2010 Project Abstract
For the Period Ending June 30, 2012

PROJECT TITLE: Science and Innovation from the Soudan Iron Mine
PROJECT MANAGER: Jeffrey A. Gralnick
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: 2010

APPROPRIATION AMOUNT: $ 545,000

Overall Project Outcome and Results

The Soudan Iron Mine near Ely, MN is home to an extreme environment where microorganisms are thriving 2300 feet below the surface in an ancient, salty brine. Though mining operations have been closed for almost 50 years, the mine is now a State Park managed by Minnesota's Department of Natural Resources. Visitors can tour the mine, learning about the history of mining at Soudan and can also tour the state-of-the-art physics laboratory built at the bottom of the mine. Just a few hundred feet away from the physics laboratory, bubbling up from holes drilled in the last days of iron mining, is strange water – an incredibly salty brine that lacks any oxygen gas – and strange microorganisms (bacteria and other single-celled microbes) living in the water. Our work has resulted in the characterization of the level 27 brine with respect to its chemical makeup, the rate that the brine mixes with surface water, cultured and uncultured microbial communities living in the brine and speciation of minerals found in the brine channel. We have also specifically cultured about two dozen microorganisms from the mine that produce potent anti-fungal compounds, several of which have been shown to have activity against fungal pathogens. We have also isolated several novel species of iron oxidizing and iron reducing bacteria, which we continue to characterize. Finally, we developed an interactive touchscreen display and presentation about subsurface microbiology and geochemistry, specifically highlighting our work from this project. The goal of this touchscreen display is to both educate citizens of Minnesota broadly about subsurface microbiology and highlight some of the most exciting results from our project in a way that is broadly accessible to non-scientists.

Findings from this project formed the basis for a follow-up project begun in 2013 – "Harnessing Soudan Mine Microbes: Bioremediation, Bioenergy, and Biocontrol" (http://www.lccmr.leg.mn/projects/2013-index.html#201303f) – that is to exploring potential applications of using the microorganisms living in Soudan Iron Mine for removing metals from mine waters, producing biofuels, and developing a biocontrol for White-Nose Syndrome, which is decimating bat populations around the country.

Project Results Use and Dissemination

Project results have been disseminated through presentations made by students and investigators supported on this project. Co-Investigator Prof. Brandy Toner has presented research from our project at an international meeting in 2011 (Goldschmidt Conference, Prague, Czech Republic) and at a national meeting in 2012 (American Geophysical Union, San Francisco, CA). Prof. Jeff Gralnick presented some of the work supported by this project at the TEDxUMN 2012 event (http://www.youtube.com/watch?v=wNXI8lJ8SyU), Students working on this project gave several poster presentations at national and local meetings (2 presentations in 2012, 4 presentations in 2013). Two scientific publications are currently in preparation (first authors Lindsey Briscoe from the Toner Lab and Benjamin Bonis from the Gralnick Lab) and one has been published in the open access journal of the American Society of Microbiology mBio (Summers, ZM, JA Gralnick and DR Bond. 2013. mBio. Cultivation of an obligate Fe(II)-oxidizing lithoautotrophic bacterium using electrodes. Jan 29;4(1)e00420-

12. http://mbio.asm.org/content/4/1/e00420-12.long). Our project was also featured by several media outlets including the Northland's Newscenter (http://www.northlandsnewscenter.com/news/local/Soudan-Mine-Researchers-Seek-Window-to-Past-127533473.html), WCCO Channel 4 in the Twin Cities (http://minnesota.cbslocal.com/2011/02/17/ancient-water-discovery-in-depths-of-iron-range-mine/), MoBio's blog (http://www.mobio.com/blog/2011/02/06/microbial-warfare-in-the-underworld-searching-for-new-antibiotics/) and the University of Minnesota College of Biological Sciences (http://www.cbs.umn.edu/cbs-highlights/field/going-down).

Our specific outreach component for this project was to purchase, design and implement an interactive touch screen display for the Visitor's Center at the Soudan Underground Mine State Park. We purchased the equipment (computer, 42 inch touch screen display, mounting bracket, security cables) and have finished the first presentation featuring work from this project. The installation will take place before the mine reopens for visitors for the 2014 season.

Environment and Natural Resources Trust Fund (ENRTF)
2010 Work Program FINAL REPORT

Date of Report: 11/27/2013
Date of Next Progress Report: Final Report
Date of Work Program Approval: June 16, 2010
Project Completion Date: 6/30/2013

I. PROJECT TITLE: Science and Innovation from the Soudan Iron Mine

Project Manager: Jeffrey A. Gralnick
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Web Site Address: http://www.cbs.umn.edu/labs/gralnick

Location: *Ramsey County, St. Paul / Hennepin County, Minneapolis / St. Louis County, Soudan (Breitung Township)*

Total ENRTF Project Budget:	**ENRTF Appropriation**	**$ 545,000**
	Minus Amount Spent:	**$ 536,876**
	Equal Balance:	**$ 8,124**

Legal Citation: ML 2010, Chap. 362, Sec. 2, 03f, 'Science and Innovation from the Soudan Underground Mine State Park'.

Appropriation Language:
f) Science and Innovation from the Soudan Underground Mine State Park

$545,000 is from the trust fund to the Board of Regents of the University of Minnesota to characterize unique microbes discovered in the Soudan Underground Mine State Park and investigate the potential application in bioenergy and bioremediation. This appropriation is available until June 30, 2012, by which time the project must be completed and final products delivered.

II. FINAL PROJECT SUMMARY AND RESULTS:

The Soudan Iron Mine near Ely, MN is home to an extreme environment where microorganisms are thriving 2300 feet below the surface in an ancient, salty brine. Though mining operations have been closed for almost 50 years, the mine is now a State Park managed by Minnesota's Department of Natural Resources. Visitors can tour the mine, learning about the history of mining at Soudan and can also tour the state-of-the-art physics laboratory built at the bottom of the mine. Just a few hundred feet away from the physics laboratory, bubbling up from holes drilled in the last days of iron mining, is strange water – an incredibly salty brine that lacks any oxygen gas – and strange microorganisms (bacteria and other single-celled microbes) living in the water. Our work has resulted in the characterization of the level 27 brine with respect to its chemical makeup, the rate that the brine mixes with surface water, cultured and uncultured microbial communities living in the brine and speciation of minerals found in the brine channel. We have also specifically cultured about two dozen microorganisms from the mine that produce potent anti-fungal compounds, several of which have been shown to have activity against fungal pathogens. We have also isolated several novel species of iron oxidizing and iron reducing bacteria, which we continue to characterize. Finally, we developed an interactive touchscreen display and presentation about subsurface microbiology and geochemistry, specifically highlighting our work from this project. The goal of this touchscreen display is to both educate citizens of Minnesota broadly about subsurface microbiology and highlight some of the most exciting results from our project in a way that is broadly accessible to non-scientists.

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III. PROGRESS SUMMARY AS OF:

January 2013 – We continue to our microbial physiological characterization of JG233. We have sequenced its genome, generated a genetic system, mapped its range of metabolic potential and are currently exploring mechanisms of iron oxidation. This work will lead to both interesting biotechnology applications of these proteins in bioenergy and bioremediation, but also enable us to approximate the activity of these organisms in the Level 27 brine iron oxidizing community. Census of Deep Life program has generated large 16s rRNA gene datasets for several sample sites for bacterial community analysis. We continue working with Prof. Gregory Dick's lab at University of Michigan for metagenomic analysis and additional 16s rRNA gene analysis of archaea DNA isolated from anoxic boreholes on Level 27. We have obtained our first isotopic measurements that appear to indicate that the water below Level 27 is more separated from surface water intrusion that some of the geochemistry had suggested earlier on. More work is being done on this front. We have also begun a collaboration with Prof. Adnina Paytan at the University of California Santa Cruz who is interested in phosphate / oxygen isotopes from the Level 27 system and also going to measure methane isotopes to determine if, and to what extent, the methane in the Level 27 system is biogenic in origin. The Salomon Lab continues to focus on scaling up cultivation of several isolates from the Soudan Iron Mine that appear to produce promising anti-fungal compounds. They are also in the process of purifying novel compounds from a fungus isolated from Level 10 of the mine. This is an interesting organism as it is typically found only in the Arctic and Antarctic regions of the planet. Two compounds made by this fungus have shown potent activity against a malarial parasite. Prof. Brandy Toner presented work from this project at the 2012 American Geophysical Union meeting (San Francisco, December, 2012). Prof. Gralnick has a publication accepted in the American Society for Microbiology Journal *mBio* on using electrochemical techniques for cultivation of iron-oxidizing bacteria. Finally, we have a draft of an interactive touchscreen presentation for the visitors center at the Soudan Underground Mine State Park. We are working with James Pointer (DNR) on this outreach project at the mine.

