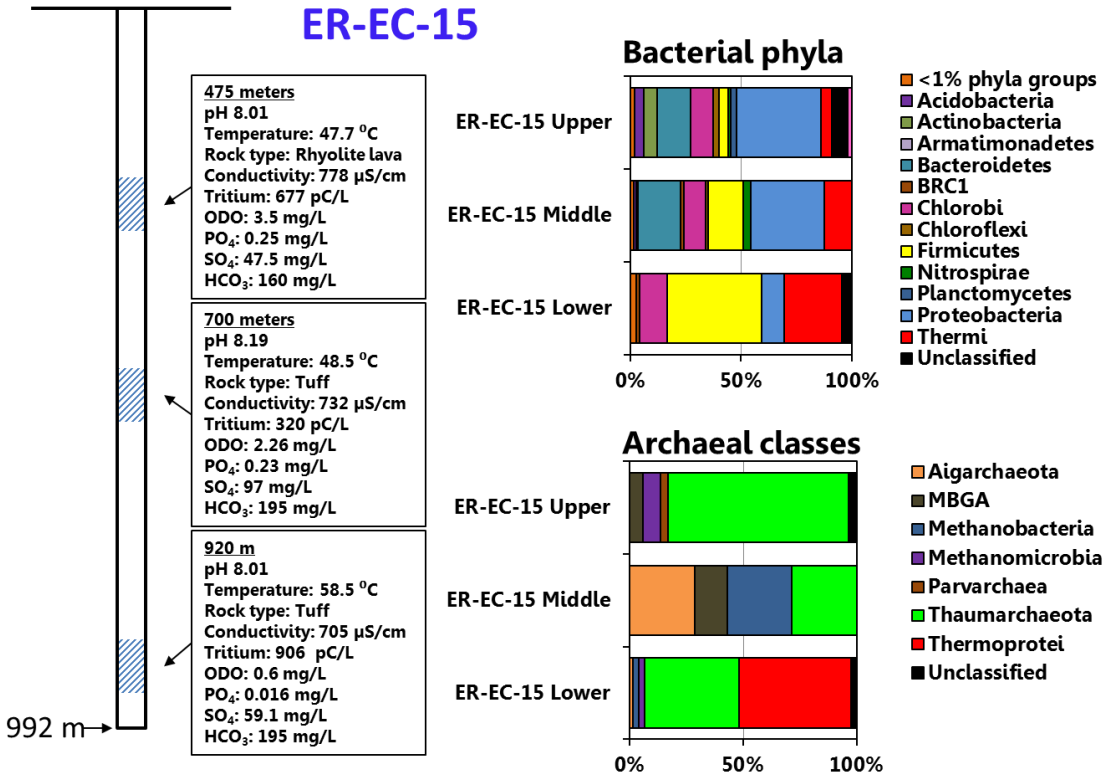


Fig. 6. Shifts in microbial populations with depth. Stacked bar graphs, normalized to percent, showing distribution of 16S rRNA gene sequences obtained by Illumina sequencing of bacterial phyla and archaeal classes in ER-EC-15 from three discrete pumped levels. A total of 81,115 and 833 DNA sequences for bacteria and archaea are represented. Note the relatively enrichment in Firmicutes (bacteria) and Thermoprotei (archaea) in the deepest, hottest, most anoxic zone near hole bottom.

with other observations from the DVRFS suggesting that patterns of archaeal diversity are more useful for tracking discrete subsurface water sources than the more diverse and variable bacteria (129). The presence of Thermprotei is also consistent with the inferred presence of the hotter end member in ER-EC-15-Lower, in that these microorganisms are generally considered hyperthermophiles and are most often associated with terrestrial hotsprings (147-150).

3.3.4. HIERARCHICAL CLUSTERING, NONMETRIC MULTIDIMENSIONAL SCALING (NMDS) AND DISTANCE-BASED REDUNDANCY ANALYSIS (DBRDA) OF COMMUNITY STRUCTURE

The microbial community structure across our MiSeq illumine dataset was assessed by hierarchical clustering, nonmetric multidimensional scaling (NMDS) and distance-based redundancy analysis (dbRDA). The results of this NMDS treatment are shown in Figure 7 for bacteria and Figure 10 for archaea. For ease of visual identification of geographically-clustered sample sites, a color code system was adopted; which differentiates well, (BLM-1) clustered separately from all other sites, both in the bacterial and archaeal analysis. This result is probably consistent with the different nature of this site in relation to the others (e.g. being in a dolomite hosted portion of the DVRFS discharge zone, along with its high

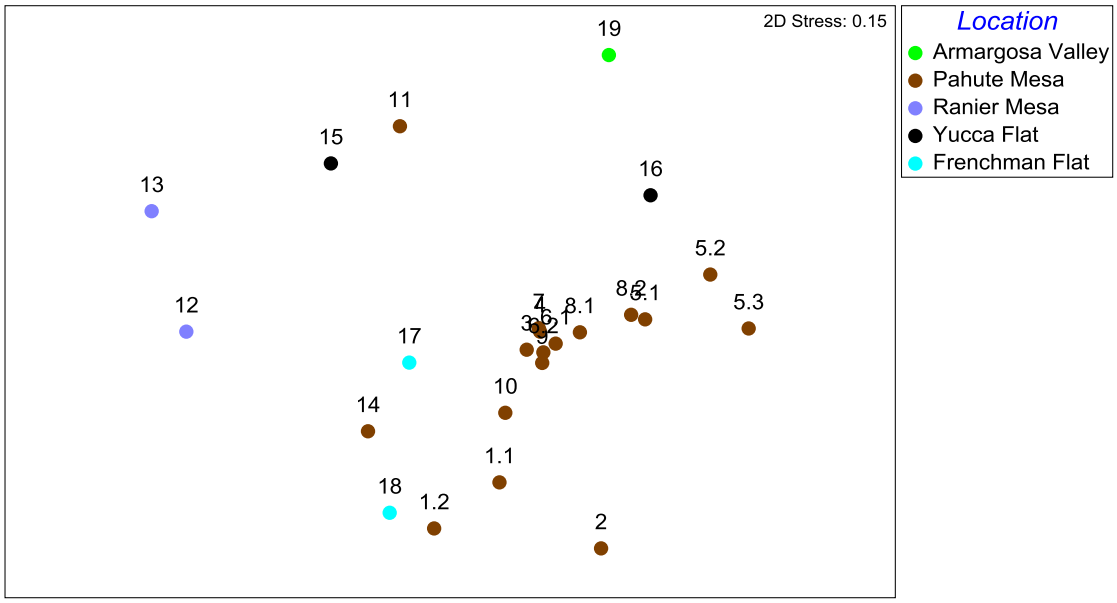


Fig. 7. Two-dimensional (2D) non-metric multidimensional scaling (NMDS) of NNSS/NTTR subsurface samples by bacterial Illumina OTUs. Interpoint distances are based upon Bray-Curtis similarity. Numbers relate to well (Fig. 1), and dot color scheme: Green = Armargosa Valley, Black = Yucca Flat, Brown = Pahute Mesa, Aqua = Frenchman Flat, and Purple = Ranier Mesa. Note the grouping of most Pahute Mesa wells within a similar statistical space. The two Ranier Mesa sites are actually mine tunnel flood water samples and their community structure may thus be controlled by sample-specific variables, rather than location.

temperature and anoxia). Overall, community grouping trends of the sampled sites may be attributable to divergent environmental or sampling conditions of each site or both (e.g. differences in oxygen saturation, rock type, temperature, and pH). Hierarchical cluster analysis for bacteria and archaea also support cohesion among the samples sites, as shown in Figures 8 and 11, respectively.

Relationships of microbial communities to selected environmental geochemistry measurements such as conductivity, dO_2, pH, temperature; as well as nitrate, chloride, sulfate, potassium, and tritium concentrations, are shown in Figure 9 for bacteria and Figure 12 for archaea. In this analysis, vectors are plotted for significant geochemical measurements, including temperature, conductivity, dO_2, pH, temperature; as well as nitrate, chloride, sulfate, potassium, and tritium. The values found in Table 2 indicate the proportions of described variation and their *p*-value significance for each geochemical measurement in relation to bacterial and archaeal community structure. In the dbRDA analysis for bacteria, the geochemical measurement of temperature most strongly explained variation within the community, especially those in the Pahute Mesa sites (~11%, Fig. 9 and Table 2). All other geochemical measurements explained smaller but similar proportions of variation in the bacterial Illumina data. As determining the role of radioactivity as a controller of microbial population structure was a stated objective of this study, the fact that tritium explained ~5% of community

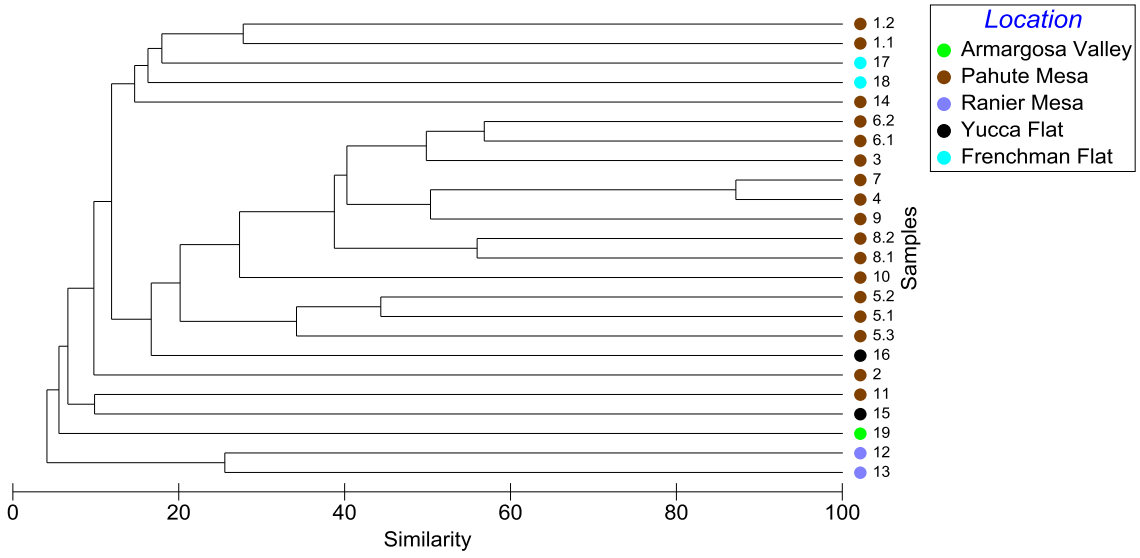


Fig. 8. Hierarchical clustering of bacterial communities in NNSS/NTTR wells sequenced using the Illumina platform. Distances are based upon Bray-Curtis similarities. Numbers relate to well (Fig. 1), and dot color scheme: Green = Armargosa Valley, Black = Yucca Flat, Brown = Pahute Mesa, Aqua = Frenchman Flat, and Purple = Ranier Mesa.

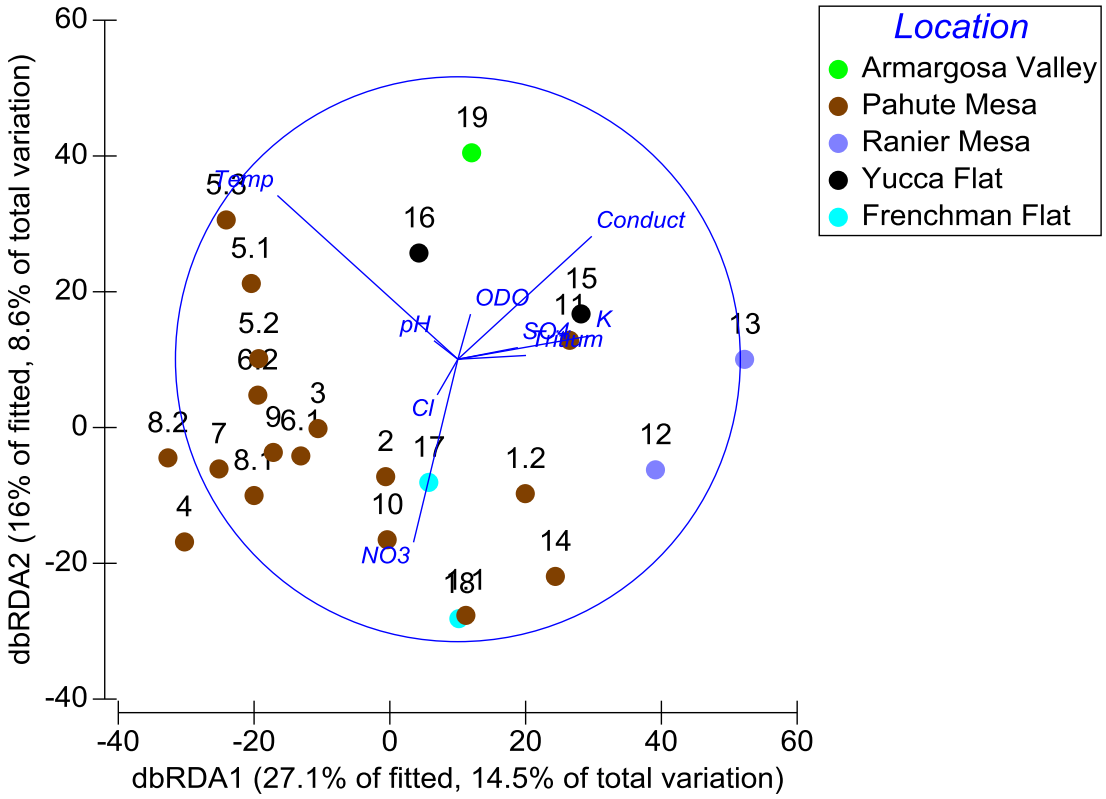


Fig. 9. Distance-based Redundancy Analysis (dbRDA) of NNSS/NTTR wells based on bacterial OTUs and selected geochemistry variables. Vectors represent temperature (Temp), conductivity (Conduct), dissolved oxygen (ODO), pH, chloride (Cl^-), nitrate (NO_3^-), sulfate (SO_4^{2-}), potassium (K^+), and tritium. Percentages describe variation explained (total and fitted) in the communities by measured geochemistry. Numbers relate to well key code (Fig. 1), and dot color scheme: Green = Armargosa Valley, Black = Yucca Flat, Brown = Pahute Mesa, Aqua = Frenchman Flat, and Purple = Ranier Mesa.

variation (Fig. 9) is of note. However, with a *p*-value of 0.1712, the significance is poor, thus failing to support any statistical relationship between whole bacterial communities and the major source of radioactivity at NNSS (Table 2). This does not rule out the possibility that individual bacterial groups could respond to tritium in the environment.

Archaeal communities responded most strongly to gradients in K^+, pH and temperature; which explained ~19%, 18% and 16% of variation in OTU data, respectively (Fig. 12 and Table 2). Although pH explained a significant proportion of variation, the "best" model (software option) for fitting data excluded this variable. Most samples appear to ordinate strongly along the temperature-potassium continuum. Although tritium accounted for ~8% of the archaeal variation, as was similar for bacterial communities, the *p*-value was 0.2224 again, failing to support any significant relationship (Table 2).

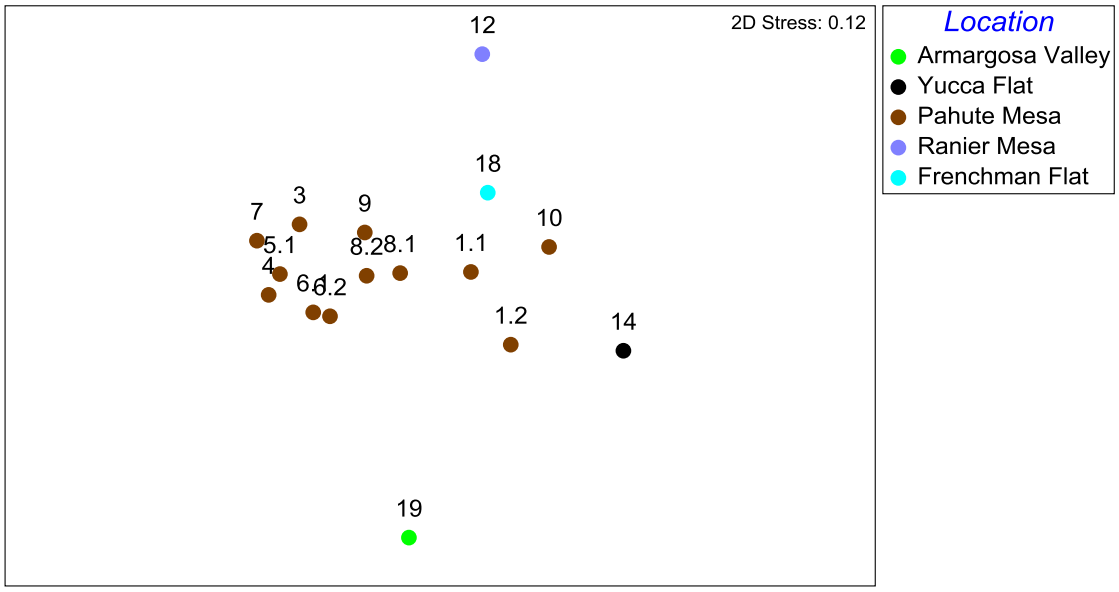


Fig. 10. Two-dimensional (2D) Non-metric Multidimensional Scaling (NMDS) of NNSS/NTTR subsurface samples by archaeal Illumina OTUs. Interpoint distances are based on Bray-Curtis similarities. Numbers relate to well (Fig. 1), and dot color scheme: Green = Armargosa Valley, Black = Yucca Flat, Brown = Pahute Mesa, Aqua = Frenchman Flat, and Purple = Ranier Mesa.

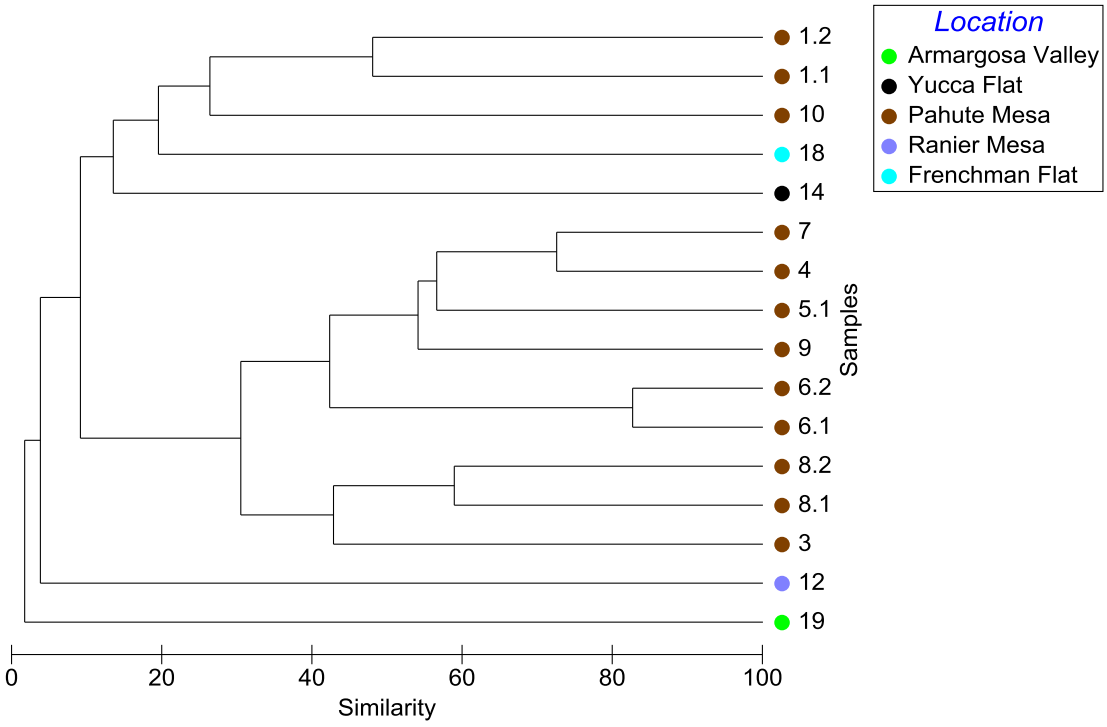


Fig. 11. Hierarchical clustering of bacterial communities in NNSS/NTTR wells sequenced using the Illumina platform. Distances are based upon Bray-Curtis similarities. Numbers relate to well (Fig. 1), and dot color scheme: Green = Armargosa Valley, Black = Yucca Flat, Brown = Pahute Mesa, Aqua = Frenchman Flat, and Purple = Ranier Mesa.

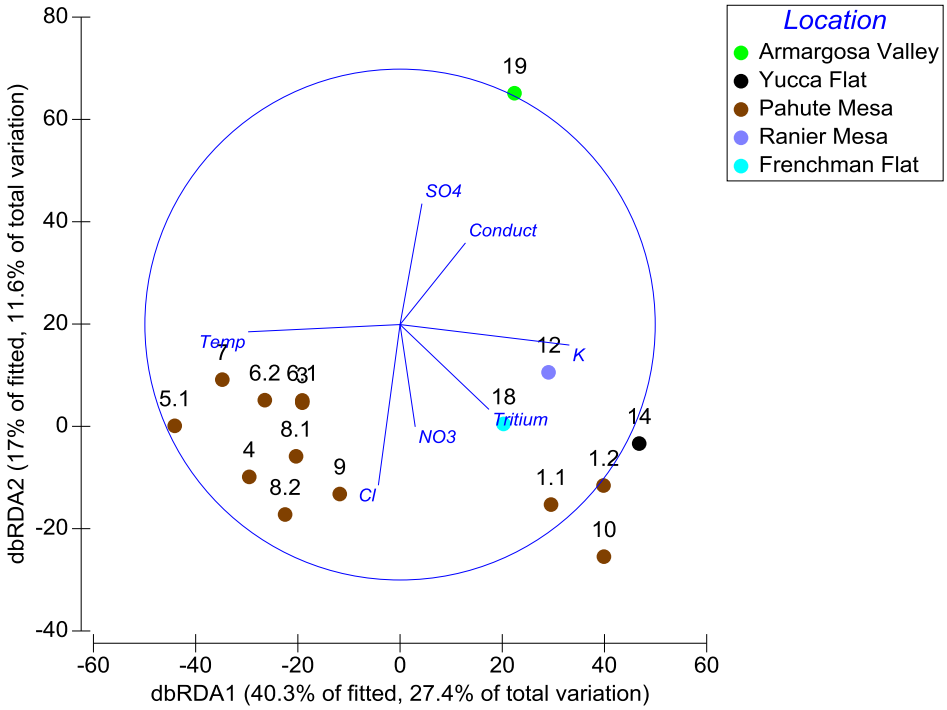


Fig. 12. Distance-based Redundancy Analysis (dbRDA) of NNSS/NTTR wells based on archaeal OTUs and measured geochemistry. Vectors represent geochemical measurements of temperature (Temp), conductivity (Conduct), chlorine (Cl), nitrate (NO_3^-), sulfate (SO_4^{2-}), potassium (K^+), and tritium. Percentages describe variation explained (total and fitted) in the communities by measured geochemistry. Numbers relate to well (Fig. 1), and dot color scheme: Green = Armargosa Valley, Black = Yucca Flat, Brown = Pahute Mesa, Aqua = Frenchman Flat, and Purple = Ranier Mesa.

Table 2. Distance-Based Linear Modeling Significance (*p*) of and Proportion of OTU Variation Explained by Chemical and Physical Variables.

	Bacterial DistLM		Archaeal DistLM	
Variable	***p****	**Proportion**	***p** **	**Proportion**
pH	0.1542	5.33E-02	**0.0011**	0.18143
Temperature	**0.0001**	0.11654	**0.0042**	0.16128
Conductivity	**0.0052**	7.52E-02	0.2022	8.18E-02
Dissolved O_2	0.1239	5.49E-02	0.1559	9.01E-02
Tritium	0.1712	5.50E-02	0.2224	8.58E-02
SO_4^{2-}	0.1965	5.19E-02	0.2017	8.36E-02
NO_3^-	**0.0442**	6.46E-02	0.1191	9.70E-02
Cl^-	0.1239	5.74E-02	0.4716	6.38E-02
K^+	**0.0004**	8.25E-02	**0.0003**	0.19611

* Bold values indicate statistical significance ($p \leq 0.05$).

3.4. PHYLOGENETICS: AMPLIFIED 16S rRNA CLONE LIBRARIES

3.4.1. BACTERIA

Twenty-one DNA samples were used to construct 1,577 complete (~1500 bp) and 121 partial (~700 bp) bacterial 16S rRNA gene sequences. Libraries from ER-EC-15-Middle and ER-EC-15 Lower were not completed in time for this report. OTUs are arranged and displayed by class in Figure 13. Overall, patterns of diversity reflect those obtained from Illumina libraries of the same sites. For example, Proteobacteria are prominent across the sample set, represented by one or more of the classes Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria. These range from 1.1% Deltaproteobacteria in U12n.10 to 85% Betaproteobacteria in PM 3-2. Correspondingly, wells that were low in dissolved oxygen had high proportions of *Clostridia* (e.g. 56.7% in U12n.10; 28.8% in U19v-PS (Almendro); and 86.2% in BLM-1). Unassigned classes were present at all sites, except ER-EC-12 and U19v-PS (Almendro); with the lowest (1.2%) in Ue-20N#1 and the highest (49%) in ER 5-5.

One possible explanation for the abundance of Proteobacteria (Gram-negative) in many of these samples may relate to sampling- or well-related contamination. It may be of significance, for example, that several of the closest rRNA gene sequence matches for Alpha- and Betaproteobacterial clones (Figure 13, (118, 120)) were from mine service water and drilling fluid detected in other studies. The presence of related sequences here might indicate at least a partial overprint of indigenous subsurface microbial populations by drilling remnants or microorganisms suited to growth on stainless steel well casings.

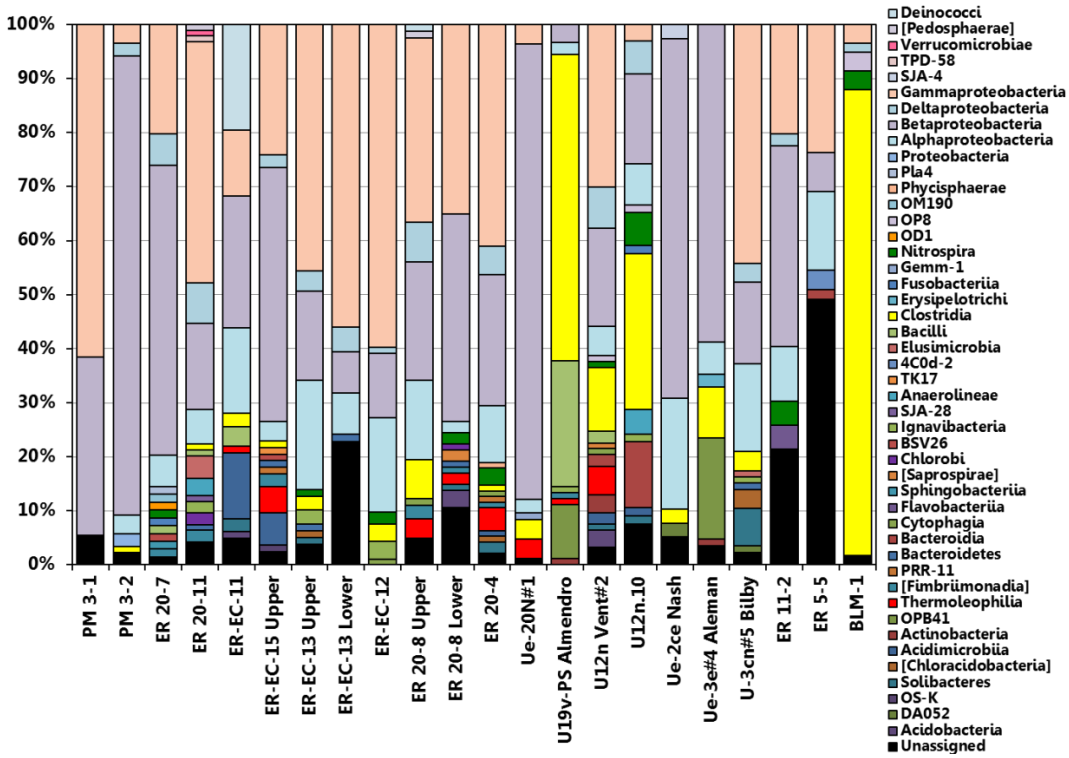


Fig. 13. Summary of bacterial 16S rRNA genes from PCR-amplified clone libraries for 22 sample sites at the class level. A total of 1,577 complete and 121 partial sequences were clustered into OTUs at the phylogenetic level of class.

Firmicutes are a large complex group of Gram-positive bacteria found in soil, the human gut, the terrestrial subsurface, and marine environments. Here, Illumina and clone library dataset show this group to be abundant when high temperatures or anaerobic conditions are present (e.g. ER-EC-15 Lower, U19V-PS Almendro, U12n.10, U-3cn#5 Bilby, and BLM-1). Present in seven of the sampled sites, is an uncultured microorganism, initially detected in fractured rock environments accessed via ultra-deep South African mines, *Candidatus Desulforudis audaxviator* ((30, 122), Section 3.9). *D. audaxviator* represents the only known organism to comprise its own ecosystem. Perhaps more cogent to this dataset, it is thought to employ a sulfate-reducing lifestyle relying upon products from the radiolytic decay of water.

3.4.2. ARCHAEA

Archaeal clone libraries from 13 sampled sites generated 77 complete (~1500 bp) and 758 partial (~700 bp) 16S rRNA gene sequences (Figure 14). As with the Illumina libraries, Thaumarcheota were abundant across this dataset, ranging from 18.1% in Ue-20N#1 to 98.6% in ER-EC-15-Upper, with the exception of U-3cn#5 (Bilby) where none were detected. This group dominated in six of the samples. Many unassigned OTUs were observed, as well, ranging from 94.2% in U-3cn#5 (Bilby) to 1.5% in ER-EC-13-Lower. Uncultured archaea belonging to the marine benthic group A (MBGA) were detected at the lowest percentage of 1.1%

in Ue-20N#1 to as high as 28.4% in ER 20-4. The Miscellaneous Crenarcheota Group (MCG) was only detected in U12n Vent#2, at 16.9%.

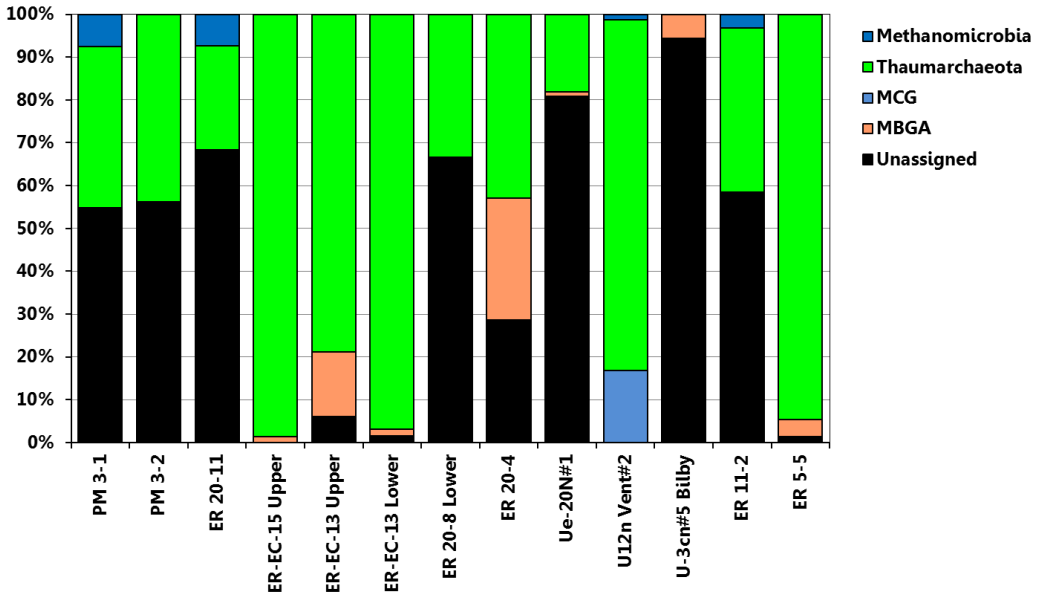


Fig. 7. Archaeal 16S rRNA genes from PCR-amplified clone libraries for 12 wells and 1 tunnel (a subset of samples). For this analysis, a total of 77 complete and 758 partial sequences were clustered into OTUs at the phylogenetic level of class. Note the high abundance of Thaumarchaeota across the dataset.

3.5. ACTINIDE ABSORPTION/DESORPTION EXPERIMENTS

Colloid-Facilitated Radionuclide Transport. As a result of Cold War weapons testing, the NNSS is contaminated with millions of Curies of radioactivity (2); with a substantial portion of that being associated with subsurface aquifers (151). Although most of the radionuclide inventory is in the form of tritium, significant quantities of certain actinides, especially isotopes of Pu (21) also exist. Whereas, it has been argued that radionuclides such as Pu are relatively immobile in the NNSS subsurface due to their low solubility in groundwater (152) and tendency to sorb to rock surfaces (153), colloids are increasingly thought to play a role in the transport of non-soluble contaminants (20, 21, 154-158).

Operationally defined as particles of less than 1 micron (μm) in diameter, colloidal particles can remain suspended in water and are ultimately transported at groundwater velocities (159, 160). Mineral colloids are well known to mediate the transport of Pu and actinides (156-158) in groundwater and have been studied as an agent influencing Pu mobility at NNSS (21, 154, 155). At the NNSS, for example, radionuclides associated with the colloid fraction, including ^{239,240}Pu from an underground test, are known to have been transported over a distance of 1.3 km in the subsurface (21). Although the colloids in this case were thought

to be mineral (e.g., zeolite and illite), other work at the NNSS (154) suggests that organic colloids may likewise be important, especially in the tunnel-associated tests (e.g. U12n.Vent#2 and U12n.10 in this work). It is known that a variety of microbially-produced or altered materials, such as citric acid, fulvic acid, and extracellular polymeric substances (EPS), complex with Pu in solution (161, 162). Furthermore, recent literature suggests that Pu(V) sorption onto mineral particles (silica) is enhanced by the presence of colloidal organic matter (163).