July 2012 – The project is now entering the third and final year of support from the Trust Fund. We are focusing on an early mine isolate, JG233, as a model organism to understand iron oxidation and species interactions at DDH 942, the far bubbler on Level 27. The Toner and Gralnick labs continue to collaborate with the Census of Deep Life program (headed by Prof. Rick Colwell, Oregon State University) and Prof. Gregory Dick (University of Michigan) to analyze the microbial communities from several sample spots on level 27 in the Mine. Two variable regions of the 16s rRNA gene have been sequenced (using 454) from PCR amplification products from DNA isolated from six sample sites. Ph.D. student Lindsey Briscoe collected new data on the iron chemistry of the Soudan microbial mats in March 2012 at the Advanced Photon Source. The Alexander Lab has performed additional salinity readings and mixing calculations aimed at determining the origin of the brine water found on Level 27. Samples have also been submitted to determine the isotopic composition for hydrogen (tritium), carbon-14 and chlorine-36, which will help determine age and potential mixing rates. Recent efforts from the Salomon Lab have been focused on culturing and testing fungi isolated from the Soudan Mine. Two species of *Cadophora* fungi were chosen for fermentation and extraction because we have recently identified several novel bioactive compounds from isolates of this genus. The Gralnick Lab (with Prof. Bond) is working on a manuscript demonstrating the first growth of a pure culture of an iron oxidizing bacterium on carbon electrodes (to be submitted by early fall). Work with this pure culture has provided important information on how to grow iron-oxidizing bacteria and work is now in progress with electrodes to enrich for bacteria with this metabolism from Level 27 of the mine.

<u>January 2012</u> – We are now able to get good quality genomic DNA from Level 27 samples, have generated several small clone libraries and submitted 6 samples for 454 high-throughput sequencing through the Census of Deep Life project. The Gralnick lab has successfully enriched microbes from Level 27 that can utilize electrodes (both cathodically as an electron donor and anodically as an electron acceptor). We are currently working to identify these microbes and isolate them in pure culture. As a side project, we have successfully grown an aerobic iron oxidizing bacteria using a cathode as electron donor instead of Fe(II) – something that has never been reported to date. The Gralnick lab is working on a manuscript describing the cathode cultivation of iron oxidizing bacteria, which will be of great interest to the field. The Toner lab is working on their first manuscript describing the mineralogy of the Level 27 brine systems. The Toner lab has presented their work at an international meeting (Goldschmidt in Prague, Czech Republic) and at the 2011 Geological Society of America meeting (Minneapolis, MN). These presentations were well attended and well received by the scientific community. The Alexander lab has successfully deployed data logging units and have already begun learning about seasonal variation in flow and chemistry that was previously unexpected. The Salomon lab continues their work with promising anti-fungal compounds candidates. The Salomon lab has established a collaboration with another lab here at the U of M who are experts in fungal cultivation (Prof. Bob Blanchette, Department of Plant Pathology) and are working on proposals to fund work to explore how some of the anti-fungal compounds produced by bacterial isolates influence the native fungal population of the mine. The Salomon lab is also exploring a collaboration with Dr. David Blehert (USGS, Madsion, WI) who is a leading expert in 'white nose' disease, currently decimating bat populations on the East Coast and Southern portions of the US.

<u>July 2011</u> – Our team continued to refine DNA extraction protocols (some of the saltier water on the west side were still proving difficult), but have recently made good progress. Samples were taken for two large-scale sequencing experiments enabled by the LCCMR project, but funded by Greg Dick's Laboratory at Michigan and by the Census of Deep Life project headed by Rick Colwell at Oregon State. This additional information will add depth to the sequencing currently underway in the Gralnick and Salomon Labs. Our team has purchased and deployed data logging units in two different boreholes on the west side of level 27. These units monitor salinity, temperature, pH and water flow to provide dynamic information related to the water. The Salomon lab continues to find a wide array of anti-fungal compounds produced by bacteria isolated from the mine and are making progress in their purification to determine if any of the compounds themselves are novel. The Toner lab has been characterizing the different kinds of iron oxide minerals found at various points in the

water on level 27 and have been collaborating with the Institute for Rock Magnetism at UMN to determine characteristics of magnetic iron minerals also found in the water.

January 2011 – DNA extraction protocols from the highly unusual mine water were perfected. The first mineralogy on samples from level 27 was characterized at the synchrotron facility. Additional samples were taken for geochemical analysis and cross-referencing maps and notes from the original drilling operations in the 1960s identified several new boreholes. Media for actinobacteria cultivation was prepared from mine water and optimized. Samples were submitted to the Census of Deep Life project (Rick Colwell at Oregon State University) for 454 pyrosequencing (leveraging this proposal to generate a significantly larger number of sequences for microbial diversity assessment from level 27 mine samples). Developing laboratory cultivation methods for iron oxidizing bacteria, which has been highly successful so far.

IV. OUTLINE OF PROJECT RESULTS*:*

RESULT 1: Basic Science – Microbiology, Mineralogy and Geochemistry

Description: Result 1 will establish fundamental parameters for understanding the level 27 brine ecosystems. The microbiology section will focus on identifying and categorizing the microbial populations found in the mine. The mineralogy portion will focus on synchnotron-enabled analysis of both structure and composition of minerals found in the Level 27 brine and on samples from iron formations. The geochemistry analysis will focus on chemical and isotopic characterization of the Level 27 brine, in addition to heavy metal analysis on Level 10 and elsewhere in the mine as directed by mine staff.

Summary Budget Information for Result 1: **ENRTF Budget: $ 389,318**
Amount Spent: $ 386,055
Balance: $ 3,263

Deliverable	Completion Date	Budget
1. Molecular phylogentic analysis and isolation / characterization of microbes from the Soudan Iron Mine and publication of this work.	June 2013	$157750
2. Mineralogical, speciation and elemental analyses	June 2013	$165,370
3. Isotope analysis, gas composition, geochemistry	June 2013	$43,740

Result Completion Date: June 2013

Result Status as of *(January 2011)*:

Deliverable 1:

Ben Bonis was hired by the Gralnick Lab on September 1, 2010 as a 100% time junior scientist (research technician). Bonis has been accepted into the PhD program in Molecular, Cellular and Structural Biology graduate program here at the U and will start this fall. Bonis has been developing DNA extraction protocols for sediment and water samples from a variety of sites on Level 27 of the Soudan Mine. Effective DNA extractions are essential to generate an unbiased (molecular) survey of the microbes living in the sediment / water of Level 27. Sampling permits for Toner and Gralnick were approved and issued in December 2010 (previously, samples were collected under the E.C. Alexander's permit). Since his hiring in September 2010, Bonis has isolated and characterized 24 new bacterial species from the mine, 15 of which may represent novel species (based on 97% 16s rDNA identity) and 6 of which may represent novel genera (based on 95% 16s rDNA identity). In January we initiated cultivation experiments for archaeal methanogens that we suspect to be present in the anaerobic zones within the boreholes. Gralnick and Toner submitted a proposal to perform next-generation high-throughput sequencing of 16s rDNA samples from sites throughout the mine. If funded, phylogenetic analysis of microbial populations would grow from 100's of sequences (as proposed and funded by this project) to 10,000's of sequences. We expect to be notified in March 2010 for this sequencing opportunity.

<u>Deliverable 2:</u>
Toner began working on this project during the summer of 2010. On October 4, 2010, co-PI Toner hired Lindsey Briscoe in a 74% technician position. Since that time Toner and Briscoe have been:

1) Analyzing iron X-ray absorption spectroscopy data collected at the Advanced Photon Source, Argonne National Laboratory, Argonne, Illinois, in August 2010 by Toner.

2) Analyzing the mineralogy, particle size distribution, and iron chemistry of the iron microbial mats collected from Level 27 of the mine. We are using instruments at the Institute for Rock Magnetism, University of Minnesota, and collaborating with Professor Bruce Moskowitz.

3) Developing a wet chemistry method for measuring iron oxidation and iron reduction rates in iron microbial mats collected from Level 27. This effort includes outfitting existing pH-controlled reactors with oxygen sensing capabilities, as well as rehabbing an existing UV-vis spectrophotometer for dissolved iron measurements using an adapted ferrozine method.