Microorganisms and some of their exudates (e.g. EPS) fall within the colloid fraction as well (164) and the interactions between microorganisms and actinides, especially U (13, 48, 66, 165, 166)and Pu (54, 68, 167) are well known. The effects of so-called "biocolloids" on actinide transport in aquatic media have also been considered, albeit to date to a limited extent (68, 168, 169). Potential biocolloids that can bind to and facilitate the transport of actinides span several orders of magnitude in size fraction and include live cells (growing or stationary phase) or dead cells (~1 µm), cell exudates (from lysed cells; 0.1-1 µm), and EPS (0.001-0.1 µm). As part of this study, we investigated the relative effects of these different biological fractions on the sorption of U and Pu. Batch experiments, performed in

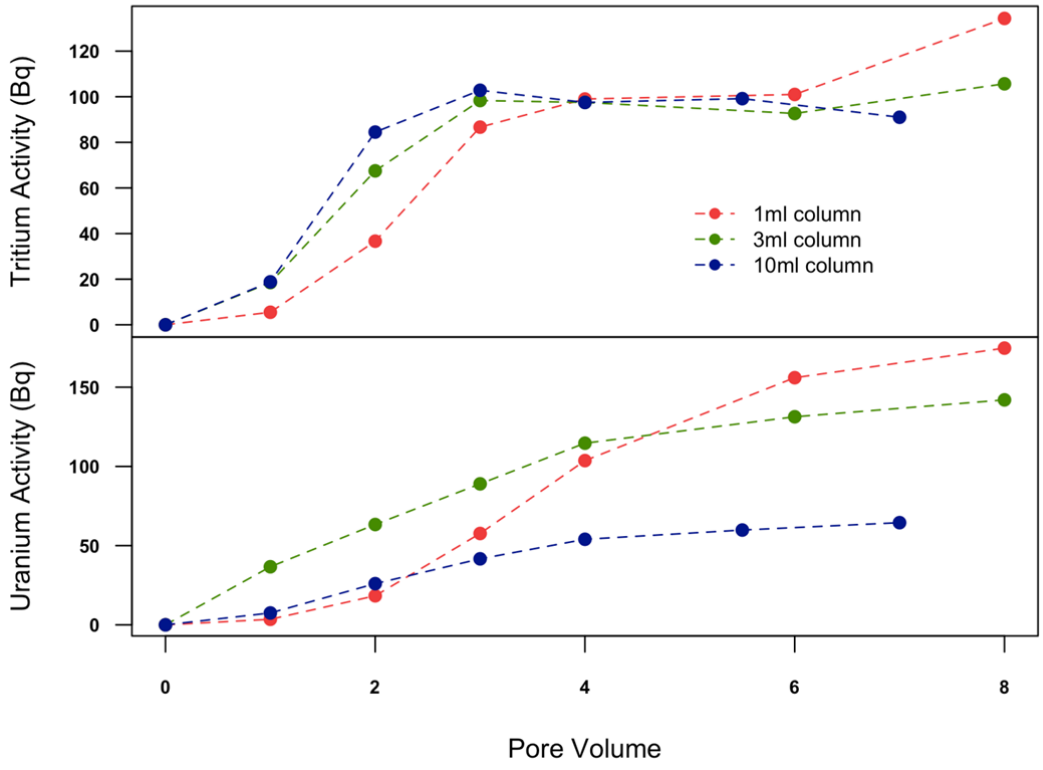


Fig. 15. Sorption of U-233 onto packed columns of NNSS tuff. Upper) Tritium was used as a passive tracer to track the movement of fluid through the column. Lower) Cumulative activity (In Bq) of U-233 removed from column attached to bacterial cells.

synthetic groundwater media, with volcanic tuff (the dominant mineral form found over most of the NNSS) and bacterial strains primarily from the site were used to constrain the potential role of microorganisms as a factor influencing radionuclide transport.

An initial set of experiments indicated that sorption of actinides to tuff may be enhanced variably under different conditions and in the presence of different bacterial species. Sorption kinetics were rapid (<24 hours) and sorption coefficients were relatively high for bacterial-amended treatments of both 233-U and 239-Pu. In these experiments, high-density microbial cultures (10^8 -10^{10} cells/mL) effectively illustrated the propensity of living cells to sorb significant amounts of U-233 in the absence of tuff (Figure 15). These cells also had a higher K_ds (on the order of 10^3-10^4 mL/g) than tuff (Table 3). Lower density cultures were also effective, but sorbed a lower percentage of the total U-233 in solution. Cell cultures also sorbed significantly different amounts of actinide among different bacterial strains (not shown), indicating that not only the density of cells, but the type of organisms present affect sorption in the environment. Incubation of cells with tuff prior to addition of U-233 significantly increased the amount of sorption, indicating that particle-attached vs. free-living cells may also determine the degree of sorption in each environment. At the concentrations of Pu(IV) (~$7x10^{-10}$ M) and cell material used (~10^9 cells/mL), the percent Pu(IV) sorbed was similar across the various treatments. A high percentage (70-90%) of the Pu present sorbed to cells almost instantly (in 0.25 h) suggesting that biological material in the environment would sorb Pu, even under transport conditions with low residence times/high flow rates. Pu remained sorbed in all treatments after 24 hours and cells of *Pseudomonas* U12n.10-R had the highest K_d of the intact cell cultures and sorbed the greatest percentage of Pu (Figure 15), which is part of the reason this isolate was selected for production of EPS described below.

Cells also effectively desorbed some uranium and facilitated removal from tuff (Figure 15), although a significant amount was still eluted when the tuff was washed with a carbonate solution. Column experiments showed that bacterial cultures in minimal salts buffer desorbed significantly more 233-U from tuff than low bicarbonate NNSS water, but less than a high concentration bicarbonate buffer. Overall, the type(s) of bacteria present, the growth phase (e.g. stationary vs. growing or senescing) of the organisms, and whether cells are free-living or attached to tuff appear to have a greater influence on sorption than the type or size of tuff present. Lysed cells of CN-32 had the highest K_d and sorbed the greatest percentage of Pu, possibly due to the increased surface area made available by compromised cell membranes. This observation is also important because it shows that cells do not need to take up Pu into the periplasm or cytoplasm to produce a biosorptive effect. These results support the hypothesis that bacteria may facilitate transport of U-233 (and possibly other actinides) in the NNSS environment. Also notable were the higher K_d values for treatments with higher EPS contents, suggesting that Pu may sorb primarily to EPS, as has been shown for other actinides.

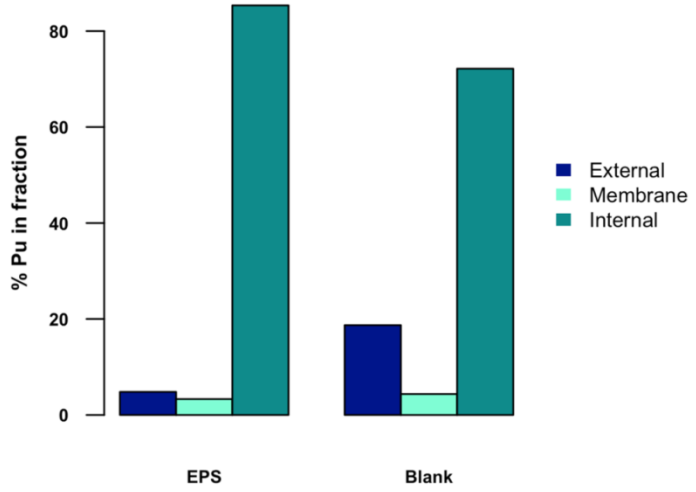


Fig. 16. Dialysis experiment showing the sorption of Pu-239 (IV) to purified exopolysaccharides (EPS) from an NNSS bacterial isolate held within a 3 kDa membrane bag. Note that most of the Pu was associated with the EPS fraction rather than external buffer or bacterial membrane fraction.

These preliminary findings were confirmed with a dialysis experiment (Fig. 16). This experiment compared the diffusion of Pu-239(IV) across a 3kD membrane, performed in the presence and absence of EPS preparations obtained from an EPS-producing *Pseaudomonas* spp. isolate (from the U12n.10 tunnel). These experiments showed enhanced retention of Pu inside the membrane when EPS was present versus the control (including purified membranes) with no EPS. Only 5% of the total Pu added reached the dialysed liquid (external fraction) in the EPS treatment, while over 18% of the Pu by mass balance was found in the dialysis liquid of the control. Slightly less Pu was bound to the membrane itself in the EPS treatment, but the difference was not significant. Overall, this work demonstrated a relatively high K_d for Pu sorption to whole cells, lysed cells and purified exopolysaccharides from this organism. In general, higher K_d values were observed in treatments with higher EPS contents, suggesting that Pu may sorb primarily to EPS as has been shown for other actinides (170, 171).

Table 3. Sorption Coefficients for Tuff and Tuff + Bacteria.

Substrate	Treatment	U-233 activity	Kd (mL/g)
UE12n15A 75-500 µm	NNSS U12n.10 water, unfiltered	1700 Bq/g tuff	30.4 ± 2.59
UE12n15A <75 µm	NNSS U12n.10 water, unfiltered	1700 Bq/g	39.9 ± 0.71
UE12n15A <75 µm	NNSS U12n.10 water, filtered	1700 Bq/g	40.1 ± 0.75
UE12n15A 75-500 µm	NaOH + bacteria	1000 Bq/g	58.2 ± 5.4
UE12t#2 <75 µm	Incubated with GASC3Ar	2000 Bq/g	13.7-42
UE12t#2 <75 µm	NNSS water + GASC3Ar	2000 Bq/g	8-11.6
UE12t#2 <75 µm	NNSS U12n.10 water, filtered	2000 Bq/g	2-3.9
UE12n#8 <75 µm	NNSS U12n.10 water, filtered	2000 Bq/g	9.7 ± 0.97
UE12n#8 <75 µm	NNSS water +GASC3Ar	2000 Bq/g	10.0 ±1.4
UE12n#8 <75 µm	NNSS water +MR-1	2000 Bq/g	16.4 ±1.3
Tuff, devitrified (75-500 µm)	J-13, CO_2	~20,000 Bq	2.1 - 14.5
Tuff, zeolitized (75-500 µm)	J-13, CO_2	~20,000 Bq	3.7 - 20.8
Tuff, vitric (75-500 µm)	J-13	~20,000 Bq	0.2 – 4.6

3.6. CULTIVATION EXPERIMENTS

Traditional cultivation-based methods remain an important tool for evaluating the physiologic potential of microbial communities. For this work, enrichments were prepared by inoculating unfiltered water from sampling sites into a range of defined microbiological growth media targeting expected physiotypes including: aerobic heterotrophs, nitrate reducers, fermenters, sulfate reducers, iron reducers, and methanogens. Given the low DOC values measured in this environment, we anticipated that oligotrophs would be disproportionately dominant in NNSS subsurface ecosystems. In our cultivation studies, despite supplementation with mM concentrations of C and energy sources, typical cell densities in our cultures failed to produce visible turbidity and ranged from 10^5 to 10^6 cells/mL. All sites produced turbid enrichments in liquid media and colonies on plates targeting aerobic heterotrophs. In liquid, cultures attained cell densities up to 10^9 cells/mL. As suggested in our statistical analysis of Illumina datasets, temperature appears to be a primary controller of microbial community structure in this environment. Thus, we tested a range of incubation temperatures for this work (25 to 60°C), with an emphasis on the inoculation's origin site temperature.

3.6.1. Isolates

Isolates were generated from enrichments by either serial dilution, plating onto agar (plates or slants), or the use of cultivation conditions focused on supporting targeted physiologies while inhibiting unwanted physiologies (e.g. aerobic vs anaerobic metabolism). Over the course of this study, a total of 40 isolates were produced from NNSS/NTTR borehole wells/tunnels and the control well BLM-1 (Table 4). All isolated microorganisms belong to the domain, Bacteria. As a considerable investment of time and effort is needed to characterize a microorganism for peer review, especially slow growing anaerobes, only two novel microbes were selected for complete characterization.

Table 4. Isolates.

DRI Isolate	Closest identification microorganism to isolate	ID (%)	Inoculum origin	Medium conditions for culturing	Culturing temperature (°C)
DRI-1	*Desulfotomaculum putei*	99	BLM-1	Lactate and SO_4	55
DRI-2	*Aeromonas salmonicida*	100	U12n vent-2	Lactate and SO_4	25
DRI-3	*Cupriavidus metallidurans*	98	ER-EC-13 upper	$N_2/CO_2/O_2$ and NH_3, NO_2, NO_3	40
DRI-4	*Methylibium petroleiphilum*	96	ER-EC-13 upper	$N_2/CO_2/O_2$ and NH_3, NO_2, NO_3	40
DRI-5	Desulfotomaculum hydrothermale strain HA2	97	ER-EC-13	H_2/CO_2 and SO_4	40
DRI-6	*Moorella glycerini*	96	BLM-1	$H_2/CO_2/N_2$ and SO_4	50
DRI-7	*Desulfotomaculum putei*	99	ER-EC-12	Glucose/tryptone and SO_4	50
DRI-8	*Thermotalea metallivorans*	98	ER-EC-12	$H_2/CO_2/N_2$ and SO_4	50
DRI-9	*Schlegelella aquatica*	99	ER-EC-13 lower	$N_2/CO_2/O_2$ and NH_3, NO_2, NO_3	37
DRI-10	*Caldimonas manganoxidans*	99	ER-EC-13 lower	$N_2/CO_2/O_2$ and NH_3, NO_2, NO_3	37
DRI-11	*Cupriavidus gilardii*	99	ER-EC-13 lower	$N_2/CO_2/O_2$ and NH_3, NO_2, NO_3	37
DRI-13	*Pelotomaculum thermopropionicum*	90	U-3cn5 (Bilby)	N_2 and Peptides	45
DRI-14	*Candidatus Desulforudis audaxviator*	91	U-3cn5 (Bilby)	H_2/CO_2 and SO_4 or CH_4 and SO_4	45
DRI-17	*Sphingopyxis alaskensis* strain RB2256	100	ER 20-8	R2A	25
DRI-18	*Phenylobacterium koreense* strain NBRC 102285	98	ER 20-8	R2A	25

DRI-19	*Phenylobacterium lituiforme* strain FaiI3	99	ER 20-8	R2A	25
DRI-20	*Blastomonas natatoria* strain D2AR31	100	ER 20-8	R2A	25
DRI-21	*Aquabacterium fontiphilum* strain CS-6	99	ER 20-8	R2A	25
DRI-22	*Phenylobacterium conjunctum* strain FWC 21	99	ER 20-8	R2A	25
DRI-23	*Caldimonas hydrothermale* strain Han-85	99	ER 20-8	R2A	25
DRI-24	*Phenylobacterium muchangponense* strain A8	99	ER 20-8	R2A	25
DRI-25	*Roseomonas lacus* strain TH-G33	97	ER 20-8	R2A	25
DRI-26	*Rubrivivax gelatinosus* strain 16	98	ER 20-8	R2A	25
DRI-27	*Schlegelella aquatica* strain wcf1	100	ER 20-8	R2A	25
DRI-28	*Aquabacterium commune* strain B8	97	ER 20-8	R2A	25
DRI-29	*Aquabacterium fontiphilum* strain CS-6	99	ER 20-8	R2A	25
DRI-30	*Thiovirga sulfuroxydans* strain SO07	99	U12n.10	R2A	25
DRI-31	*Sulfuritalea hydrogenivorans* strain sk43H	95	U12n.10	R2A	25
DRI-32	Candidatus *Contubernalis alkalaceticum* clone Z-7904	90	U12n.10	R2A	25
DRI-33	*Hydrogenophaga caeni* strain EMB71	98	U12n.10	R2A	25
DRI-34	*Hyphomicrobium aestuarii* strain ATCC 27483	95	U12n.10	R2A	25
DRI-35	*Holospora curviuscula* strain 02AZ16	85	U12n.10	R2A	25
DRI-36	*Malikia granosa* strain P1	97	U12n.10	R2A	25
DRI-37	*Cytophaga fermentans* strain NBRC 15936	84	U12n.10	R2A	25
DRI-38	*Propionigenium modestum* strain Gra Succ 2	92	U12n.10	R2A	25
DRI-39	*Delftia acidovorans* strain IARI-NIAW1-20	99	U12n.10	R2A	25
DRI-40	*Pedomicrobium manganicum* strain ATCC 33121	95	U12n.10	R2A	25

3.6.2. Characterization of Isolates: DRI-13 and DRI-14

Strain DRI-13 (Table 4) was isolated from the well U-3cn#5 (Bilby) (Figure 17c). Anaerobic unfiltered water samples were taken in 120 mL serum bottles (Fig. 19b) and an enrichment culture was prepared by injecting 1 mL of inoculum into anaerobically prepared 0.1% w/v peptone in SMC medium and incubating at 45°C. The culture was transferred three times after two weeks of growth each time and densities of 10^8 cells/mL could be reached. Genomic DNA was isolated, the 16S rRNA gene was amplified by PCR using universal bacterial primers (section 2.4), and the resulting amplicon was sequenced. Phylogenetic analysis indicates that DRI-13 belongs to the phylum, Firmicutes, and that the closest cultivated relative (90% identity) is *Pelotomaculum thermopropionicum* (172), followed by *Pelotomaculum schinkii,* at 89% (173) (Fig. 18). When the full length 16S rRNA gene sequence from DRI-13 is compared by a BLAST analysis (NCBI), the closest related environmental DNA sequences (uncultured/unpublished bacterial sequences) were related (95-99% identity) to subsurface microorganisms from anaerobic groundwater (e.g. a deep well in Japan and to

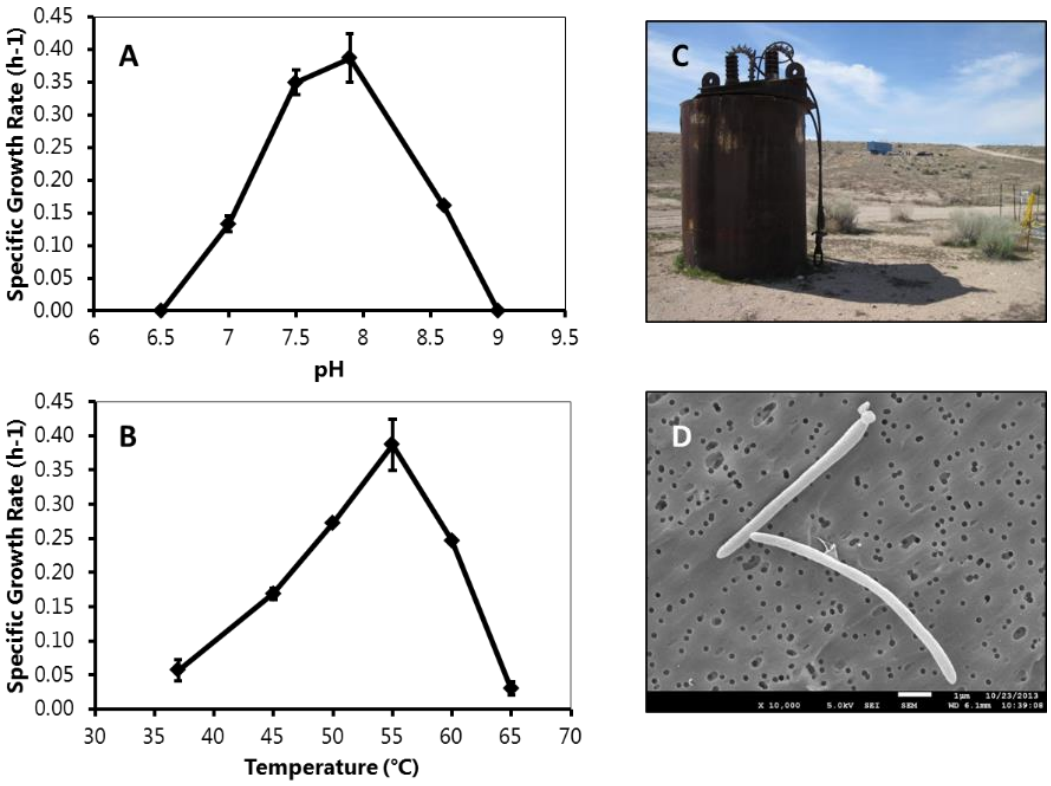


Fig. 17. Some growth characteristics of isolate, DRI-13 under fermentative conditions (e.g. 10 mM fumerate). (A) pH and (B) temperature were plotted vs. specific growth rate. (C) Location, U-3cn#5 (Bilby), where isolate was obtained. Actual sampling operation is visible in the distance. (D) DRI-13 under scanning electron microscopy (SEM). Bar in bottom panel shows relative scale of 1 µm.

those from a Danish geothermal heating system (174)). Scanning electron microscopy (SEM) was used to acquire images of DRI-13 (Fig. 17d).