4) Building a collaboration with Dr. F. Marc Michel, an expert in the use of synchrotron radiation X-ray scattering measurements for poorly ordered iron minerals. Total X-ray scattering measurements were made on subsamples of iron microbial mats from Level 27 by Dr. Michel at the Advanced Photon Source in August 2010. Briscoe is currently working with Dr. Michel to interpret the data.

In addition to these activities, Briscoe has been accepted into the Ph.D. program in the Department of Geology and Geophysics at the University of Minnesota for Fall 2011. The Soudan LCCMR research will form the basis of her dissertation research.

<u>Deliverable 3:</u>

Calvin Alexander's group's work in the first six months focused on locating and characterizing as many of the 1960s diamond drill holes (DDH) on the 27th Level of the Soudan Mine as are still accessible. Waters trickling from these old DDHs are our current best access points to the anoxic calcium chloride brines in the bottom of the mine. We have been sampling these DDHs for water chemistry and isotope measurements as we locate them. The location and sampling effort has identified the DDH emitting the most concentrated brine yet located. Data from the initial survey is consistent with a singe brine composition beneath the mine which being diluted with varying amounts of surface water infiltration. The stable isotope results are compatible with this two-component model. We have also been providing geochemical analyses of waters from sites sampled by Toner, Gralnick and the Salomon groups.

Result Status as of *(July 2011)*:

Deliverable 1:

Ben Bonis has formally started the BMBB PhD program and will continue his work on this project for part of his thesis. From the previous update we have primarily worked with isolating and identifying pure strains from samples taken from the mine using 16S analysis from the Feb. 2011 day-long sampling trip to the mine. Surprisingly, some isolates from the East side of level 27 are related to organisms known to have photosynthetic metabolisms. The presence or absence of genes involved in photosynthesis may help us use biology (so-called 'biological clock') to help estimate the time the organisms have been in the mine (or permanently dark conditions). Therefore, we have begun experiments to elucidate photosynthetic activity and the test for genes they may have retained using molecular techniques. Several isolates were of interest to the Salomon lab and were sent to her group for secondary metabolite screening. After the DNA isolation technique was better developed, we started making bacterial and archaeal libraries of the 16S gene; though with limited success on the archaeal front as of yet. Recently we have started looking into techniques for the study of iron oxidation in several of these strains. We have also established a collaboration with the Census of Deep Life project (headed by Dr. Rick Colwell, Oregon State University) which will help us generate larger datasets. CoDL will pay for the costs of deep sequencing for microbial diversity (a fragment of the 16s rRNA genes), while our project will cover the cost for sample acquisition and DNA isolation.

New isolates and new sequences generated. Note, when closest related species is 'unknown' and/or when % identity is below ~ 97%, these represent potentially new bacterial species.

New Cultured Isolates

ID	% Identity	Closest Related Genus	Closest Related Species
23W1.1	96	Pseudomonas	fluorescens
27M1.3	95	Rheinheimera	chironomi
920W1.1	98	Devosia	Unknown
926W2.3	95	Paracoccus	MOLA 22
932W1.1	96	Marinobacter	Unknown
932W2.3	97	Bacillus	firmus
942W2.2	98	Marinobacter	gudaonensis
942W2.4	97	Intrasporangiaceae	Ornithinimicrobium
942W2.5	89	Proxlibacter/Sphingobacterium	Unknown
942W4.3	99	Aspergillus	candidus
942W10.1	99	Streptomyces	sampsonii
944W1.2	98	Marinobacter	kribbensis
944W1.5	99	Pseudonocardiaceae	pseudonocardia
944W3.1	96	Rhodobacter	litoralis
944W3.2	100	Ralstonia	pickettii
949E3.1	96	Algoriohagus	aquimarinus
951W4.3	99	Dermacoccus	barathri
951W4.5	98	Marinobacter	lipolyticus
951W5.3	99	Exophiala	GHP 1205

951W8.1	99	Bacillus	thuringiensis
951W8.2	97	Bacillus	pumilus
951W8.3	99	Bacillus	cereus
951W8.4	100	Bacillus	cereus
951W8.5	95	Martelella	mediterranea
952E1.1	99	Porphyrobacter	sanguineus
952E1.3	97	Lutibacterium	anuloederans
952E1.4	97	Erythrobacteraceae	Unknown
952E1.5	96	Agrobacterium	sanguineum
952E1.6	97	Lutibacterium	anuloederans
952E2.1	99	Sphingopyxis	Unknown
952E2.2	94	Maricaulis	maris
952E2.3	92	Pelagibius	litoralis
962E1.1	100	Rhodobacteraceae	Unknown
962E1.2	99	Roseovarius	mucosus
962E1.4	99	Enterococcus	faecalis
962E1.7	94	Maricaulis	Unknown
962E1.8	99	Roseovarius	Unknown
962E2.4	96	Roseovarius	tolerans
962E4.1	99	Devosia	Unknown
962E4.3	98	Sphingomonas	sp. R13-12
962E5.1	98	Streptomyces	verne
962E5.2	98	Streptomyces	microflavus
962E5.3	97	Streptomyces	Unknown
962E5.4	95	Streptomyces	xanthophaeus
962E5.5	99	Streptomyces	nojiriensis

Library 16S Clones

DDH932 W1

Rhodovulum; iodosum (95% BLAST identity 950nt)
Marinobacter; sp. CF6-10 (98% BLAST identity 757nt)
Marinobacter; gudaonensis (97% BLAST identity 1056nt)
Rhodovulum; sp. 1R7 (97% BLAST identity 1039nt)
Marinobacter; guinea (99% BLAST identity 1035nt)
Marinobacter; sp. SCSWB27 (96% BLAST identity 839nt)
Rhodovulum; iodosum (95% BLAST identity 1037nt)
Rhodovulum; iodosum (95% BLAST identity 1040nt)
Rhodovulum; iodosum (95% BLAST identity 1037nt)
Unknown Actinomycete

DDH942 W12

Desulfobulbaceae; catecholicum (94% BLAST identity 1072nt)
Martelella; mediterranea (92% BLAST identity 1057nt)
Rhodovulum; imhoffii (89% BLAST identity 1063nt)
Methylobacteriaceae; Methylobacterium (98% BLAST identity 1197nt)
Pseudomonas; Unkown (87% BLAST identity 1246nt)
Rhodovulum; Unknown (97% BLAST identity 1042nt)

DDH942 W13

Roseovarius; tolerans (97% BLAST identity 1047nt)
Truepera; radiovictrix DSM 17093 (91% BLAST identity 1052nt)
Thalassobacter sp. W-2-2 (95% BLAST identity 1040nt)
Rhodobacteraceae, Unknown (96% BLAST identity 1040nt)
Rhodovulum; iodosum (95% BLAST identity 1029nt)

Rhodovulum; iodosum (95% BLAST identity 1029nt)
Thiohalophilus; thiocyanatoxydans (98% BLAST identity 1031nt)
Martelella; mediterranea (98% BLAST identity 1031nt)
Halothiobacillus (100% BLAST identity 1045nt)
Rhodobacteraceae, Unknown (95% BLAST identity 1022nt)
Rhodovulum; iodosum (95% BLAST identity 1017nt)
Rhodovulum; sp. 1R7 (97% BLAST identity 1034nt)
Rhodovulum; sp. 1R7 (97% BLAST identity 1045nt)
Paracoccus; sp. dtb77 (97% BLAST identity 625nt)
Rhodovulum; sp. 1R7 (97% BLAST identity 1028nt)
Halothiobacillus; sp. NP36 (99% BLAST identity 1029nt)
Martelella; mediterranea (96% BLAST identity 943nt)
Rhodovulum; robiginosum (94% BLAST identity 1029nt)
Rhodobacteraceae; Unknown (95% BLAST identity 1024nt)
Halothiobacillus; sp. NP36 (100% BLAST identity 1027nt)