Characterization of substrate utilization patterns for DRI-13 indicated that H_2/CO_2, formate, lactate, acetate, pyruvate, CH_4, methanol, and propionate, combined with nitrite, nitrate, hydrous ferric oxide (HFO), MnO_2, sulfate, thiosulfate, sulfite, and inorganic sulfur as electron acceptors fail to promote growth in any combination or at any concentration. Glucose, casamino acids, peptone, and yeast extract produce marginal support for growth (doubling time 23 h, data not shown). When grown on fumarate, however, DRI-13 responds robustly, with a doubling time of 3.4 hours. For growth characterizations, 10 mM fumarate was used as a fermentative substrate for optimal temperature and pH characterizations (Figs. 17a and 17b).

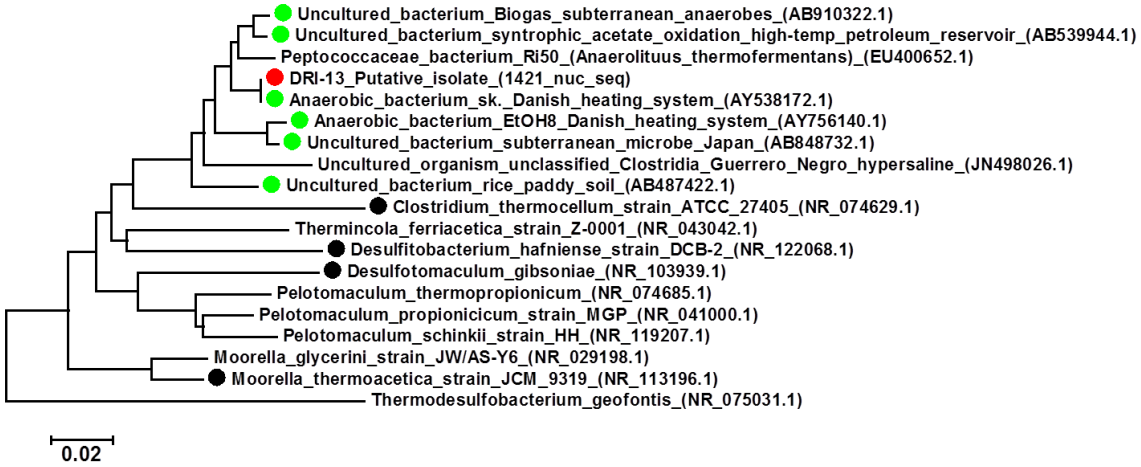


Fig. 18. Phylogenetic position (16S rRNA gene) of bacterial putative isolate, DRI-13 (●). Microorganisms marked with (●) indicate publically-available genomes. Environmental rRNA gene sequences marked by (●) indicate terrestrial subsurface origin. Bootstrap values were based on 1,000 permutations. The scale bar represents 0.02% identity dissimilarity between sequences.

High-quality genomic DNA was extracted from DRI-13 and submitted for sequencing by the Joint Genome Institute (JGI). The completed genome of DRI-13 is 3,649,665 bp with 87.32% coding regions, and a G+C content of 45.14%. The genome contains 3,671 predicted ORFs, 78.63% of which can be attributed to protein coding genes with a known function; whereas, 19.29% of the ORFs have no known functional prediction.

Strain DRI-14 (Table 4) was also isolated from the well U-3cn#5 (Bilby) (Fig. 17c). To obtain this isolate, anaerobic basal medium (25 mL in a 160-mL serum bottle) was inoculated with 1 mL of sample and prepared with a headspace of 99% pure CH_4 at 2 bar, incubated at 45°C. The only cellular morphology that appeared in the subsequent enrichment was a tiny curved rod of ~0.5 to 1.0 µm length. The

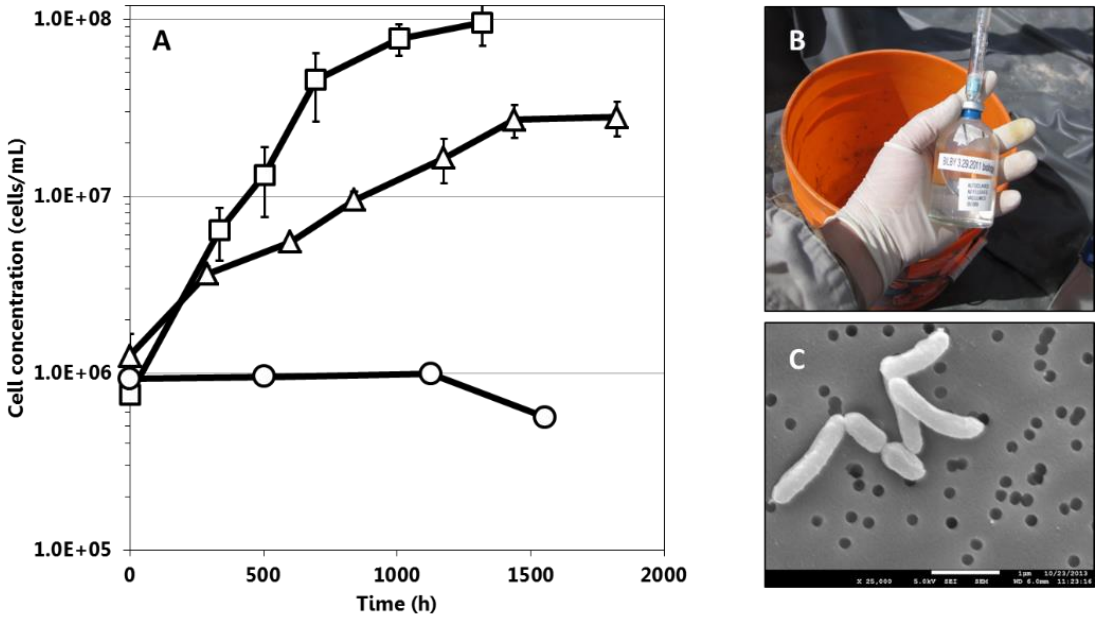


Fig. 19. Growth characteristics of isolate, DRI-14. This strain shows exponential growth in sulfate minimal salt medium with a headspace of 80%:20% hydrogen/carbon dioxide (□), and 100% methane (Δ). There is no growth with a pure nitrogen control (o). (B) Collection of the sample that resulted in DRI-14 enrichment. (C) Scanning electron microscopy (SEM). Bar in bottom panel shows relative scale of 1 µm.

primary enrichment was transferred three times following incubations of 30-50 days. DNA was extracted and the full length 16S rRNA gene was amplified by PCR using universal bacterial primers and then sequenced. Phylogenetic analysis revealed that DRI-14 belongs to the phylum, Firmicutes; with *Ammonifex degensii* as the closest cultivated relative (87% identity, Fig. 20). DRI-14's nearest uncultured neighbor (91% identity) is *Candidatus Desulforudis audaxviator* (122). By BLAST comparison the only other close relatives are uncultured environmental bacteria (94-99%) from subsurface origins in the Southern Nevada desert, Kalahari Shield of Southern Africa, and a Danish geothermal heating system (174, 175).

DRI-14 is a fastidious, strict anaerobe. In our hands, growth is only supported by H_2/CO_2 (80:20), pure CH_4, or formate combined with sulfate or thiosulfate as electron acceptors (Fig. 19a). Substrates tested that do not support growth include fumarate, lactate, acetate, pyruvate, methanol, propionate, glucose, casamino acids, and peptone in combination with a range of potential electron acceptors, including nitrate, nitrite, sulfate, thiosulfate, sulfite, hydrous ferrous oxide (HFO), MnO_2, and S^o. When growing anaerobically with sulfate and in the presence of H_2/CO_2 at 50°C, a doubling time of 5.3 days was obtained; whereas, growth with CH_4 as C source and electron donor required 16 days. SEM images revealed rod-shaped cells of about 0.5 to 1.0 µm long that tend to grow as diplobacilli (Fig. 19c).

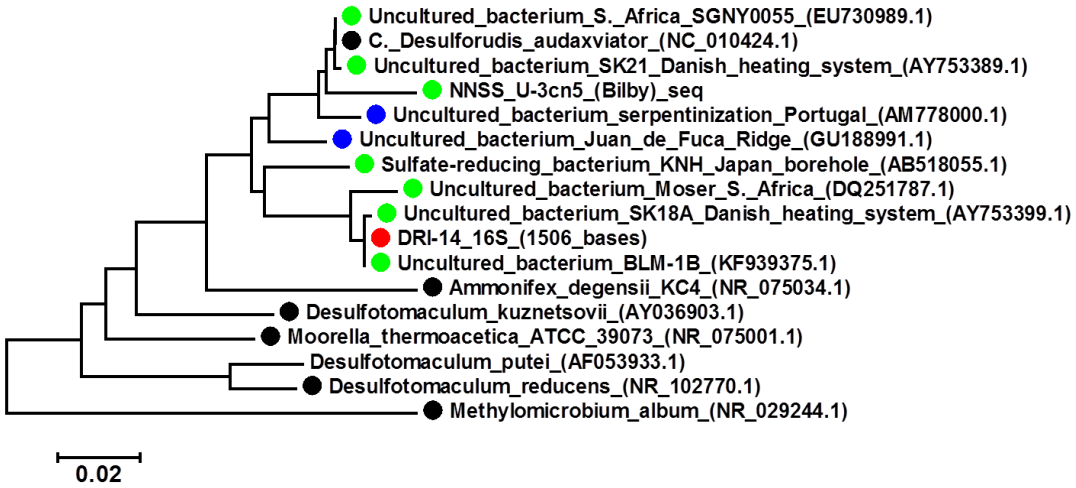


Fig. 20. Phylogenetic position (16S rRNA gene) of bacterial putative isolate, DRI-14 (●). Organisms marked with (●) indicate publically available genomes. Environmental rRNA gene sequences marked by (●) indicate terrestrial subsurface origin. Environmental DNA sequences marked by (●) indicate marine subsurface origin. Bootstrap values were based on 1,000 permutations. The scale bar represents 0.02% dissimilarity between sequences.

A preliminary assessment of DRI-14 contigs from the draft genome revealed subunits from the anaerobic respiratory CoB-CoM hetrodisulfide reductase (EC 1.8.98.1). These gene products are associated with the last metabolic step of archaeal methanogenesis. This is an exciting result, if substantiated, as it has been hypothesized that an anaerobic methanotroph could utilize the methanogenesis pathway in reverse (176-180).

3.7. *CANDIDATUS DESULFORUDIS AUDAXVIATOR*

Over the past 15 or so years, work by members of this team (Co-I Onstott and PI Moser, among others) has revealed the presence of very deep, isolated continental microbial biosystems in several locations around the world (30, 118, 181-183). Coincident with these discoveries, the near-invariant dominance of a single predicted sulfate-reducing phylotype (Fig. 21a, from corresponding fracture fluids, has been reported on a number of occasions (30, 120, 130, 184). In one long-running study, this member of the bacterial phylum, Firmicutes, was found in all flowing borehole samples from below ~1.5 km below land surface (kmbls), across large swaths of the South African Witwatersrand Basin (130). As the presence of this, to date uncultivable, microorganism (denoted *Candidataus Desulforudis audaxviator* (122)) has been detected or inferred as deep as 4 - 5 kmbls (30), it is today regarded as one of the deepest life forms known. In the Witwatersrand project, C. *D. audaxviator* was present at 97 - 99.9% of the total in microbial communities from boreholes at 2.8 – 3.3 kmbls from Mponeng and Driefontein Mines. Because of its near monoculture abundance, it was possible to reconstruct its complete genome from environmental DNA extracts (122). A

subsequent survey of the gene content from *C. D. audaxviator*, considered within the context of the geological and geochemical setting, provided corroborative evidence for the feasibility of a proposed radiolytic mechanism for sustaining isolated subsurface microbial ecosystems (122).

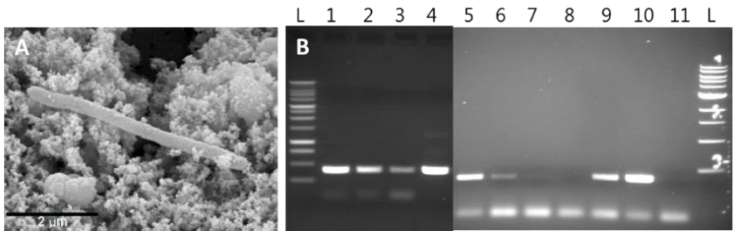


Fig. 21. A) Electron microscopy image of C. *Desulforudis audaxviator* from Chivian *et al* 2008 (122). Bar in bottom of SEM panel shows relative scale of 2 µm. B) PCR assay (16S rRNA gene) for C. *D. audaxviator* applied against DNA extracts from select project samples. Samples probed, from left to right, include: 1) South Africa gold mine, 2) U19ad PS#1A Chancellor, 3) ER-EC-11a, 4) BLM-1, 5) ER-EC-11b, 6) ER 20-4, 7) ER 20-8 Upper, 8) ER 20-8 Lower, 9) ER-EC-12, 10) U-3cn#5 (Bilby), and 11) No template control. (L) is 1 kb DNA ladder.

The central hypothesis that resulted from the African work was that once below the zone of photosphere influence; energy from the decay of radioactive elements could be coupled to biological activity via the radiolysis of water. In South Africa, fracture water samples from below about 2 km depth usually contained high concentrations of H_2 and sulfate (up to 2 M (181) and 40 mM (185), respectively). This observation, coupled with isotopic data and available knowledge about the hosting lithology, supported a model whereby radiolytic dissociation of H_2O during radioactive decay of U, Th, and K accounted for potentially large accumulations of H_2, given sufficient time (181, 186). In addition, this mechanism was expected to produce fluxes of O_2, $OH^{\cdot}$ and H_2O_2, which in turn would be consistent with the production of SO_4^{2-} from readily available FeS_2 and H_2S (181). Whereas, other mechanisms for sulfate production in the subsurface are possible (i.e., the dissolution of barite, if present (184)), the co-generation of both a relevant reductant and oxidant, coupled to radioactive decay in the subsurface represents a credible mechanism for continuous replenishment of the key energy substrates for at least sulfate-reducing bacteria.

It was thus of interest when a DOE Environmental Remediation Sciences (ERSP) Exploratory project (*Characterization of Microbial Communities in Subsurface Nuclear Blast Cavities of the Nevada Test Site*) revealed the presence of 16S rRNA gene sequences with about 98% identity to the African type strain of C. *D. audaxviator* in fluids sampled in 2008 from a tunnel at the NNSS (U12n.10). The observation of the one deep microorganism known to live through the utilization of radiogenic H_2 was invoked as a justification for the proposal that subsequently supported this work.

This line of study has expanded significantly during the course of our SBR project. One early project activity was to develop and test a presence/absence assay for C. *D. audaxviator*. This assay utilized specific PCR primers, designed to amplify a 450 bp segment of the 16S rRNA gene of C. *D. audaxviator*. The primers were

validated on DNA extracts from South African environmental samples known to contain C. *D. audaxviator*. DNA extracts from a subset of NNSS/NTTR sites were subsequently probed and included U19ad PS#1A (Chancellor), ER-EC-11, ER 20-4, ER 20-8 Upper, ER 20-8 Lower, ER-EC-12, U-3cn#5 (Bilby), and BLM-1 (Fig. 21b). The fact that C. *D. audaxviator* was detected in all sites, except ER 20-8 Upper and Lower, suggests that this microorganism is present across much of the DVRFS, at least as a member of the rare biosphere.

C. *D. audaxviator* and close relatives were ultimately detected in amplified rRNA gene libraries from five boreholes and two tunnels addressed by this study. Since the 96-well format utilized by this method only detects relatively abundant microorganisms, an exciting result of this study is that C. *D. audaxviator* and its allies are among the more abundant taxa across this collection of NNSS subsurface aquifer samples. Figure 22 summarizes these data and assesses the current global distribution of C. *D. audaxviator*-related sequences in the NCBI database. One conclusion that remains true, as of this writing, is that the submission of C. *D. audaxviator* to NCBI from environmental samples remains relatively rare. The inference from this observation that C. *D. audaxviator* is correspondingly rare in the environment outside of South Africa and the NNSS/NTTR, however, may be unsupported if one considers that most environmental bacterial surveys these days rely upon short Illumina and 454 Pyrotag sequences. This factor reinforces the value of completing full-length PCR-amplified 16S rRNA gene libraries, such as were conducted here. Figure 22 also shows the phylogenetic relationship of DRI-14 (section 3.8.2) to C. *D. audaxviator.* To the best of our knowledge, this strain is the closest cultivated microorganism to C. *D. audaxviator.*

One objective of this study was to examine the relationship between our detection of C. *D. audaxviator*-like rRNA gene sequences to radiation and ultimately radiogenic H_2 and SO_4^{2-} production. Even though the first two sites from which C. *D. audaxviator* was detected during this project were indeed radioactive (U12n.10 and ER-EC-11), ultimately, this hypothesis appears to be unsupported. Firstly, several of the wells in which C. *D. audaxviator* was detected (ER-EC-12, ER-EC-13, and Ue-3cn#5 (Bilby) have low or background concentrations of tritium (Table 1). Moreover, C. *D. audaxviator* was not detected in libraries from some of the most radioactive wells (ER 20-7, ER 20-11, U19Vps#1(Almendro), UE-2ce (Nash), and UE-3E#4(Aleman)). In fact, fluids from BLM-1, with conditions of temperature, sulfate, and anaerobicity similar to the African mines (except for much lower H_2 concentrations (0.008 vol/%, Table 1)), had more rRNA library clones affiliated with the genus *Desulforudis* than any other sample (~50% of all clones). BLM-1 is far from the NNSS and presumably free of anthropogenic radioactive contamination. However, this site has never been surveyed for natural radioactivity.