DDH944 W10
Desulfotomaculum; Unknown (90% BLAST identity 1042nt)
Rhodovulum; sp.1R7 (96% BLAST identity 896nt)
Desulfotomaculum; sp. 175 (90% BLAST identity 1033nt)
Desulfotomaculum; geothermicum (86% BLAST identity 928nt)
Desulfotomaculum; geothermicum (86% BLAST identity 917nt)
Pelotomaculum; thermopropionicum SI (89% BLAST identity 683nt)
Desulfotomaculum; salinum (88% BLAST identity 986nt)
Carboxydocella; sp. SLM61 (91% BLAST identity 520nt)
Desulfotomaculum; sp. Mechichi-2001 (90% BLAST identity 1021nt)
Desulfotomaculum; salinum (88% BLAST identity 1024nt)
Carboxydocella; sp. SLM61 (92% BLAST identity 512nt)
Maritimibacter; alkaliphilus (97% BLAST identity 353nt)
Vibrio; sp. K3-01 (94% BLAST identity 271nt)
Marinobacter; gudaonensis (95% BLAST identity 910nt)
Rhodovulum, sp. 1R7 (97% BLAST identity 939nt)
Desulfitibacter; alkalitolerans (89% BLAST identity 950nt)
Rhodobacteraceae; Rhodovulum, sp. 1R7 (97% BLAST identity 907nt)
Desulfotomaculum; geothermicum (86% BLAST identity 720nt)
Desulfotomaculum; geothermicum (86% BLAST identity 810nt)

DDH951 W10
Anaerovorax; odorimutans (92% BLAST identity 1020nt)
Clostridium; aminobutyricum (93% BLAST identity 1015nt)
Dehalobacter; sp. 1,1-DCA1 (96% BLAST identity 1046nt)
Halocella; cellulolsilytica (93% BLAST identity 1043nt)
Desulfobacterium; corrodens (97% BLAST identity 1045nt)
Spirochaeta; sp. MET-E (95% BLAST identity 927nt)
Spirochaeta; sp. MET-E (99% BLAST identity 1053nt)
Incertae Sedis Anaerovorax; odorimutans (92% BLAST identity 1054nt)
Marinobacter; sp. S16-2-1 (97% BLAST identity 1057nt)
Clostridium; sp. DY192 (92% BLAST identity 1045nt)
Spirochaeta; sp. MET-E (98% BLAST identity 1031nt)
Spirochaeta; sp. MET-E (99% BLAST identity 1035nt)
Desulfobacterium; corrodens (97% BLAST identity 1040nt)
Clostridium; aminobutyricum (93% BLAST identity 1044nt)
Marinilabilia; salmonicolor (96% BLAST identity 1048nt)
Marinilabilia; salmonicolor (95% BLAST identity 1036nt)
Desulfobulbaceae; catecholicum (94% BLAST identity 1041nt)
Peptostreptococcaceae; Unknown (98% BLAST identity 1036nt)
Desulfobacterium; corrodens (97% BLAST identity 1033nt)
Clostridium; aminobutyricum (92% BLAST identity 1019nt)

Deliverable 2:

During this period, we have begun to prepare data for the mineralogy of the iron mats for conference presentations and peer-review journal publication. A permit for B. Toner also obtained from the DNR for sampling in the Soudan Iron Mine, on Level 27 (previous sampling visits were under E. C. Alexander's DNR permit).

1) Toner has an invited talk at an international geochemistry conference, Goldschmidt, in Prague, Czech Republic in August 2011. The talk is titled, *Iron microbial mat formation from deep continental brines* and is co-authored by several LCCMR research team members and an external collaborator from Stanford University: Brandy M. Toner, Lindsey J. Briscoe, F. Marc Michel, Scott C. Alexander, E. Calvin Alexander Jr., Jeffrey A. Gralnick.

2) Briscoe has a conference talk accepted at the Geological Society of America meeting in Minneapolis, MN in October 2011 that will emphasize the work she has been doing at the Institute for Rock Magnetism. The title of this presentation is, *Iron mineral formation in microbial mats formed from shield brines along an oxidation-reduction gradient* and is co-authored by Lindsey J. Briscoe, E. Calvin Alexander Jr., Scott C. Alexander, Thelma D. Berquo, Jeffrey A. Gralnick, F. Marc Michel, Bruce K. Moskowitz, Christine E. Salomon, and Brandy M. Toner. This co-author list includes all of the LCCMR PIs, as well as an expanded set of external collaborators having expertise in magnetism and Mossbauer techniques.

3) Toner and Briscoe were invited to submit a manuscript to a special issue of the journal Frontiers in Microbiological Chemistry. The special issue is titled, *The microbial ferrous wheel: iron cycling in terrestrial, freshwater, and marine environments* and is edited by David Emerson, Eric Roden, and Benjamin Twining. Our plan is to contribute all of the initial observations of iron mat mineralogy to this issue with Briscoe as the lead author in October 2011.

4) The next phase of the research for the Toner group will be measuring the rates and products of iron oxidation (and reduction) in controlled conditions. We are currently developing these experiments and will shift our research efforts to this entirely come January 2012.

<u>Deliverable 3:</u>

To develop an understanding of the waters, and dependent microbiologic communities, existing in 27th level of the Soudan Underground Mine we have collected and analyzed existing borehole and geologic information. Dean Peterson and the Precambrian Research Institute have been of invaluable assistance in this effort. Accurate locations for diamond core drill holes have led to the rediscovery of many important water sources into the lowest level of the mine. This includes the highest salinity boreholes found to date as well as understanding the distribution of salinity within the mine. Figure 1 is cross-section of the 27th level with all the borehole information collapsed into a North-South line.

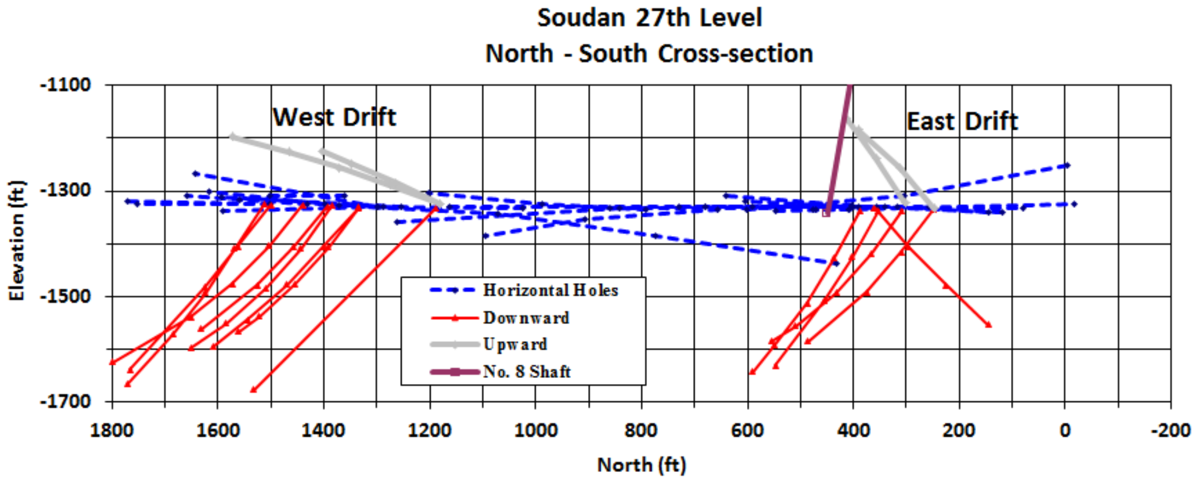


Figure 1

The boreholes can be grouped into three types: Horizontal, Downward, and Upward. The downward trending holes have the highest salinities although there is not an absolute correlation of depth to salinity. The horizontal holes have intermediate salinities and produce extensive suites of mineralogical and biological formations. Upward trending boreholes have the lowest salinity but are still classified as brackish. By locating and sampling individual boreholes we have now found waters with salinity levels four times higher than seawater. We are developing plans to access additional boreholes that surrounded by delicate formations, particularly on the East Drift.

Figure 2 is a conceptual model of groundwater flow into the 27th level. The current results suggest that the salinity at each individual borehole is a mixture of fresher waters, originating ultimately from the surface, and deeper, older, continental shield brines. We have collected and are currently analyzing samples to help estimate the ages of these source waters.