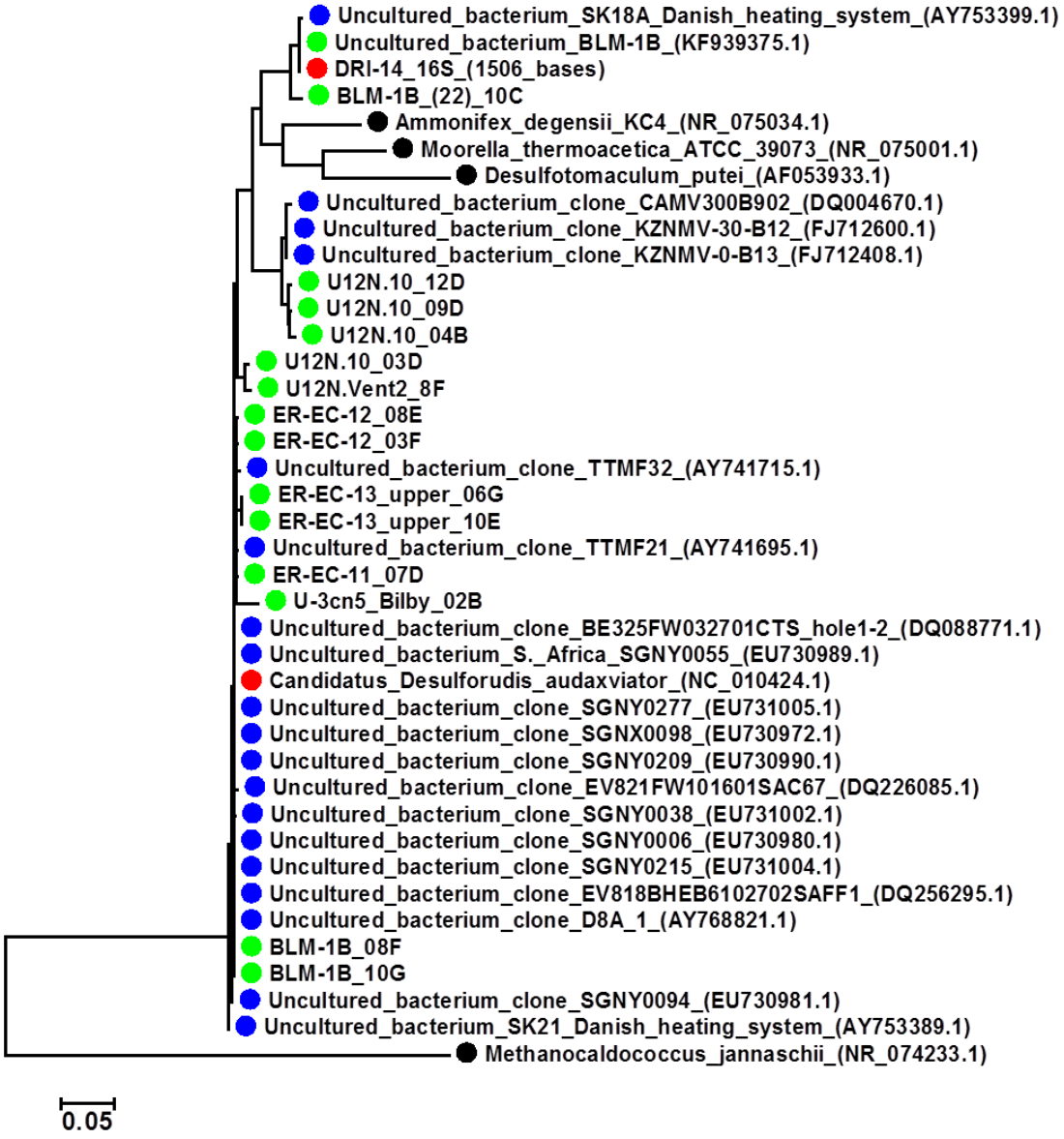


Fig. 22. Nearest neighbor phylogenetic tree of NNSS/NTTR bacterial 16S rRNA gene clones related to C. *Desulforudis audaxviator* and isolate DRI-14 (●). Clones from environmental samples associated with this study are marked with (●), Environmental sequences from NCBI are marked with (●), and cultured type organisms are marked with (●). Bootstrap values were based on 1,000 permutations. The scale bar represents 0.05% dissimilarity between sequences.

Overall, no correlation between the presence or abundance of C. *D. audaxviator* (positive or negative) and any environmental variable other than dO_2 (inversely correlated) was evident from this dataset. Thus, whereas, the presence of this enigmatic microbe from a range of sites at the NNSS was unexpected and intriguing, we do not know which factor explains its appearance here.

4.0 CONCLUSIONS

The work described here revealed the presence of diverse microbial communities located across 19 subsurface sites at the NNSS/NTTR and nearby locations. Overall, the diversity of microorganisms was high for subsurface habitats and variable between sites. As of this writing, preparations are being made to combine the Illumina sequences and 16S rRNA clone libraries with other non-NNSS/NTTR well sites of Southern Nevada Regional Flow System for a publication manuscript describing our very broad landscape scale survey of subsurface microbial diversity.

Isolates DRI-13 and DRI-14 remain to be fully characterized and named in accordance with the conventions established by Bergey's Manual of Systematic Bacteriology. In preparation to be published, these microorganisms will be submitted to the American Type Culture Collection (ATCC) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).It is anticipated that the data resulting from this study in combination with other data sets that will allow us to produce a number of publications that will be impactful to the subsurface microbiology community.

5.0 PROJECT TASKS and OUTCOMES

As with any long-running, field-intensive project, project scope shifts, in response to sampling opportunities, are likely. This was the case with this project. Specifically, as the project progressed, we encountered a much higher number of high-quality sampling opportunities than was initially anticipated. As a result, to take full advantage of this historic opportunity, the descriptive portion of this project took on a correspondingly great proportion of emphasis. Listed below are the stated objectives as they appeared in our original proposal and brief backward-looking commentary relating to our ultimate ability to meet these goals.

Task 1. Characterize radiation environment and corresponding geochemical features of select NNSS sites. A comprehensive profiling of major radioisotopes from the well fluids was compiled, primarily utilizing data provided by the Underground Test Area Activity and its affiliates (see Table 1). As these analyses were conducted by other entities, their completion and reporting is beyond our control. For the most part, we now have access to these data, but in some cases these have not been reported yet, often the result of budget cuts.

Task 2. Model production of microbial substrates via radiochemical reactions, and predict dominant microbial reactions from a thermo dynamic perspective. Geochemists Workbench Professional was obtained and is located in the lab of funded collaborator, TC Onstott. Given that some of the required

inputs for the model were not available to us (see Task 1), this task was not fully implemented.

Task 3. Employ gas compositional analysis and stable isotope measurements of water and gases to constrain the origin of H_2 and hydrocarbon gases and their role in supporting the microbial community. This task ultimately could not be pursued because of concerns about radioactivity damaging equipment or permanently contaminating entire laboratories at the University of Toronto, where this work was to be performed.

Task 4. Characterize microbial community structure of radioactive sites in relation to non-radioactive reference sites with particular reference to *Desulforudis audaxviator*. Given the high sample volume ultimately obtained, addressing this "low-hanging fruit" task ultimately became the major analytical focus of our project. To fully address this task, several 16S rRNA gene-based analyses, including PCR-amplified clone libraries and next generation (Illumina) sequencing, were conducted across the entire sample set. A major output of this work was the statistical analyses (Figs. 9 and 12) pinpointing controllers of comparative microbial diversity between wells based on chemical data.

Task 5. Identify major functional genes driving these systems. GeoChip analysis targeting functional genes used by microbes in nutrient cycling was initially proposed as a possible means of exploring the potential relevant functions of microorganisms at the site. Ultimately, we determined that characterization of select isolates from the site would be more cost effective and provide detailed understanding of individual unique metabolism instead of a generalized spectrum of metabolite profiling.

Task 6. Obtain and characterize collection of relevant microorganisms from NTS subsurface habitats. Water samples from a number of boreholes were utilized for cultivation exercises and a collection of 40 representative microorganisms, encompassing a range of relevant physiotypes, was obtained (Table 3). Up to nine of the isolates are potentially novel species, four are new genera, and one is a possibly a new order or family of bacteria. Focus was given to two microorganisms isolated from borehole U-3cn#5 associated with the Bilby nuclear test, one representing a new genus and the other potentially a new order or family in the phylum, Firmicutes.

Task 7 (Optional). Establish radiation-driven microcosm demonstration. In the proposal, the construction of an artificial radiation-fueled microcosm for the enrichment of organisms that could survive from radiolysis byproducts was forwarded as an optional activity. Although we remain dedicated to this concept, the experimental set-up turned out to be untenable due to logistical limitations in the facility where this work was to be performed and several long shut-downs of this facility that were beyond our control.

6.0 PRESENTATIONS

*,[1]**Moser, D.P.** 2009. Desert windows into a deep wet biosphere. Devils Hole Workshop, Furnace Creek, CA, 05/06/2009. Contributed Talk ([2]10%).

*,[1]**Moser. D.P.** 2009. Deep Earth life and the Nevada Test Site connection. Community Environmental Monitoring Program (CEMP) workshop, Ely, NV. 07/29/2009. Invited Talk (30%).

*,[1]**Fisher, J.C., S.A. Faye, K.R. Czerwinski, D.P. Moser.** 2009. Microbial effects on the sorption of uranium: kinetics and distribution ratios for tuff samples from the Nevada Test Site. Migration 2009, Richland, WA. 08/09/2009. Contributed Poster (30%).

***Moser, D.P.** 2009. Finding common ground, C-DEBI Workshop, Big Island, HI, 10/19/2009. Contributed Talk (30%).

***Moser, D.P.** 2010. Ash Meadows unseen: microbial biogeochemistry of a new biodiversity hotspot. Ash Meadows Workshop, Pahrump, NV, 02/10/2010, Contributed Talk (25%).

***Moser, D.P.** 2010. Earth's deep biosphere and the Nevada Test Site connection. Lake Mead Chapter, Health Physics Society, 02/13/2010. Invited Talk (90%).

Bruckner J.C., J.C. Fisher, M. Zavarin, K. Czerwinski, M.J. Daly, R.E. Lindvall, M. Marshall, C. Russell, and *D.P. Moser. 2010. Characterization of microbial communities in subsurface nuclear blast cavities of the Nevada Test Site. DOE-Environmental Remediation Sciences Program (ERSP) 4th Annual PI meeting. Washington, D.C. 03/29/2010. Poster (100%).

Alfred P. Sloan Foundation, Deep Carbon Observatory. Deep Life Directorate White Paper: Deep subsurface microbiology and the Deep Carbon Observatory. 2010. Co-chairs: Mitchell Sogin (MBL), Katrina Edwards (USC), Steve D'Hondt (URI). Moser, D.P. and Bartlett, D. Group Leaders (10%).

***Moser, D.P.** 2010. Earth's deep continental biosphere: a previously unrecognized rock-hosted biome? Origins Lecture. McMaster University, Hamilton, ON. 04/05/2010. Invited Talk (40%).

***Moser, D.P.** 2010. Edge of the abyss: new windows into the deep continental biosphere. Arizona-Nevada Branch American Society for Microbiology. Las Vegas, NV. 04/22/2010. Contributed Talk (50%).

Kersting, A., *M. Zavarin, B. Powell, D. Moser, S. Carroll, R. Maxwell, Z. Dai, R. Williams, S. Tumey, P. Zhao, R. Tinnacher, P. Huang, R. Kips. 2010. Environmental transport of plutonium: biogeochemical processes at femtomolar

concentrations and nanometer scales. UGTA TIE Annual Meeting, Desert Research Institute, Las Vegas, NV, 05/2010, Poster (5%).

***Moser, D.P., J. Bruckner, J. Fisher, K. Czerwinski, R.E. Lindvall, C. Russell, M. Zavarin.** 2010. Characterization of microbial communities in subsurface nuclear detonation cavities of the Nevada Test Site. UGTA TIE Annual Meeting, Desert Research Institute, Las Vegas, NV, 05/2010, Contributed Poster (100%).

***Moser, D.P., Bruckner, J.C., Ehrsam, M., Fisher, J.C., Gihring, T., Lin, L.H., Newburn, J., Onstott, T.C.** 2010. Poster. Globally-distributed lineages of putative sulfate reducing firmicutes from varied deep subsurface habitats. 137A. International Society for Microbial Ecology (ISME). Seattle, WA. 08/23/2010. Contributed Poster (50%).

***Fisher, J., R. Tinnacher, M. Zavarin, A. Kersting, K. Czerwinski, D. Moser.** 2010. Microbially-produced organic matter and its role in facilitating plutonium transport in the deep vadose zone. American Geophysical Union Fall Meeting, San Francisco, CA, 12/17/2010, Contributed Talk (100%).

***Moser, D.P.** 2011. Globally-distributed lineages of deep subsurface microoganisms: what are *Desulforudis* and SAGMA trying to tell us? Deep Carbon Observatory Conference. Bloemfontein South Africa. 01/20/2011. Contributed Poster (25%).

***Moser, D.P.** 2011. The Dark Planet: Explorations of Earth's intraterrestrial biosphere. Public lecture and Garden Route Botanical Garden fundraiser at Fancourt Country Club, George, South Africa. 02/2011. Invited Talk (50%).

***Moser, D.P.** 2011. Deep subsurface microbial ecology. Guest lecture for UNLV School of Life Sciences Microbial Ecology Class. Penny Amy = instructor. 02/10/12. Invited Talk (35%).

***Moser, D.P.** 2011. The Dark planet: explorations of Earth's intraterrestrial biosphere. Center for Biofilm Enineering (CBE), Montana State University. 04/2011. Invited Talk (50%).

***Moser, D.P., J. Bruckner, J. Fisher, M. Zavarin, C. Russell, K. Czerwinski, R.E. Lindvall, S. Roberts, T. Onstott, B. Sherwood Lollar.** 2011. Radiochemically-supported microbial communities: a potential mechanism for biocolloid production of importance to actinide transport. DOE Subsurface Biogeochemical Research (SBR) PI Meeting. Washington D.C. 04/26-28/2011 (100%).

***Wheatley, A.** 2011. Microbial explorations of a new window into the Death Valley Deep Hydrological Flow System. Southern Nevada Regional High School Science Fair. UNLV. 04/2011. Contributed Poser (25%).

***Wheatley, A., King. M., Moser, D.P.** 2011. Microbial communities of deep wells of Amargosa and Death Valley. Devils Hole Workshop, Pahrump, NV,05/05/2011. Contributed Poster (25%).

***Moser, D.P.** 2011. Status Report: Desert windows into deep microbial ecosystems of Death Valley, Amargosa Valley, and the Nevada National Security Site. Devils Hole Workshop. Pahrump. NV, 05/05/2011. Invited Talk (50%).

***Wheatley, A.** 2011. Microbial explorations of a new window into the Death Valley Deep Hydrological Flow System. Intel International High School Science Fair. Los Angeles, CA. 05/11/2011. Contributed Poster (25%).

***Moser, D.P.** 2011. Looking for life in all the wrong places: how basic and applied geomicrobiology at DRI are leading to new discoveries at the fringe of biology. Part I. DRI Foundation Retreat. Carmel Valley, CA. 05/13/2011. Invited Talk (50%).

***Moser, D.P.** 2011. Looking for life in all the wrong places: how basic and applied geomicrobiology at DRI are leading to new discoveries at the fringe of biology. Part II. DRI Foundation Retreat. Carmel Valley, CA. 05/13/2011. Invited Talk (50%).

***Moser, D.P.** 2011. Deep life and the NNSS connection. Community Environmental Monitoring Program (CEMP) Workshop, Brian Head, UT, 07/26/2011. Invited Talk (100%).

***Moser, D.P.** 2011. Some new windows into terrestrial deep subsurface microbial Ecosystems. American Geophysical Union. San Francisco, CA, 12/08/2011. Invited Talk (40%).

***Moser, D.P.** 2011. Microbial ecosystems from the deepest regions of the terrestrial deep biosphere. American Geophysical Union. B54B Life Under Stress III. Special session in memory of Dr. James H. Scott. San Francisco, CA, 12/09/2011. Invited Talk (25%).

Moser, D.P., C. Russell, J. Thomas, G. Miller, G. Slater. 2011. Assessing the possible role of microorganisms in altering ^{14}C and ^{13}C signatures in deep fractured rock aquifers. Deep Carbon Observatory Concept Paper (10%).

Ramunas Stepanauskas, William P. Hanage, Tullis. C. Onstott, Esta van Heerden, Duane Moser, Beth Orcutt. 2011. Charting the evolution of subsurface microbes by single cell sequencing and population genetics. Deep Carbon Observatory Concept Paper (10%).

***Moser, D.P.** 2012. Deep subsurface microbial ecology. Guest lecture for UNLV School of Life Sciences Microbial Ecology Class. Brian Hedlund = instructor. 02/10/12. Invited Talk (35%).

***Moser, D.P.** 2012. Looking for life in all the wrong places: radiation-fueled deep life at NNSS and beyond. Air Monitoring Users Group, Palace Station, Las Vegas, NV, 04/27/12. Invited Talk (90%).

***Moser , D., J. Fisher, J. Bruckner, M. Zavarin, C. Russell, K. Czerwinski, R. E. Lindvall, S. Roberts, T. Onstott, B. Sherwood Lollar.** 2012 Radiochemically-supported microbial communities: a potential mechanism for biocolloid production of importance to actinide transport. DOE Subsurface Biogeochemical Research (SBR) Annual PI Meeting. Washington, DC. 04/30/2012 – 05/02/12Poster (100%).

***Moser, D.P., B. Sherwood Lollar, G.F. Slater, T.C. Onstott, J. Bruckner, J. Fisher, J. Reihle.** 2012. Finding the biotic fringe in the continental deep subsurface. Session: The Limits of Microbial Life. American Society for Microbiology, Annual Meeting, San Francisco, CA 06/19/12. Invited Talk (40%).

***Moser, D.P.** 2012. Status: continental sites. NASA Astrobiology Institute, Life Underground All Hands Meeting. Catalina Island, CA 10/31/12. Talk (35%).

***Moser, D.P.** 2012. Underground Observatories. Deep Carbon Observatory Deep Life Mini workshop. Hilton Hotel, San Francisco, CA 12/02/12. Invited talk (20%).

***Moser, D.P.** 2012. Deep microbial ecosystems of the U.S. Great Basin: a second home for *Desulforudis audaxviator*? Invited talk. Session: A Census of Deep Life: Putting a Face on the Subsurface Biosphere. American Geophysical Union, San Francisco, CA. 12/07/12. Invited Talk (50%).

***Moser, D.P., Sherwood Lollar, T.C., Kieft, T., Fisher, J.C., Bruckner, J. Hamilton-Brehm, S., Wheatley, A.** 2013. Enigmatic deep life: insights from microbial "sightings" in continental fractured rock habitats. Alfred P. Sloan Foundation's Deep Carbon Observatory Annual Science Conference. National Academy of Sciences, Washington DC. 03/04/13. Deep Life Directive Keynote. Invited Talk (50%).

***Moser, D.P.** 2013. Deep subsurface microbiology. Guest lecture for UNLV School of Life Sciences Microbial Ecology Class. Penny Amy = instructor. 04/01/13. Invited Talk (35%).

***Moser, D.P.** 2013. Enigmatic deep and atmospheric life. Air Monitoring Users' Group. Annual Conference. Palace Station Casino. 04/30/13. Keynote Address, Invited Talk (50%).

***Hamilton-Brehm, S.D., J. Fisher, G. Zhang, and D.P. Moser.** 2013. Cultivation of cosmopolitan deep subsurface microbial lineages from the Southern Great Basin and Ultradeep African Gold Mines. Devils Hole Workshop. Furnace Creek,

CA, 05/02/13. Contributed Poster (100%).

***Moser, D.P.**. 2013. Underground microbial oases of the Southern Great Basin, USA, 2013 Devils Hole Workshop. Furnace Creek, CA, 05/03/13. Keynote Address, Invited Talk (30%).

***Thomas, J.M., D.P. Moser, J.C. Fisher, J. Reihle, A. Wheatley, R.L. Hershey, C. Baldino, D. Weissenfluh.** 2013. Using water chemistry, isotopes and microbiology to evaluate groundwater sources, flow paths and geochemical reactions in the Death Valley Flow System, USA. Water Rock Interactions Conference. Grenoble, France, 06/13/13 (25%).

***Moser, D.P.** 2013. Microbiology at NNSS. Invited presentation and progress report to Bill Wilborn, Underground Test Area Program Lead. 07/19/13. Invited Talk (100%).

***Stewart, L., S.D. Hamilton-Brehm, A.J. McKenna and D.P. Moser.** 2013. Culturing bacteria from environments in the desert deep subsurface and hypersaline playas. Student Science Fair. UNLV. 08/03/13. Contributed Poster (100%).

***Moser, D.P. S. Hamilton-Brehm, G. Zhang, A. Wheatley, Willever, K., McKenna, A., Khan, M., Devita, M.** 2013. Discussions with the unseen witness: DRI's Environmental Microbiology and Astrobiology Labs. DRI Executive Staff Meeting, Features Faculty Member. 11/18/13. Invited Talk (35%).

***Moser, D.P.** 2014. Deep subsurface microbial ecology. Guest lecture for UNLV School of Life Sciences Microbial Ecology Class. Brian Hedlund = instructor. 04/10/14. Invited Talk (35%).

***Moser, D.P.** 2014. The Moser Lab: recent discoveries and future aspirations. DRI. DEES Environmental Microbiology Workshop. Las Vegas, NV 05/08/14. Invited Talk (35%).

***Moser, D.P., Kruger, B., Hamilton-Brehm, S., Sackett, J.** 2014. Moser lab overview. NASA Astrobiology Institute, Life Underground All Hands Meeting. Catalina Island, CA 09/15/14. Talk (15%).

*** Moser, D.P., S.D. Hamilton-Brehm, G. Zhang, J. Fisher, B. Kruger, J. Thomas, A. Wheatley, L. Stewart, T.C. Onstott, B. Sherwood Lollar.** 2014. Natural and manmade windows into deep microbial communities of the Death Valley Regional Flow System, USA. International Symposium for Subsurface Microbiology (ISSM), Asilomar, CA 10/07/14. Contributed Talk (50%).

Moser, D.P. 2014, The Death Valley Regional Flow System: a fault-controlled deep life oasis of geological, seismic, and societal significance. Deep Carbon

Observatory Deep Life Workshop to Develop and Intercontinental Drilling Program Project. GeoForschungZentrum, Potsdam, Germany (25%).

**Presenting author.*

[1]Delivered prior to official start of project, but contained significant content describing this project. All presentations were either cleared in their entirety or contained only previously-cleared material.

[2]Goals and analysis relating to this project at times overlapped with other projects focused on the same sites. Percentages denote proportion of the work that directly corresponded to this project.

7.0 PUBLICATION

Thomas, J.M., Moser, D.P., Fisher, J.C., Reihle, J., Wheatley, A., Hershey, R.L., Baldino, C., Weissenfluh, D. 2013. Using water chemistry, isotopes and microbiology to evaluate groundwater sources, flow paths and geochemical reactions in the Death Valley Flow System, USA. Procedia Earth and Planetary Science 7, 842-845.

8.0 PLANNED PUBLICATIONS

Hamilton-Brehm, S.D., Fisher J., Bruckner J., Campbell J.H., Collins C., Russel C., Onstott T., and Moser D. (2015). *Region-scale distribution of microbial communities across a deep fractured rock aquifer*. [Microbial Ecology, In preparation].

Hamilton-Brehm, S.D., Sackett J., Campbell J.H., and Moser D. (2015). *Chacterization of three vertically-segregated, hard rock microbial ecosystems accessed via a single deep borehole.* [Applied and Environmental Microbiology, In preparation].

Hamilton-Brehm, S.D., Stewart L., Grzymski J., and Moser D. (2015). *Peptococcaceae thermofumoratis* sp. novel anaerobic fumarate fermenting moderately thermophilic bacterium isolated from 800 meters beneath the Southern Nevada Desert. [International Journal of Systematic and Environmental Microbiology, In preparation].

Hamilton-Brehm, S.D., Orphan V., and Moser D. (2015) Anaerobic oxidization of methane by a single bacterial strain from the deep continental subsurface. [Nature, In preparation].

Hamilton-Brehm, S.D., Stewart L., Orphan V., and Moser D. (2015) *Desulfogeovirga methanotrophicus* sp. a novel, thermophilic, anaerobic methane oxidizing bacterium isolated from the deep continental subsurface" [International Journal of Systematic and Environmental Microbiology, In preparation]

9.0 REFERENCES

1. **Norris RS, Arkin WM.** 1996. Known nuclear tests worldwide, 1945-1995. Bulletin of the Atomic Scientists **52:**61-63.
2. **Bowen S, Finnegan D, Thompson J, Miller C, Baca P, Olivas L, Geoffrion C, Smith D, Goishi W, Esser B.** 2001. Nevada Test Site Radionuclide Inventory, 1951-1992. Los Alamos National Laboratory. LA-13859.
3. **Wills C.** 2014. Nevada National Security Site Environmental Report 2013: Attachment A: Site Description. U.S. Deprtment of Commerce.
4. **Lin X, Kennedy D, Fredrickson J, Bjornstad B, Konopka A.** 2012. Vertical stratification of subsurface microbial community composition across geological formations at the Hanford Site. Environmental Microbiology **14:**414-425.
5. **Moser DP, Fredrickson JK, Geist DR, Arntzen EV, Peacock AD, Li SMW, Spadoni T, McKinley JP.** 2003. Biogeochemical processes and microbial characteristics across groundwater-surface water boundaries of the Hanford Reach of the Columbia River. Environmental Science & Technology **37:**5127-5134.
6. **Fredrickson JK, Zachara JM, Balkwill DL, Kennedy D, Li SMW, Kostandarithes HM, Daly MJ, Romine MF, Brockman FJ.** 2004. Geomicrobiology of high-level nuclear waste-contaminated vadose sediments at the Hanford Site, Washington State. Appl. Environ. Microbiol. **70:**4230-4241.
7. **Wu W, Carley JM, Watson DB, Gu B, Brooks SC, Kelly SD, Kemner KM, Van Nostrand J, Wu L, Zhou J.** 2011. Bioreduction and immobilization of uranium in situ: a case study at a USA Department of Energy radioactive waste site, Oak Ridge, Tennessee. Acta Scientiae Circumstantiae **31**.
8. **Kostka JE, Kerkhof L, Chin K-J, Keller M, Stucki JW.** 2011. Structure and Function of Subsurface Microbial Communities Affecting Radionuclide Transport and Bioimmobilization. Florida State University, Tallahassee, Florida 32306.
9. **Cardenas E, Wu WM, Leigh MB, Carley J, Carroll S, Gentry T, Luo J, Watson D, Gu BH, Ginder-Vogel M, Kitanidis PK, Jardine PM, Zhou JZ, Criddle CS, Marsh TL, Tiedje JM.** 2010. Significant Association between Sulfate-Reducing Bacteria and Uranium-Reducing Microbial Communities as Revealed by a Combined Massively Parallel Sequencing-Indicator Species Approach. Appl. Environ. Microbiol. **76:**6778-6786.
10. **Strandberg GW, Shumate SE, Parrott JR.** 1981. Microbial-Cells as Biosorbents for Heavy-Metals - Accumulation of Uranium by Saccharomyces-Cerevisiae and Pseudomonas-Aeruginosa. Appl. Environ. Microbiol. **41:**237-245.
11. **N'Guessan AL, Vrionis HA, Resch CT, Long PE, Lovley DR.** 2008. Sustained removal of uranium from contaminated groundwater following stimulation of dissimilatory metal reduction. Environmental Science & Technology **42:**2999-3004.

12. **Williams KH, Kemna A, Wilkins MJ, Druhan J, Arntzen E, N'Guessan AL, Long PE, Hubbard SS, Banfield JF.** 2009. Geophysical monitoring of coupled microbial and geochemical processes during stimulated subsurface bioremediation. Environmental Science & Technology **43:**6717-6723.
13. **Anderson RT, Vrionis HA, Ortiz-Bernad I, Resch CT, Long PE, Dayvault R, Karp K, Marutzky S, Metzler DR, Peacock A, White DC, Lowe M, Lovley DR.** 2003. Stimulating the in situ activity of *Geobacter* species to remove uranium from the groundwater of a uranium-contaminated aquifer. Appl. Environ. Microbiol. **69:**5884-5891.
14. **Vrionis HA, Anderson RT, Ortiz-Bernad I, O'Neill KR, Resch CT, Peacock AD, Dayvault R, White DC, Long PE, Lovley DR.** 2005. Microbiological and geochemical heterogeneity in an in situ uranium bioremediation field site. Appl. Environ. Microbiol. **71:**6308-6318.
15. **Teller E, Talley, W.K., Higgins, G.H. Johnson, G.W. .** 1968 The Constructive Uses of Nuclear Explosives. McGraw-Hill, New York.
16. **Chapman J.** 1995. Classification of groundwater and the Nevada Test Site. Report. DOE/NV/103 84-28.
17. **Oberlander PL, McGraw, D. and C. E. Russell.** 2007. Hydraulic conductivity with depth for Underground Test Area (UGTA) wells. Report. DOE/NV/26383-08. Publication No. 45228.
18. **Carroll RWH, Hershey, R.L., and G.M. Pohll.** 2006. Numerical simulation of groundwater withdrawal from proposed pumping near the Southeastern Nevada Test Site. Report, DOE/NV/13609-47, Publication N. 45217.
19. **Hassan A, Chapman, J., Bekhit, H., Lyles, B., and K. Pohlmann.** 2006. Validation analysis of the groundwater flow and transport model of the Central Nevada Test Area. Report. DOE/NV/13609-51. Publication No. 45221.
20. **Kersting A, Wang LM, You LP, Utsunomiya S, Zhao PH, Ewing R.** 2002. Characterization of mineral colloids in ground water at the Nevada test site by advanced tem techniques. Abstracts of Papers of the American Chemical Society **223:**U616-U616.
21. **Kersting AB, Efurd DW, Finnegan DL, Rokop DJ, Smith DK, Thompson JL.** 1999. Migration of plutonium in ground water at the Nevada Test Site. Nature **397:**56-59.
22. **Haldeman DL, Amy PS.** 1993. Bacterial heterogeneity in deep subsurface tunnels at Rainier Mesa, Nevada Test Site. Microbial Ecol **25:**183-194.
23. **Kieft TL, W. P. Kovacik Jr., D. B. Ringelberg, D. C. White, D. L. Haldeman, P. S. Amy, L. E. Hersman.** 1997. Factors limiting microbial growth and activity at a proposed high-level nuclear repository, Yucca Mountain, Nevada. Appl. Environ. Microbiol. **63::**3128–3133.
24. **Story SP, Amy PS, Bishop CW, Colwell FS.** 1995. Bacterial transport in volcanic tuff cores under saturated flow conditions. Geomicrobiology Journal **13:**249-264.
25. **Haldeman DL, Amy PS, White DC, Ringelberg DB.** 1994. Changes in Bacteria Recoverable from Subsurface Volcanic Rock Samples during Storage at 4 degrees C. Appl Environ Microbiol **60:**2697-2703.

26. **Amy PS, and D.L. Haldeman.** 2001. **The Microbiology of Yucca Mountain: A Proposed High Level Nuclear Waste Repository**. John Wiley and Sons, Inc. , **New York**.
27. **Russell CE, R. Jacobson., D.L. Haldeman, and P.S. Amy.** 1994 Heterogeneity of deep subsurface microorganisms and correlations to hydrological and geochemical parameters at Rainier Mesa, Nevada Test Site. Geomicrobiol. J. **12:**37-51.
28. **Fredrickson JK, McKinley JP, Bjornstad BN, Long PE, Ringelberg DB, White DC, Krumholz LR, Suflita JM, Colwell FS, Lehman RM, Phelps TJ, Onstott TC.** 1997. Pore-size constraints on the activity and survival of subsurface bacteria in a late Cretaceous shale-sandstone sequence, northwestern New Mexico. Geomicrobiology Journal **14:**183-202.
29. **Pedersen K.** 2001. Diversity and activity of microorganisms in deep igneous rock aquifers of the Fennoscandian shield. John Wiley, New York.
30. **Moser DP, Gihring TM, Brockman FJ, Fredrickson JK, Balkwill DL, Dollhopf ME, Lollar BS, Pratt LM, Boice E, Southam G, Wanger G, Baker BJ, Pfiffner SM, Lin LH, Onstott TC.** 2005. *Desulfotomaculum* and *Methanobacterium* spp. dominate a 4- to 5-kilometer-deep fault. Appl. Environ. Microbiol. **71:**8773-8783.
31. **Peterson FF.** 1981 Landforms of the Basin and Range Province defined for soil survey Nevada Agricultural Bulletin. No. 28, Max C. Fleischmann College of Agriculture, University of Nevada, Reno, Nev., 53 p.
32. **Rustad ME.** 2013. Earth's Hottest Place and Other Earth Science Records. Capstone.
33. **http://geomaps.wr.usgs.gov/parks/deva/weather.html.**
34. **http://earthquake.usgs.gov/earthquakes/states/nevada/history.php.**
35. **Harris AGT, E.; Tuttle, S. D.** 1997. Geology of National Parks (5th ed.). Iowa: Kendall/Hunt Publishing. ISBN 0-7872-5353-7.
36. **Collier M.** 1990. An Introduction to the Geology of Death Valley. Death Valley, California: Death Valley Natural History Association. LCN 90-081612.
37. **Hopper RH.** 1947. Geologic section from the Sierra Nevada to Death Valley, California. Geol Soc Am Bull **58:**393-432.
38. **Winograd IJ.** 2001. Interbasin groundwater flow in south central Nevada: A further comment on the discussion between Davisson et al. [1999a, 1999b] and Thomas [1999]. Water Resour Res **37:**431-433.
39. **Winograd IJ, C. J. Fridrich, D. Sweetkind, W. R. Belcher, and J. M. Thomas** 2005. Comment on "testing the interbasin flow hypothesis at Death Valley, California",. EOS, Transactions of the American Geophysical Union **86:**295–296.
40. **Eakin TE.** 1966. A regional interbasin groundwater system in the White River area, southeastern Nevada. Water Resour Res **2:**251-271.
41. **Harrill JR, Gates JS, Thomas JM.** 1988. Major ground-water flow systems in the Great Basin region of Nevada, Utah, and adjacent states. The Survey.
42. **Belcher WR, M. S. Bedinger , J. T. Back, D. S. Sweetkind.** 2009. Interbasin flow in the Great Basin with special reference to the southern Funeral Mountains and the source of Furnace Creek springs, Death Valley, California, U.S. J. Hydrol. **369:**30-43.