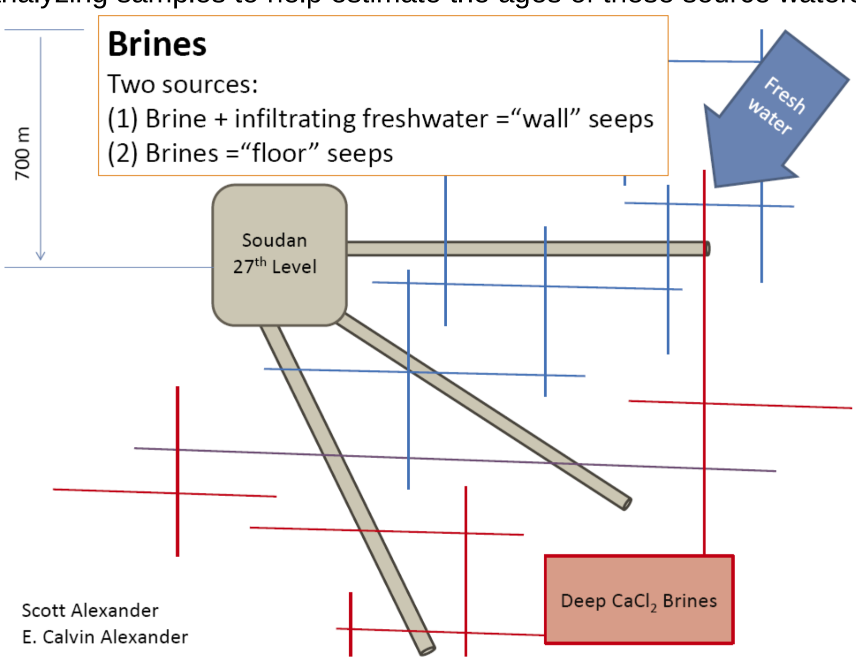


Figure 2

To date 31 samples for major and trace element, and isotopic analysis have been collected. Several boreholes have been sampled at up to four different times throughout the past year to look for seasonal variation in the waters. In addition, two data loggers have been deployed to monitor salinity, temperature and flow variations in borehole waters. Figure 3 shows preliminary results from one of the loggers that was installed into horizontal drill hole 920 in January 2011. The water levels, and therefore the flow rates, have been relatively stable and are close to the resolution of the sensor. Temperatures show a slight warming while the air ventilation systems were shut down following the March shaft fire. There are also small daily variations due to the on/off cycling of the ventilation system. Conductivity does show a significant decrease bottoming out in late February before rising back to its original level. The aperiodic drops in conductance are likely due to methane bubble trapping on the sensor. As the time period of monitoring increases we hope to tie changes in conductivity to surface recharge events, i.e. spring snowmelt and fall rains.

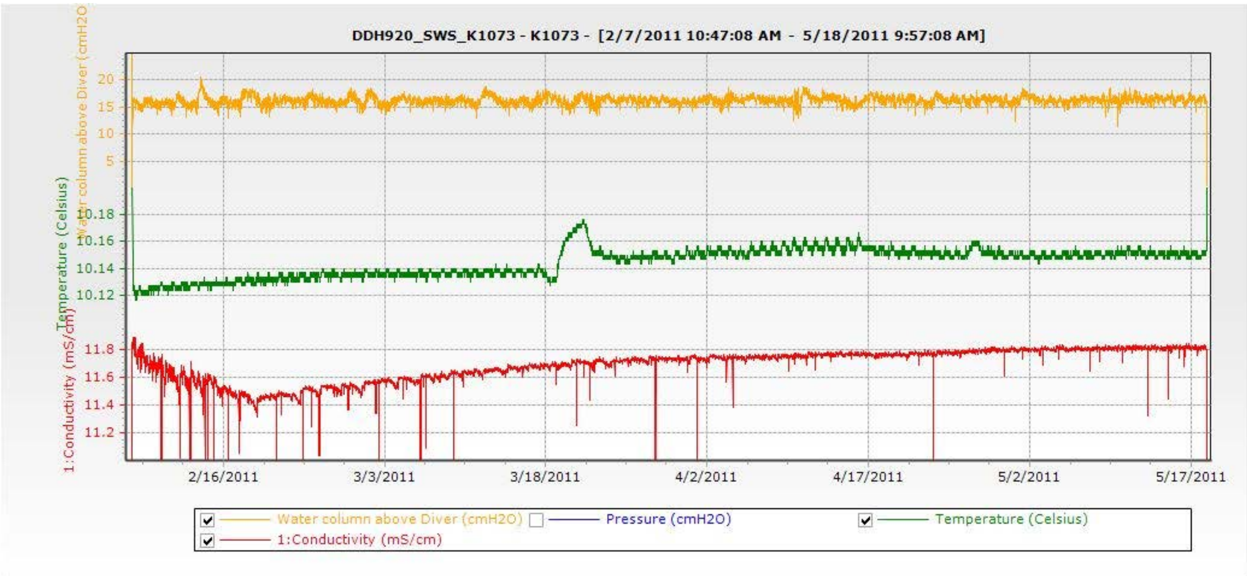


Figure 3

Result Status as of *(January 2012)*:

Deliverable 1:

The Gralnick Lab has continued to work with isolates described in our previous update and have begun testing a subset of isolates for their ability to mediate iron oxidation. Several of the isolates are related to bacteria that should have phototrophic lifestyles. We are currently pursuing this aspect as a way to potentially quantify how long the microbes have been ‘trapped’ in the subsurface, as genes involved in photosynthetic pathways should have no evolutionary benefit to an organism living in complete darkness. Our lab has also refined DNA preparation protocols sufficiently to send samples to the Census of Deep Life (CoDL) project headed by Prof. Rick Colwell at Oregon State University. These samples were submitted at the end of December and we will be able to report our results in the next update. Ben Bonis transitioned from a junior scientist to a PhD student in the Biochemistry, Molecular Biology and Biophysics graduate program and has just finished rotating in three different labs (though he will be joining the Gralnick lab to continue his work on the Soudan Mine poject).

Deliverable 2:

(1) **Personnel.** During this period, Lindsey Briscoe transitioned from a laboratory technician to a Ph.D. student in the Earth Sciences program at the University of Minnesota-Twin Cities.
(2) **Communicating Findings.** During the fall semester, we presented two conference talks on mineralogy research for the iron microbial mats from Level 27 of the mine (citations below). In addition, PI Gralnick and co-PI Toner hosted a Deep Biosphere symposium at the Geological Society of America Meeting in Minneapolis in October. The symposium brought together six keynote speakers that represent leaders in the field, and allowed us to highlight our research efforts at Soudan.
(3) **Science.** We have discovered that the iron microbial mats contain mixtures of iron oxyhydroxide minerals. We have observed abundant akaganeite (beta-FeOOH) forming the mats hosted by the most concentrated brines. This is a relatively rare iron oxyhydroxide that often incorporates chloride (Cl-) into its structure. Lindsey is in the process of generating synthetic versions of the mineral so that we may better interpret our data. In support of these efforts, we earned competitively awarded instrument access at Argonne National Laboratory for March 2012. This instrument time will be used to develop the methods needed for measuring the rates and products of iron oxidation and reduction in the mats.
(4) **Publications.** We are in the final stages of data collection. Preparation of a journal article that will describe the mineralogy of the Soudan iron mats is underway, and will be the first chapter if Lindsey's dissertation.
(5) **Challenges.** Our biggest challenge at present is method development for the iron oxidation and reduction rate experiments. We must first determine whether sub-samples from our experiments can be stored (frozen under inert gas) without artifacts. If we discover sample storage artifacts, then the kinetic experiments will be conducted at Argonne National Laboratory and measurements will be made in real-time.

Briscoe, L. J., Alexander, E. C., Jr, Alexander, S. C., Berquo, T. S., Gralnick, J. A., Michel, F. M., Moskowitz, B., Salomon, C. E., and Toner, B. M., 2011. Iron mineral formation in microbial mats formed from shield brines along an oxidation-reduction gradient. Talk at the *Geological Society of America Meeting,* Minneapolis, MN. (October, 2011)

Toner, B. M., Briscoe, L. J., Michel, F. M., Alexander, S., Alexander, C., and Gralnick, J. A., 2011. Iron Microbial Mat Formation from Deep Continental Brines. Talk at the *Goldschmidt Internation Geochemistry Conference*. Prague, Czech Republic. (August, 2011)

<u>Deliverable 3:</u>

Geochemical and Isotopic Update

To develop an understanding of the waters, and dependent microbiologic communities, existing in 27th level of the Soudan Underground Mine we have collected and analyzed existing borehole and geologic information. Dean Peterson and the Precambrian Research Institute have been of invaluable assistance in this effort. Accurate locations for diamond core drill holes have led to the rediscovery of many important water sources into the lowest level of the mine. This includes the highest salinity boreholes found to date as well as understanding the distribution of salinity within the mine. Figure 1 is cross-section of the 27th level with all the borehole information collapsed into a North-South line.