43. **Nelson ST, Anderson K, Mayo AL.** 2004. Testing the interbasin flow hypothesis at Death Valley, California. Eos, Transactions American Geophysical Union **85:**349-356.
44. **Anderson K, Nelson S, Mayo A, Tingey D.** 2006. Interbasin flow revisited: The contribution of local recharge to high-discharge springs, Death Valley, CA. Journal of Hydrology **323:**276-302.
45. **Bushmann M, Nelson, S.T., Tingeya, D., Eggett, D.** 2010. Regional groundwater flow in structurally-complex extended terranes: An evaluation of the sources of discharge at Ash Meadows, Nevada. J. Hydrol. **386:**118-129.
46. **Suzuki Y, Kelly SD, Kemner KM, Banfield JF.** 2004. Enzymatic U(VI) ireduction by Desulfosporosinus species. Radiochimica Acta **92:**11-16.
47. **Fredrickson JK, Zachara JM, Kennedy DW, Duff MC, Gorby YA, Li SMW, Krupka KM.** 2000. Reduction of U(VI) in goethite (alpha-FeOOH) suspensions by a dissimilatory metal-reducing bacterium. Geochim Cosmochim Ac **64:**3085-3098.
48. **Lovley DR, Phillips EJP, Gorby YA, Landa ER.** 1991. Microbial Reduction of Uranium. Nature **350:**413-416.
49. **Payne RB, Gentry DA, Rapp-Giles BJ, Casalot L, Wall JD.** 2002. Uranium reduction by *Desulfovibrio desulfuricans* strain G20 and a cytochrome c3 mutant. Appl. Environ. Microbiol. **68:**3129-3132.
50. **Spear JR, Figueroa LA, Honeyman BD.** 2000. Modeling reduction of uranium U(VI) under variable sulfate concentrations by sulfate-reducing bacteria. Appl. Environ. Microbiol. **66:**3711-3721.
51. **Liu C, Gorby YA, Zachara JM, Fredrickson JK, Brown CF.** 2002. Reduction kinetics of Fe(III), Co(III), U(VI), Cr(VI), and Tc(VII) in cultures of dissimilatory metal-reducing bacteria. Biotechnol Bioeng **80:**637-649.
52. **Fredrickson JK, Kostandarithes HM, Li SW, Plymale AE, Daly MJ.** 2000. Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by *Deinococcus radiodurans* R1. Appl. Environ. Microbiol. **66:**2006-2011.
53. **Wildung RE, Gorby YA, Krupka KM, Hess NJ, Li SW, Plymale AE, McKinley JP, Fredrickson JK.** 2000. Effect of electron donor and solution chemistry on products of dissimilatory reduction of technetium by Shewanella putrefaciens. Appl. Environ. Microbiol. **66:**2451-2460.
54. **Neu MP, Icopini GA, Boukhalfa H.** 2005. Plutonium speciation affected by environmental bacteria. Radiochimica Acta **93:**705-714.
55. **Icopini GA, Lack JG, Hersman LE, Neu MP.** 2005. The influence of metal reducing bacteria on plutonium speciation. Geochim Cosmochim Ac **69:**A472-A472.
56. **Giesy JP, Paine D.** 1977. Effects of Naturally Occurring Aquatic Organic Fractions on Am-241 Uptake by *Scenedesmus obliquus* (Chlorophyceae) and *Aeromonas hydrophila* (Pseudomonadaceae). Appl. Environ. Microbiol. **33:**89-96.
57. **Rittmann BE, Banaszak JE, Reed DT.** 2002. Reduction of Np(V) and precipitation of Np(IV) by an anaerobic microbial consortium. Biodegradation **13:**329-342.

58. **Campos J, M. Martinez-Pacheco, C. Cervantes.** 1995. Hexavalentchromium reduction by a chromate-resistant *Bacillus* sp. strain. Antonie Leeuwenhoek **68:**203-208.
59. **Lloyd JR, Ridley J, Khizniak T, Lyalikova NN, Macaskie LE.** 1999. Reduction of technetium by *Desulfovibrio desulfuricans*: Biocatalyst characterization and use in a flowthrough bioreactor. Appl. Environ. Microbiol. **65:**2691-2696.
60. **Lloyd JR, Nolting HF, Sole VA, Bosecker K.** 1998. Technetium reduction and precipitation by sulfate-reducing bacteria. Geomicrobiology Journal **15:**45-58.
61. **Rusin PA, Quintana L, Brainard JR, Strietelmeier BA, Tait CD, Ekberg SA, Palmer PD, Newton TW, Clark DL.** 1994. Solubilization of Plutonium Hydrous Oxide by Iron-Reducing Bacteria. Environmental Science & Technology **28:**1686-1690.
62. **Lloyd JR, Thomas GH, Finlay JA, Cole JA, Macaskie LE.** 1999. Microbial reduction of technetium by *Escherichia coli* and *Desulfovibrio desulfuricans*: Enhancement via the use of high-activity strains and effect of process parameters. Biotechnol. and Bioeng. **66:**122-130.
63. **Wildung RE, Li SW, Murray CJ, Krupka KM, Xie Y, Hess NJ, Roden EE.** 2004. Technetium reduction in sediments of a shallow aquifer exhibiting dissimilatory iron reduction potential. FEMS Microbiol. Ecol. **49:**151-162.
64. **Francis A.** 2002. Microbial transformations of radionuclides in transuranic wastes.
65. **Francis A.** 2007. Microbial mobilization and immobilization of plutonium. Journal of Alloys and Compounds **444:**500-505.
66. **Hu MZC, Norman JM, Faison BD, Reeves ME.** 1996. Biosorption of uranium by Pseudomonas aeruginosa strain CSU: Characterization and comparison studies. Biotechnology and Bioengineering **51:**237-247.
67. **Texier AC, Andres Y, Le Cloirec P.** 1999. Selective biosorption of lanthanide (La, Eu, Yb) ions by Pseudomonas aeruginosa. Environmental Science & Technology **33:**489-495.
68. **Gillow JB, Dunn M, Francis AJ, Lucero DA, Papenguth HW.** 2000. The potential of subterranean microbes in facilitating actinide migration at the Grimsel Test Site and Waste Isolation Pilot Plant. Radiochimica Acta **88:**769-774.
69. **Hersman LE.** 1997. Subsurface microbiology: Effects on the transport of radioactive waste in the vadose zone. CRC Press, Boca Raton.
70. **Ohnuki T, Yoshida T, Ozaki T, Samadfam M, Kozai N, Yubuta K, Mitsugashira T, Kasama T, Francis AJ.** 2005. Interactions of uranium with bacteria and kaolinite clay. Chem Geol **220:**237-243.
71. **Ohnuki T, Yoshida T, Ozaki T, Kozai N, Sakamoto F, Nankawa T, Suzuki Y, Francis AJ.** 2007. Chemical speciation and association of plutonium with bacteria, kaolinite clay, and their mixture. Environ Sci Technol **41:**3134-3139.
72. **Lollar BS, Lacrampe-Couloume G, Slater GF, Ward J, Moser DP, Gihring TM, Lin LH, Onstott TC.** 2006. Unravelling abiogenic and biogenic sources of methane in the Earth's deep subsurface. Chem Geol **226:**328-339.
73. **Lollar BS, Slater G, Moser DP, Gihring TM, Telling J, Lacrampe-Couloume G, Lin LH, Onstott TC.** 2005. Abiogenic gases, H2 autotrophy and

methanogenesis in the deep subsurface. Abstracts of Papers of the American Chemical Society **229:**U888-U888.

74. **Lollar BS, Ward J, Slater G, Lacrampe-Couloume G, Hall J, Lin LH, Moser D, Onstott TC.** 2002. Hydrogen and hydrocarbon gases in crystalline rock: Implications for the deep biosphere. Geochim Cosmochim Ac **66:**A706-A706.
75. **Lau MC, Cameron C, Magnabosco C, Brown CT, Schilkey F, Grim S, Hendrickson S, Pullin M, Sherwood Lollar B, van Heerden E, Kieft TL, Onstott TC.** 2014. Phylogeny and phylogeography of functional genes shared among seven terrestrial subsurface metagenomes reveal N-cycling and microbial evolutionary relationships. Frontiers in microbiology **5:**531.
76. **Lane DJ.** 1991. 16S/23S rRNA Sequencing. *In* Stackebrandt EaMG (ed.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley and Sons, Hoboken, NJ.
77. **DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL.** 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. **72:**5069-5072.
78. **Wright ES, Yilmaz LS, Noguera DR.** 2012. DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. Appl. Environ. Microbiol. **78:**717-725.
79. **Tamura K, Dudley, J., Nei, M., and Kumar, S.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol **24:**1596 -1599.
80. **Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R.** 2010. QIIME allows analysis of high-throughput community sequencing data. Nature methods **7:**335-336.
81. **Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM.** 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. **37:**D141-D145.
82. **Hou WG, Wang S, Dong HL, Jiang HC, Briggs BR, Peacock JP, Huang QY, Huang LQ, Wu G, Zhi XY, Li WJ, Dodsworth JA, Hedlund BP, Zhang CL, Hartnett HE, Dijkstra P, Hungate BA.** 2013. A Comprehensive Census of Microbial Diversity in Hot Springs of Tengchong, Yunnan Province China Using 16S rRNA Gene Pyrosequencing. PloS one **8**.
83. **Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF.** 2009. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. Appl. Environ. Microbiol. **75:**7537-7541.
84. **Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD.** 2013. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for

Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. Appl. Environ. Microbiol. **79:**5112-5120.

85. **Shakya M, Quince C, Campbell JH, Yang ZK, Schadt CW, Podar M.** 2013. Comparative metagenomic and rRNA microbial diversity characterization using archaeal and bacterial synthetic communities. Environmental microbiology **15:**1882-1899.
86. **Clarke KR, Warwick RM.** 2001. Change in Marine Communities: An Approach to Statistical Analysis and Interpretation. PRIMER-E Ltd, Plymouth.
87. **Anderson MJ, Gorley RN, Clarke KR.** 2008. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods. PRIMER-E Ltd, Plymouth.
88. **Steve Rozen aHJS** 1998, posting date. Primer3. [Online.]
89. **Bruckner JC, Fisher, J. C., Lindval, R., Zavarin, M., Czerwinski, K., Russell, C., and D.P. Moser.** 2009. Microbial communities of underground nuclear blast cavities. American Society for Microbiology, Philadelphia, PA.
90. **Miller TL, Wolin MJ.** 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl. Microbiol. **27:**985-987.
91. **Sambrook J, Russell DW.** 2001. Molecular Cloning: A Laboratory Manual, Third Edition ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
92. **Markowitz VM, Chen IM, Palaniappan K, Chu K, Szeto E, Pillay M, Ratner A, Huang J, Woyke T, Huntemann M, Anderson I, Billis K, Varghese N, Mavromatis K, Pati A, Ivanova NN, Kyrpides NC.** 2014. IMG 4 version of the integrated microbial genomes comparative analysis system. Nucleic acids research **42:**D560-567.
93. **Chao YQ, Zhang T.** 2011. Optimization of fixation methods for observation of bacterial cell morphology and surface ultrastructures by atomic force microscopy. Appl. Microbiol. Biotechnol. **92:**381-392.
94. **Cao B, Ahmed B, Kennedy DW, Wang Z, Shi L, Marshall MJ, Fredrickson JK, Isern NG, Majors PD, Beyenal H.** 2011. Contribution of extracellular polymeric substances from Shewanella sp. HRCR-1 biofilms to U(VI) immobilization. Environmental science & technology **45:**5483-5490.
95. **Cao B, Shi L, Brown RN, Xiong Y, Fredrickson JK, Romine MF, Marshall MJ, Lipton MS, Beyenal H.** 2011. Extracellular polymeric substances from Shewanella sp. HRCR-1 biofilms: characterization by infrared spectroscopy and proteomics. Environ. Microbiol. **13:**1018-1031.
96. **http://www.clrsearch.com/Amargosa-Valley-Demographics/NV/Weather-Forecast-Temperature-Precipitation** 2014, posting date. [Online.]
97. **Mase CW, Galanis, S.P., Jr., Monroe, R.J., .** 1979 Near surface heat flow in Saline Valley, California US Geological Survey Open-File Report 79-1136, 52p.
98. **Steinkampf WC, Werrell, W.L. .** 2001 Ground-water flow to Death Valley as inferred from the chemistry and geohydrology of selected springs in Death Valley National Park, California and Nevada. US Geological Survey Water-Resources Investigations Report 98-4114, 37p.
99. **Gillman G.** 1980. The effect of crushed basalt scoria on the cation exchange properties of a highly weathered soil. Soil Science Society of America Journal **44:**465-468.

100. **Gislason SR, Eugster HP.** 1987. Meteoric water-basalt interactions. II: A field study in NE Iceland. Geochim Cosmochim Ac **51:**2841-2855.
101. **Grenthe I, Stumm W, Laaksuharju M, Nilsson A, Wikberg P.** 1992. Redox potentials and redox reactions in deep groundwater systems. Chem Geol **98:**131-150.
102. **Rose S, Long A.** 1988. Monitoring dissolved oxygen in ground water: Some basic considerations. Groundwater Monitoring & Remediation **8:**93-97.
103. **Chapelle FH, McMahon PB, Dubrovsky NM, Fujii RF, Oaksford ET, Vroblesky DA.** 1995. Deducing the distribution of terminal electron-accepting processes in hydrologically diverse groundwater systems. Water Resources Research **31:**359-371.
104. **Christensen TH, Bjerg PL, Banwart SA, Jakobsen R, Heron G, Albrechtsen H-J.** 2000. Characterization of redox conditions in groundwater contaminant plumes. J Contam Hydrol **45:**165-241.
105. **Rose S, Long A.** 1988. Dissolved oxygen systematics in the Tucson basin aquifer. Water Resour Res **24:**127-136.
106. **Herczeg A, Dogramaci S, Leaney F.** 2001. Origin of dissolved salts in a large, semi-arid groundwater system: Murray Basin, Australia. Marine and Freshwater Research **52:**41-52.
107. **Iwatsuki T, Yoshida H.** 1999. Groundwater chemistry and fracture mineralogy in the basement granitic rock in the Tono uranium mine area, Gifu Prefecture, Japan-Groundwater composition, Eh evolution analysis by fracture filling minerals. Geochemical Journal **33:**19-32.
108. **Banwart S, Tullborg E-L, Pedersen K, Gustafsson E, Laaksoharju M, Nilsson A-C, Wallin B, Wikberg P.** 1996. Organic carbon oxidation induced by large-scale shallow water intrusion into a vertical fracture zone at the Äspö Hard Rock Laboratory (Sweden). J Contam Hydrol **21:**115-125.
109. **Acworth R.** 1987. The development of crystalline basement aquifers in a tropical environment. Quarterly Journal of Engineering Geology and Hydrogeology **20:**265-272.
110. **Winograd IJ, Robertson FN.** 1982. Deep oxygenated groundwater - anomaly or common occurrence. Science **216:**1227-1230.
111. **Lovley DR, Chapelle FH, Woodward JC.** 1994. Use of dissolved H2 concentrations to determine distribution of microbially catalyzed redox reactions in anoxic groundwater. Environmental Science & Technology **28:**1205-1210.
112. **Smith MS, Tiedje JM.** 1979. Phases of denitrification following oxygen depletion in soil. Soil Biology and Biochemistry **11:**261-267.
113. **Lovley DR, Goodwin S.** 1988. Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. Geochim Cosmochim Ac **52:**2993-3003.
114. **MacGregor BJ, Moser DP, Baker BJ, Alm EW, Maurer M, Nealson KH, Stahl DA.** 2001. Seasonal and spatial variability in Lake Michigan sediment small-subunit rRNA concentrations. Appl. Environ. Microbiol. **67:**3908-3922.
115. **McMahon S, Parnell J.** 2014. Weighing the deep continental biosphere. FEMS Microbiology Ecology **87:**113-120.

116. **D'Hondt S, Rutherford S, Spivack AJ.** 2002. Metabolic activity of subsurface life in deep-sea sediments. Science **295:**2067-2070.
117. **Parkes RJ, Cragg BA, Bale SJ, Getliff JM, Goodman K, Rochelle PA, Fry JC, Weightman AJ, Harvey SM.** 1994. DEEP BACTERIAL BIOSPHERE IN PACIFIC-OCEAN SEDIMENTS. Nature **371:**410-413.
118. **Onstott TC, Moser DP, Pfiffner SM, Fredrickson JK, Brockman FJ, Phelps TJ, White DC, Peacock A, Balkwill D, Hoover R, Krumholz LR, Borscik M, Kieft TL, Wilson R.** 2003. Indigenous and contaminant microbes in ultradeep mines. Environ. Microbiol. **5:**1168-1191.
119. **Moser D, Fisher, J.C, Bruckner, J., Reihle, J., Edmiston, P., Wheatley, A., Jacovides, C., Hamilton-Brehm, S., Dodsworth, J., Hedlund, B. .** 2014. Microbial Biogeochemistry of Ash Meadows Springs. Desert Research Institute.
120. **Moser DP, Onstott TC, Fredrickson JK, Brockman FJ, Balkwill DL, Drake GR, Pfiffner SM, White DC, Takai K, Pratt LM, Fong J, Lollar BS, Slater G, Phelps TJ, Spoelstra N, Deflaun M, Southam G, Welty AT, Baker BJ, Hoek J.** 2003. Temporal shifts in the geochemistry and microbial community structure of an ultradeep mine borehole following isolation. Geomicrobiol. J. **20:**517-548.
121. **Pedersen KH, L. Arlinger, J. Jahromi, N. and Erlandson, A-C. .** 1997. Investigation of the potential for microbial contamination of deep granitic aquifers during drilling using molecular and culturing methods J. Microbiol. Methods **30** 179-192.
122. **Chivian D, Brodie EL, Alm EJ, Culley DE, Dehal PS, Desantis TZ, Gihring TM, Lapidus A, Lin LH, Lowry SR, Moser DP, Richardson PM, Southam G, Wanger G, Pratt LM, Andersen GL, Hazen TC, Brockman FJ, Arkin AP, Onstott TC.** 2008. Environmental genomics reveals a single-species ecosystem deep within Earth. Science **322:**275-278.
123. **Baker BJ, Moser DP, MacGregor BJ, Fishbain S, Wagner M, Fry NK, Jackson B, Speolstra N, Loos S, Takai K, Lollar BS, Fredrickson J, Balkwill D, Onstott TC, Wimpee CF, Stahl DA.** 2003. Related assemblages of sulphate-reducing bacteria associated with ultradeep gold mines of South Africa and deep basalt aquifers of Washington State. Environ. Microbiol. **5:**267-277.
124. **Nyyssönen M, Bomberg M, Kapanen A, Nousiainen A, Pitkänen P, Itävaara M.** 2012. Methanogenic and sulphate-reducing microbial communities in deep groundwater of crystalline rock fractures in Olkiluoto, Finland. Geomicrobiology Journal **29:**863-878.
125. **Basso O, Lascourreges J-F, Le Borgne F, Le Goff C, Magot M.** 2009. Characterization by culture and molecular analysis of the microbial diversity of a deep subsurface gas storage aquifer. Research in microbiology **160:**107-116.
126. **Onstott T, Phelps TJ, Colwell F, Ringelberg D, White D, Boone D, Mckinley JP, Stevens TO, Long PE, Balkwill D.** 1998. Observations pertaining to the origin and ecology of microorganisms recovered from the deep subsurface of Taylorsville Basin, Virginia. Geomicrobiology Journal **15:**353-385.
127. **Boone DR, Liu Y, Zhao Z-J, Balkwill DL, Drake GR, Stevens TO, Aldrich HC.** 1995. *Bacillus infernus* sp. nov., an Fe (III)-and Mn (IV)-reducing anaerobe from the deep terrestrial subsurface. International journal of systematic bacteriology **45:**441-448.