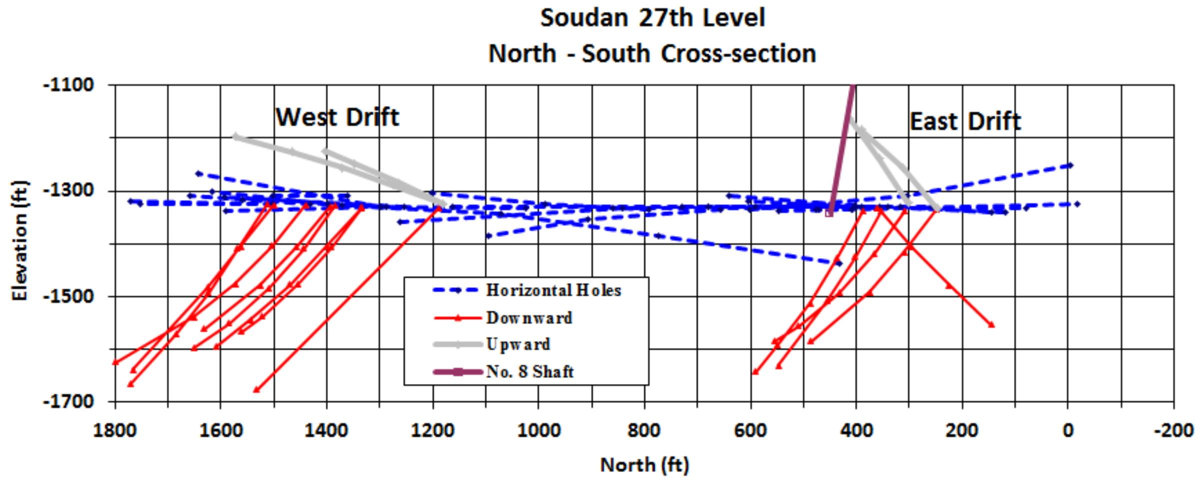


Figure 1

The boreholes can be grouped into three types: Horizontal, Downward, and Upward. The downward trending holes have the highest salinities although there is not an absolute correlation of depth to salinity. The horizontal holes have intermediate salinities and produce extensive suites of mineralogical and biological formations. Upward trending boreholes have the lowest salinity but are still classified as brackish. By locating and sampling individual boreholes we have now found waters with salinity levels four times higher than seawater. We are developing plans to access additional boreholes that surrounded by delicate formations, particularly on the East Drift.

Figure 2 is a conceptual model of groundwater flow into the 27th level. The current results suggest that the salinity at each individual borehole is a mixture of fresher waters, originating ultimately from the surface, and deeper, older, continental shield brines. We have collected and are currently analyzing samples to help estimate the ages of these source waters.

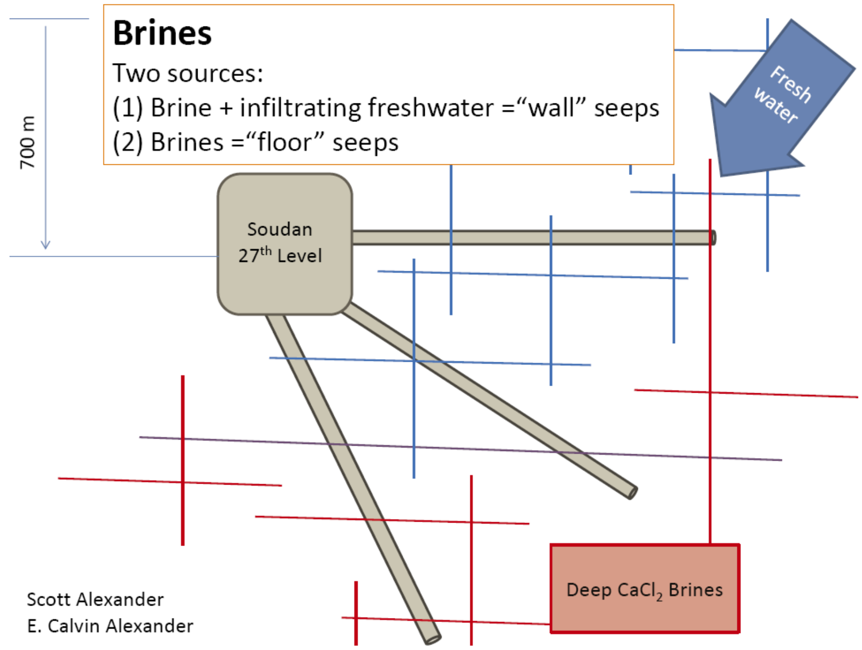


Figure 2

To date 35 samples for major and trace element, and isotopic analysis have been collected. Several boreholes have been sampled at up to four different times throughout the past year to look for seasonal variation in the waters. In addition, two data loggers have been deployed to monitor salinity, temperature and flow variations in borehole waters. Figure 3 shows preliminary results for the loggers installed into horizontal drill hole 920 in January 2011 and downward drill hole 951 in August 2011. Air temperatures on the 27^{th} level show a slight warming while the air ventilation systems were shut down following the March shaft fire. The temperature in the horizontal drill hole (920) slightly lag the air temperatures and are slightly warmer. The downward drill hole (951) shows almost no temperature change. Conductivity in the horizontal drill hole (920) does show a significant decrease bottoming out in late February before rising back to its original level along with a secondary dip in September. As the time period of monitoring increases we hope to tie changes in conductivity to surface recharge events, i.e. spring snowmelt and fall rains. The water levels (which are not plotted in Figure 3), have been relatively stable and are close to the resolution of the sensors and therefore the flow rates have had only nominal changes. The logger data, so far, support the simple model presented in Figure 2. The deepest hole, 951, is the most stable while the horizontal hole shows larger seasonal trends.

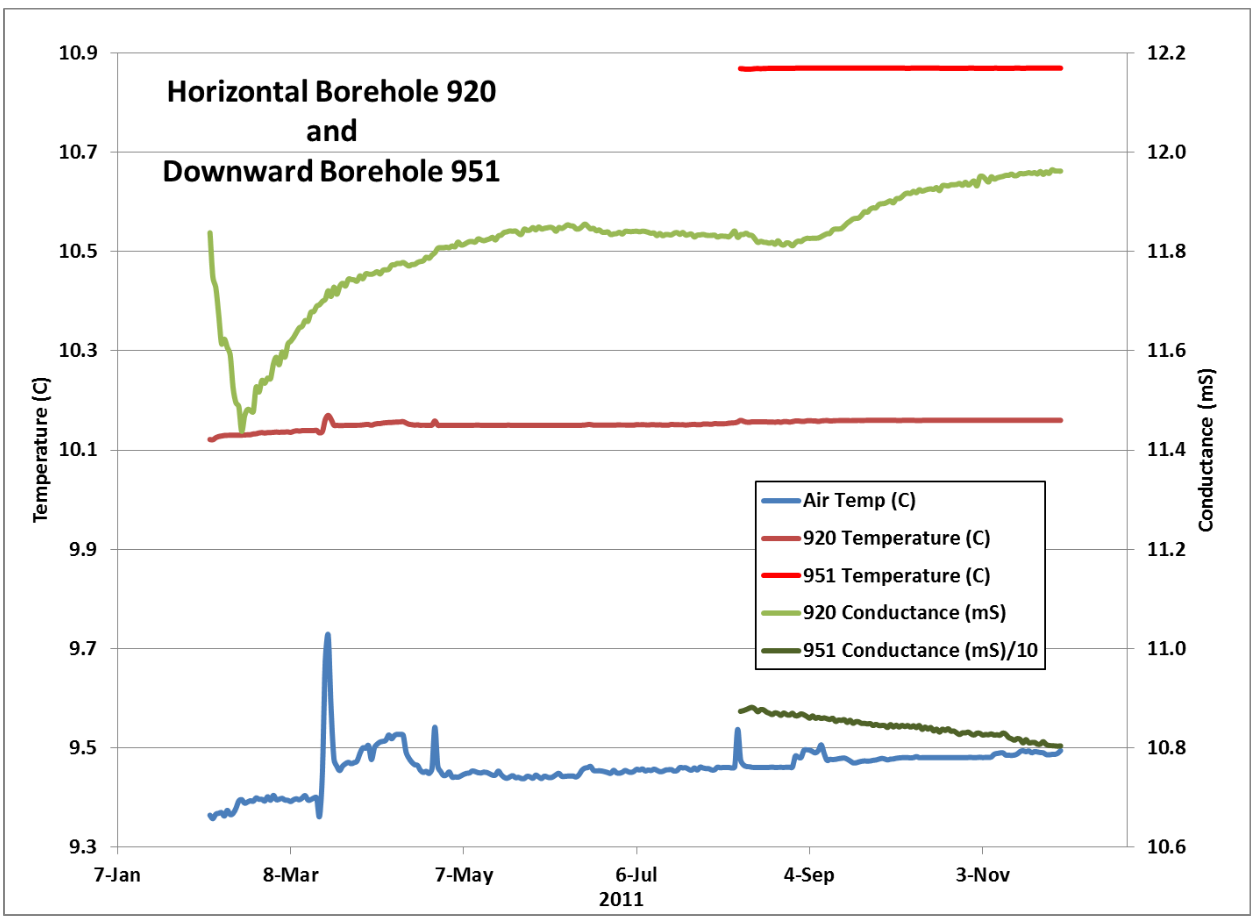


Figure 3

Result Status as of *(July 2012)***:**

Deliverable 1:

We have been working on developing both a defined media for the growth of mine isolate JG233 (DDH 942, 'Far Bubbler' and determining the conditions under which it clearly and definitively displays iron-oxidizing activity. We have also been attempting to show iron-oxidizing activity in *Marinobacter aquaeolei* VT8 as a control strain for our experiments. Methods include traditional gradient tubes, small-scale gradient tubes, zero valent Fe plates of different varieties, and CFU counts from the various conditions. We have also been working on the deletion of a c-type cytochrome from VT8 to verify and validate methods of mutagenesis in *Marinobacter* strains (including mine isolates). We have also begun working with putative photosynthetic mine isolates, starting iron gradient tubes to determine if they can grow via iron oxidation.

The Gralnick and Toner labs continue to collaborate with the Census of Deep Life program (headed by Prof. Rick Colwell, Oregon State University) and Prof. Gregory Dick (University of Michigan) to analyze the microbial communities from several sample spots on level 27 in the Mine. Two variable regions of the 16s rRNA gene have been sequenced (using 454) from PCR amplification products from DNA isolated from six sample sites. Co-PI Alexander oversaw measurements of basic geochemistry from these sites, as described below.

Table of sample site information for CoDL DNA extractions and community analysis.

Sample	DDH	Drift	DDH Orientation	pH	Salinity (mS)	Temp. (C^o)
920W2	920	West	Horizontal SE	6.09	15.06	11.7
932W3	932	West	Horizontal NNW	6.11	131.9	11.6
942W14	942	West	Downward NNW	6.35	94.6	11.5
944W11	944	West	Downward NNW	5.47	93	11.1
951W11	951	West	Downward NNW	6.29	97.4	11.7
964W1	964	West	Horizontal N	Unknown	Unknown	Unknown

Deliverable 2:

Publications

1) Co-PI Toner published a paper on marine iron microbial mats that provides a methodological framework for the Soudan microbial mats research:

Toner, B. M., Berquo, T. S., Michel, F. M., Sorensen, J. V., Templeton, A. S., and Edwards, K. J., 2012. Mineralogy of iron microbial mats from Loihi Seamount. Frontiers in Microbiological Chemistry 3, 1-18.

2) Ph.D. student Lindsey Briscoe is currently preparing a manuscript for (peer review) publication on the iron chemistry of the Soudan microbial mats. In support of that activity, she participated in training workshops here at the University of Minnesota:

- Mössbauer Spectroscopy Analysis Workshop, June 2012, Institute for Rock Magnetism
- "How to Write a Great Scientific Research Paper, and Get it Accepted by a Good Journal", March 2012, The Graduate School, UMN. Anthony Newman, Elsevier Publishing

Research Activities

3) The Census of Deep Life data set became available, and will help our team understand the

microbial composition and diversity of bacteria living the oxygen-free brines of the Soudan Mine. Collaborators Greg Dick and Cody Sheik (University of Michigan-Ann Arbor) are mentoring Co-PI Toner and Ph.D. student Lindsey Briscoe in data analysis. An abstract to the scientific meeting American Geophysical Union will be prepared for the August 8, 2012 deadline.

4) New data on the iron chemistry of the Soudan microbial mats was collected by Ph.D. student Lindsey Briscoe in March 2012 at the Advanced Photon Source, Argonne National Laboratory, Argonne, Illinois. These data were collected to support the microbial composition and diversity study described in #5.

5) New data on the microbial composition and diversity of the iron-rich oxygen-rich microbial mats was generated through DNA sequencing in collaboration with Greg Dick and Cody Sheik (University of Michigan-Ann Arbor)

Deliverable 3:
(Alexander Lab) Forty one samples for major element chemistry and a suite of trace elements have been collected and analyzed. In particular, we have analyzed a series of samples from 4 downward sloping boreholes and 2 horizontal boreholes to look for variations in time; six or more samples have been collected from the same boreholes. Salinities have varied by more than 20% in the boreholes from sample to sample indicating a variation in the mixing fresher surface waters and deeper brine solutions as hypothesized in the Figure 2 model (see above). We are now working with pumping records from the mine looking for correlations in pumping with seasonal recharge events from snowmelt and precipitation.

We have also collected samples for a variety of isotopic analyses. Stable isotopes of hydrogen and oxygen show a mixture of meteoric waters, derived from the surface, with deeper brines solutions. Similarly, stable isotopes of strontium show a stronger interaction with the rock in the deep brine solutions. Samples for age dating have been collected and submitted for analysis. Tritium, a radioactive isotope of hydrogen, has a short half-life of 12.7 years and is very sensitive to recent recharge of surficial waters. Carbon-14 has a longer half-life of 5,730 years and Chlorine-36 has an even longer half-life of 301,000 years allowing us to estimate the fraction of older waters present. We are currently awaiting results on the radiogenic isotopes.

Result Status as of *(January 2013)*:

Deliverable 1

A main project in the Gralnick Lab is continuing the characterization of a bacterium isolated from the Soudan Iron Mine that is capable of iron-oxidation. We are working on different growth systems where autotrophic growth of *Marinobacter* sp. strain JG233 on Fe(II) could be demonstrated. We used gradient systems in 2mL tubes where the entire tube is sacrificed for CFU plating in a hope to get more accurate cell counts. We are also exploring demonstrating growth by showing plasmid-loss over time, as plasmids are only lost during cell division. However, this method has proven difficult. To date, we are convinced that these bacteria can mediate iron oxidation, but we are unconvinced of their ability to use this metabolism for growth, which clearly points to other methods of growth for these abundant microbes in the Soudan Iron Mine brines on Level 27.

In an effort to better understand the metabolism of the JG233 isolate, we also worked on characterizing and optimizing growth conditions. Temperature, salinity, pH, carbon sources, and antibiotic sensitivity were determined and optimized to refine the defined media. Growth occurred at temperatures ranging from 2-37^{o}C, with an optimum temperature of 30^{o}C, and NaCl concentrations from 1-10%, with an optimum of 5%. JG233 tolerates a pH range of 5-10 with an optimum of 6, and is able to grow anaerobically respiring nitrate to nitrite. JG233 was also shown to be able to utilize arabinose, glycerol, glucose, and sucrose; differing from what is published for other *Marinobacter* isolates.

Finally we have developed a genetic system in JG233. As a demonstration, we have successfully deleted the flagellin genes *fla*B and *fla*G via allelic exchange. The flagellin genes where selected as good targets for a proof of principle gene deletion. Flagellin should not be essential for growth, has an easily observable phenotype, and may be required for locating and maintaining the cells in the optimum location in gradient tubes.

Deliverable 2

1) Ph.D. student Lindsey Briscoe is working with Scott Alexander to build a geochemical model in the software program Geochemist's Work Bench. The main effort is in correcting the thermodynamic database for the high salt concentration of the Soudan groundwater. The geochemical modeling will be part of Lindsey's first manuscript, which is still in preparation from last the last report.

2) Ph.D. student Ben Bonis re-sampled the Level 27 groundwaters for DNA-based analysis of the Archaeal microbial communities. These samples will be sequenced at the University of Michigan by collaborators Greg Dick and Cody Sheik. The data set will be paired with Bacterial microbial community data from Census of Deep Life program. PI Toner is preparing a manuscript that will report on the Archaeal and Bacterial microbial communities of the Level 27 brines and mats.

3) PI Toner presented a talk at the American Geophysical Union meeting in San Francisco, CA, USA in December 2012:

Toner, B.M., Sheik, C., Bonis, B., Briscoe, L.J., Alexander, E.C. Jr., Alexander, S., Dick, G.J., and Gralnick, J. Terrestrial deep biosphere observatory: the Soudan Iron Mine. American Geophysical Union, Fall Meeting, San Francisco, CA, B41F-06 (2012) [talk].