128. **Sahl JW, Schmidt R, Swanner ED, Mandernack KW, Templeton AS, Kieft TL, Smith RL, Sanford WE, Callaghan RL, Mitton JB, Spear JR.** 2008. Subsurface microbial diversity in deep-granitic-fracture water in Colorado. Appl. Environ. Microbiol. **74:**143-152.
129. **Thomas JM, Moser DP, Fisher JC, Reihle J, Wheatley A, Hershey RL, Baldino C, Weissenfluh D.** 2013. Using Water Chemistry, Isotopes and Microbiology to Evaluate Groundwater Sources, Flow Paths and Geochemical Reactions in the Death Valley Flow System, USA. Procedia Earth and Planetary Science **7:**842-845.
130. **Gihring TM, Moser DP, Lin LH, Davidson M, Onstott TC, Morgan L, Milleson M, Kieft TL, Trimarco E, Balkwill DL, Dollhopf ME.** 2006. The distribution of microbial taxa in the subsurface water of the Kalahari Shield, South Africa. Geomicrobiology Journal **23:**415-430.
131. **Moser DP, Hamilton-Brehm, S., Zhang, G., Fisher, J., Hughes, K., Wheatley, A., Stewart, L., McKenna, A., Russell, C., Thomas, J., Kryder, L., McRae, R., Walker, J., King, M., Federwisch, M., Onstott, T.C., Sherwood Lollar, B.** 2013. Microbial ecology of a regional flow system: deep, aerobic, fractured rock aquifers of the US Basin and Range. American Geophysical Union, San Francisco, CA, December, 2013
132. **Rinke C, Schwientek P, Sczyrba A, Ivanova NN, Anderson IJ, Cheng J-F, Darling A, Malfatti S, Swan BK, Gies EA.** 2013. Insights into the phylogeny and coding potential of microbial dark matter. Nature.
133. **Brim H, Venkateswaran A, Kostandarithes HM, Fredrickson JK, Daly MJ.** 2003. Engineering *Deinococcus geothermalis* for bioremediation of high-temperature radioactive waste environments. Appl Environ Microb **69:**4575-4582.
134. **Ghosal D, Omelchenko MV, Gaidamakova EK, Matrosova VY, Vasilenko A, Venkateswaran A, Zhai M, Kostandarithes HM, Brim H, Makarova KS, Wackett LP, Fredrickson JK, Daly MJ.** 2005. How radiation kills cells: Survival of *Deinococcus radiodurans* and *Shewanella oneidensis* under oxidative stress. FEMS Microbiol. Rev. **29:**361-375.
135. **Brochier-Armanet C, Boussau B, Gribaldo S, Forterre P.** 2008. Mesophilic Crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota. Nature reviews. Microbiology **6:**245-252.
136. **Alonso-Saez L, Andersson A, Heinrich F, Bertilsson S.** 2011. High archaeal diversity in Antarctic circumpolar deep waters. Env Microbiol Rep **3:**689-697.
137. **Karner MB, DeLong EF, Karl DM.** 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature **409:**507-510.
138. **Kubo K, Lloyd KG, J FB, Amann R, Teske A, Knittel K.** 2012. Archaea of the Miscellaneous Crenarchaeotal Group are abundant, diverse and widespread in marine sediments. The ISME journal **6:**1949-1965.
139. **Huang Q, Dong CZ, Dong RM, Jiang H, Wang S, Wang G, Fang B, Ding X, Niu L, Li X, Zhang C, Dong H.** 2011. Archaeal and bacterial diversity in hot springs on the Tibetan Plateau, China. Extremophiles : life under extreme conditions **15:**549-563.

140. **Hedlund BP, Dodsworth JA, Cole JK, Panosyan HH.** 2013. An integrated study reveals diverse methanogens, Thaumarchaeota, and yet-uncultivated archaeal lineages in Armenian hot springs. Antonie van Leeuwenhoek **104:**71-82.
141. **Pester M, Schleper C, Wagner M.** 2011. The Thaumarchaeota: an emerging view of their phylogeny and ecophysiology. Current opinion in microbiology **14:**300-306.
142. **Konneke M, Bernhard AE, de la Torre JR, Walker CB, Waterbury JB, Stahl DA.** 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature **437:**543-546.
143. **Hatzenpichler R, Lebedeva EV, Spieck E, Stoecker K, Richter A, Daims H, Wagner M.** 2008. A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. Proc. Natl. Acad. Sci. U. S. A. **105:**2134-2139.
144. **Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB.** 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proc. Natl. Acad. Sci. U. S. A. **102:**14683-14688.
145. **Wuchter C, Abbas B, Coolen MJ, Herfort L, van Bleijswijk J, Timmers P, Strous M, Teira E, Herndl GJ, Middelburg JJ, Schouten S, Sinninghe Damste JS.** 2006. Archaeal nitrification in the ocean. Proceedings of the National Academy of Sciences of the United States of America **103:**12317-12322.
146. **Leininger S, Urich T, Schloter M, Schwark L, Qi J, Nicol GW, Prosser JI, Schuster SC, Schleper C.** 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature **442:**806-809.
147. **Song Z-Q, Chen J-Q, Jiang H-C, Zhou E-M, Tang S-K, Zhi X-Y, Zhang L-X, Zhang C-LL, Li W-J.** 2010. Diversity of Crenarchaeota in terrestrial hot springs in Tengchong, China. Extremophiles **14:**287-296.
148. **Itoh T, Iino T.** 2013. Phylogeny and biological features of thermophiles, p. 249-270, Thermophilic Microbes in Environmental and Industrial Biotechnology. Springer.
149. **Song Z, Wang L, Chen J, Zhou E, Zhang C, Li W.** 2013. [Diversity of crenarchaeota in terrestrial hot springs and their surrounding environments in Kamchatka, Russia]. Wei sheng wu xue bao= Acta microbiologica Sinica **53:**569-576.
150. **Wilson MS, Siering PL, White CL, Hauser ME, Bartles AN.** 2008. Novel archaea and bacteria dominate stable microbial communities in North America's largest hot spring. Microbial. Ecology. **56:**292-305.
151. **Vartabedian R.** 2011. State seeks compensation for Nevada Test Site contamination. Los Angeles Times **June 09**.
152. **Nitsche H, Roberts, K., Prussin, T, Muller, A., Becraft, K., Keeney, D, Carpenter, S. A., and Gatti, R. C.** 1993. Measured solubilities and speciations of Neptunium, Plutonium and Americium in a typical groundwater J-13 from the Yucca Mountain region. In: Milestone Report 3010-WBS. Los Alamos LA-125-62-MS.
153. **Triay IR, Cotter, C. R., Kraus, S. M., Huddleston. M. H., Chipera, S. J., Bish, D. L. .** 1996. Radionuclide sorption in Yucca Mountain tuffs with J-13 well water: neptunium, uranium, and plutonium. Report LA-12956-MS, Los Alamos National Laboratory.

154. **Zavarin M, Maxwell RM, Kersting A.** 2004. Radionuclide migration at the Nevada test site - Evaluating mechanisms controlling colloid-facilitated transport. Abstracts of Papers of the American Chemical Society **227:**U1113-U1114.
155. **Reimus PW, Ware SD, Lu NP, Kung KS, Neu MP, Reilly SD, Kersting AB.** 2003. Colloid-facilitated transport of plutonium in saturated fractures from the Nevada Test Site. Abstracts of Papers of the American Chemical Society **225:**U984-U984.
156. **Zhuang J, Flury M, Jin Y.** 2003. Colloid-facilitated Cs transport through water-saturated Hanford sediment and Ottawa sand. Environmental Science & Technology **37:**4905-4911.
157. **Novikov AP, Kalmykov SN, Utsunomiya S, Ewing RC, Horreard F, Merkulov A, Clark SB, Tkachev VV, Myasoedov BF.** 2006. Colloid transport of plutonium in the far-field of the Mayak Production Association, Russia. Science **314:**638-641.
158. **Kaplan DI, Bertsch PM, Adriano DC, Orlandini KA.** 1994. Actinide Association with Groundwater Colloids in a Coastal-Plain Aquifer. Radiochim Acta **66-7:**181-187.
159. **Bundschuh T, Yun, J. I., Knopp, R. .** 2001 Determination of size, concentration and elemental composition of colloids with laser-induced breakdown detection/spectroscopy (LIBD/S). Fresenius Journal of Analytical Chemistry **371**.
160. **Casey WH.** 1993. Chemistry of the Solid Water Interface - Processes at the Mineral Water and Particle Water Interface in Natural Systems - Stumm,W. Nature **363:**222-222.
161. **Kantar C. HBD.** 2005 Plutonium (IV) complexation with citric and alginic acids at low Pu-T concentrations Radiochimica Acta **93:**757-766.
162. **Harper R. M. KC, Honeyman B. D. .** 2008. Binding of Pu(IV) to galacturonic acid and extracellular polymeric substances (EPS) from *Shewanella Putrefaciens*, *Clostridium* sp. and *Pseudomonas fluorescens*. Radiochimica Acta **96:**753-762.
163. **Roberts K. A. SPH, and Honeyman B. D. .** 2008 Pu(V) reduction and enhancement of particlewater partitioning by exopolymeric substances. . Radiochim. Acta **96:**739-745.
164. **Harvey RW, Garabedian SP.** 1991. Use of colloid filtration theory in modeling movement of bacteria through a contaminated sandy aquifer. Environmental science & technology **25:**178-185.
165. **Abdelouas A, Lu YM, Lutze W, Nuttall HE.** 1998. Reduction of U(VI) to U(IV) by indigenous bacteria in contaminated ground water. J Contam Hydrol **35:**217-233.
166. **Barton LL, Choudhury K, Thomson BM, Steenhoudt K, Groffman AR.** 1996. Bacterial reduction of soluble uranium: The first step of in situ immobilization of uranium. Radioact Waste Manag **20:**141-151.
167. **John SG, Ruggiero CE, Hersman LE, Tung CS, Neu MP.** 2001. Siderophore mediated plutonium accumulation by Microbacterium flavescens (JG-9). Environmental science & technology **35:**2942-2948.
168. **Francis A, Gillow J, Dodge C, Dunn M, Mantione K, Strietelmeier B, Pansoy-Hjelvik M, Papenguth H.** 1998. Role of bacteria as biocolloids in the

transport of actinides from a deep underground radioactive waste repository. Radiochimica Acta **82:**347-354.
169. **Francis A.** 1998. Biotransformation of uranium and other actinides in radioactive wastes. Journal of Alloys and Compounds **271:**78-84.
170. **Xu C, Santschi PH, Schwehr KA, Hung C-C.** 2009. Optimized isolation procedure for obtaining strongly actinide binding exopolymeric substances (EPS) from two bacteria (< i> Sagittula stellata</i> and< i> Pseudomonas fluorescens</i> Biovar II). Bioresource Technology **100:**6010-6021.
171. **Cruz-Perez P, Stetzenbach LD, Alvarez AJ.** 1996. PCR detection of groundwater bacteria associated with colloidal transport. Nevada Univ., Las Vegas, NV (United States). Harry Reid Center for Environmental Studies. Funding organisation: USDOE, Washington, DC (United States).
172. **Imachi H, Sekiguchi Y, Kamagata Y, Hanada S, Ohashi A, Harada H.** 2002. *Pelotomaculum thermopropionicum* gen. nov., sp nov., an anaerobic, thermophilic, syntrophic propionate-oxidizing bacterium. Int. J. Syst. Evol. Microbiol. **52:**1729-1735.
173. **de Bok FAM, Harmsen HJM, Plugge CM, de Vries MC, Akkermans ADL, de Vos WM, Stams AJM.** 2005. The first true obligately syntrophic propionate-oxidizing bacterium, Pelotomaculum schinkii sp nov., co-cultured with Methanospirillum hungatei, and emended description of the genus Pelotomaculum. Int. J. Syst. Evol. Microbiol. **55:**1697-1703.
174. **Kjeldsen KU, Kjellerup BV, Egli K, Frolund B, Nielsen PH, Ingvorsen K.** 2007. Phylogenetic and functional diversity of bacteria in biofilms from metal surfaces of an alkaline district heating system. FEMS Microbiol Ecol **61:**384-397.
175. **Davidson MM, Silver BJ, Onstott TC, Moser DP, Gihring TM, Pratt LM, Boice EA, Lollar BS, Lippmann-Pipke J, Pfiffner SM, Kieft TL, Seymore W, Ralston C.** 2011. Capture of Planktonic Microbial Diversity in Fractures by Long-Term Monitoring of Flowing Boreholes, Evander Basin, South Africa. Geomicrobiology Journal **28:**275-300.
176. **Beal EJ, House CH, Orphan VJ.** 2009. Manganese- and Iron-Dependent Marine Methane Oxidation. Science **325:**184-187.
177. **Caldwell SL, Laidler JR, Brewer EA, Eberly JO, Sandborgh SC, Colwell FS.** 2008. Anaerobic oxidation of methane: Mechanisms, bioenergetics, and the ecology of associated microorganisms. Environmental science & technology **42:**6791-6799.
178. **Milucka J, Ferdelman TG, Polerecky L, Franzke D, Wegener G, Schmid M, Lieberwirth I, Wagner M, Widdel F, Kuypers MMM.** 2012. Zero-valent sulphur is a key intermediate in marine methane oxidation. Nature **491:**541-+.
179. **Friedrich MW, Finster KW.** 2014. Geochemistry. How sulfur beats iron. Science **344:**974-975.
180. **Haroon MF, Hu S, Shi Y, Imelfort M, Keller J, Hugenholtz P, Yuan Z, Tyson GW.** 2013. Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. Nature **500:**567-570.
181. **Lin LH, Hall J, Lippmann-Pipke J, Ward JA, Lollar BS, DeFlaun M, Rothmel R, Moser D, Gihring TM, Mislowack B, Onstott TC.** 2005.

Radiolytic H-2 in continental crust: Nuclear power for deep subsurface microbial communities. Geochem. Geophys. Geosys. **6**.

182. **Kieft TL, McCuddy SM, Onstott TC, Davidson M, Lin LH, Mislowack B, Pratt L, Boice E, Lollar BS, Lippmann-Pipke J, Pfiffner SM, Phelps TJ, Gihring T, Moser D, van Heerden A.** 2005. Geochemically generated, energy-rich substrates and indigenous microorganisms in deep, ancient groundwater. Geomicrobiology Journal **22:**325-335.

183. **Moser DPA, C.; Bang, S.; Jones, T. L.; Boutt, D.; Kieft, T.; Sherwood Lollar, B.; Murdoch, L. C.; Pfiffner, S. M.; Bruckner, J.; Fisher, J. C.; Newburn, J.; Wheatley, A.; Onstott, T. C.** 2010. First Microbial Community Assessment of Borehole Fluids from the Deep Underground Science and Engineering Laboratory (DUSEL). American Geophysical Union, Fall Meeting 2010, abstract #H13F-1049.

184. **Lin LH, Wang PL, Rumble D, Lippmann-Pipke J, Boice E, Pratt LM, Sherwood Lollar B, Brodie EL, Hazen TC, Andersen GL, DeSantis TZ, Moser DP, Kershaw D, Onstott TC.** 2006. Long-term sustainability of a high-energy, low-diversity crustal biome. Science **314:**479-482.

185. **Onstott TC, Lin LH, Davidson M, Mislowack B, Borcsik M, Hall J, Slater G, Ward J, Lollar BS, Lippmann-Pipke J, Boice E, Pratt LM, Pfiffner S, Moser D, Gihring T, Kieft TL, Phelps TJ, Vanheerden E, Litthaur D, Deflaun M, Rothmel R, Wanger G, Southam G.** 2006. The origin and age of biogeochemical trends in deep fracture water of the Witwatersrand Basin, South Africa. Geomicrobiology Journal **23:**369-414.

186. **Lin L-H, G. F. Slater, B. Sherwood-Lollar, G. Lacrampe-Couloume , and T. C. Onstott.** 2005. The yield and isotopic composition of radiolytic H_2, a potential energy source for the deep subsurface biosphere. Geochem. Cosmochim. Acta. **69:**893–903.