4) Research fellow Rebecca Sims has started the iron oxidation and reduction rate experiments by planning an iron analytical method called the "ferrozine" method. These experiments are the final major research activity proposed in the original grant. In consultation with Professor Lee Penn (University of Minnesota, Department of Chemistry), we have decided to do some initial experiments with synthetic minerals (goethite and akaganeite) based on the mineralogical characterization of the mats (on-going, Ph.D. student Lindsey Briscoe). With a method developed for a simple system, experiments with the iron mats will be conducted.

5) Ph.D. student Lindsey Briscoe has been assisting Scott Alexander in the final sampling for the stable and radiogenic isotope studies that will address the residence time of the groundwater and the degree of interaction between the groundwater and fractured rocks.

Deliverable 3

To develop an understanding of the waters, and dependent microbiologic communities, existing in 27th level of the Soudan Underground Mine we have collected and analyzed existing borehole and geologic information. Dean Peterson and the Precambrian Research Institute have been of invaluable assistance in this effort. Accurate locations for diamond core drill holes have led to the rediscovery of many important water sources into the lowest level of the mine.

This includes the highest salinity boreholes found to date as well as understanding the distribution of salinity within the mine. Figure 1 is cross-section of the 27th level with all the borehole information collapsed into a North-South line.

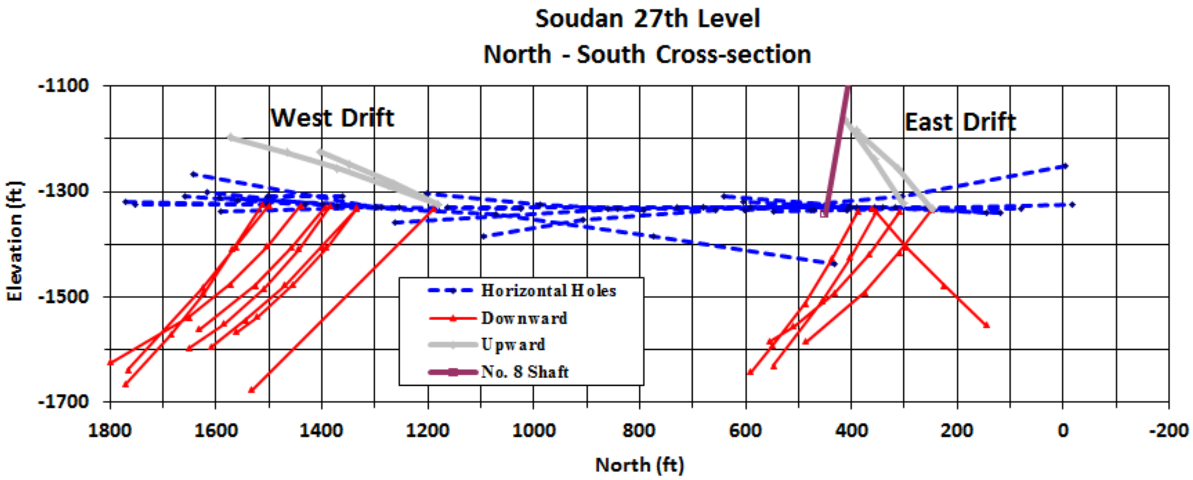


Figure 1

The boreholes can be grouped into three types: Horizontal, Downward, and Upward. The downward trending holes have the highest salinities although there is not an absolute correlation of depth to salinity. The horizontal holes have intermediate salinities and produce extensive suites of mineralogical and biological formations. Upward trending boreholes have the lowest salinity but are still classified as brackish. By locating and sampling individual boreholes we have now found waters with salinity levels four times higher than seawater. We are developing plans to access additional boreholes that surrounded by delicate formations, particularly on the East Drift.

Figure 2 is a conceptual model of groundwater flow into the 27th level. The current results suggest that the salinity at each individual borehole is a mixture of fresher waters, originating ultimately from the surface, and deeper, older, continental shield brines. We have collected a suite of chemical and isotopic samples to help define the inter mixing of these waters. Results to date include tritium analysis of water from both horizontal and downward drill holes. Since there is no detectable tritium we now know that the even the fresh waters mixing with the deeper brines have been underground more than 50 years (no detectable tritium). Analysis of the chloride isotopic content of the downward borehole shows almost no detectable Chlorine 36 indicating ages greater than more than a million years for the chloride. We are awaiting results of Carbon 14 analysis that should be define the ages of the fresh water component (Carbon 14 has a 5,730 year half-life that should help define the ages beyond the greater than 50 years old based on tritium.

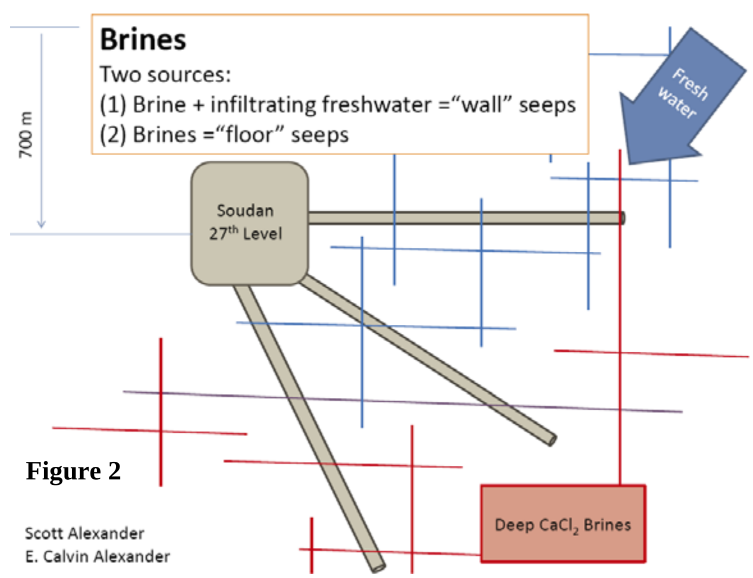


Figure 2

Fifty-one samples for major element chemistry and a suite of trace elements have been collected and analyzed. In particular, we have analyzed a series of samples from 4 downward sloping boreholes and 2 horizontal boreholes to look for variations in time; six or more samples have been collected from the same boreholes. Salinities have varied by more than 20% in the boreholes from sample to sample indicating a variation in the mixing fresher surface waters and deeper brine solutions as hypothesized in the Figure 2 model. We are now working with pumping records from the mine looking for correlations in pumping with seasonal recharge events from snowmelt and precipitation.

An additional synoptic series of 16 water samples have been collected vertically through the mine to investigate the pathways of freshwater movement downward to the base of the mine. Preliminary results show a non-linear range of freshwater chemistries down to the 22nd level of the mine. The freshest waters emerge from the 12 East drift originating near the Alaska shaft which provides a direct surface connection. From the 23rd level to the bottom 27th level salinity increases rapidly.

We have also collected samples for a variety of isotopic analyses. Stable isotopes of hydrogen and oxygen show a mixture of meteoric waters, derived from the surface, with deeper brines solutions. Similarly, stable isotopes of strontium show a stronger interaction with the rock in the deep brine solutions.

In addition, five data loggers have been deployed to monitor salinity, temperature and flow variations in borehole waters. Additional loggers have been placed in the east and west drains of the 2th level to monitor any changes do to recharge from spring melt water. Figure 3 shows preliminary results for the loggers installed into horizontal drill hole 920 in January 2011 and downward drill hole 951 in August 2011. Air temperatures on the 27th level show a slight warming while the air ventilation systems were shut down following the March shaft fire. The temperature in the horizontal drill hole (920) slightly lag the air temperatures and are slightly warmer. The downward drill hole (951) shows almost no temperature change. Conductivity in the horizontal drill hole (920) does show a significant decrease bottoming out in late February before rising back to its original level along with a secondary dip in September. As the time period of monitoring increases we hope to tie changes in conductivity to surface recharge events, i.e. spring snowmelt and fall rains. The water levels (which are not plotted in Figure 3), have been relatively stable and are close to the resolution of the sensors and therefore the flow rates have had only nominal changes. The logger data, so far, support the simple model presented in Figure 2. The deepest hole, 951, is the most stable while the horizontal hole shows larger seasonal trends.

Final Report Summary:

<u>Deliverable 1 - Microbial community / cultivation</u>

Molecular Microbial Community Analysis:

Collaborators Greg Dick and Cody Sheik (University of Michigan, not funded by LCCMR) are planning to sequence Archaeal DNA from Level 27 brines to complete the dataset started by Gralnick, Toner and Dick in the Census of Deep Life program. At last communication, November 8, 2013, the samples collected by Toner and Briscoe were in the cue to be sequenced in November. These data will be analyzed with data collected with funds from the

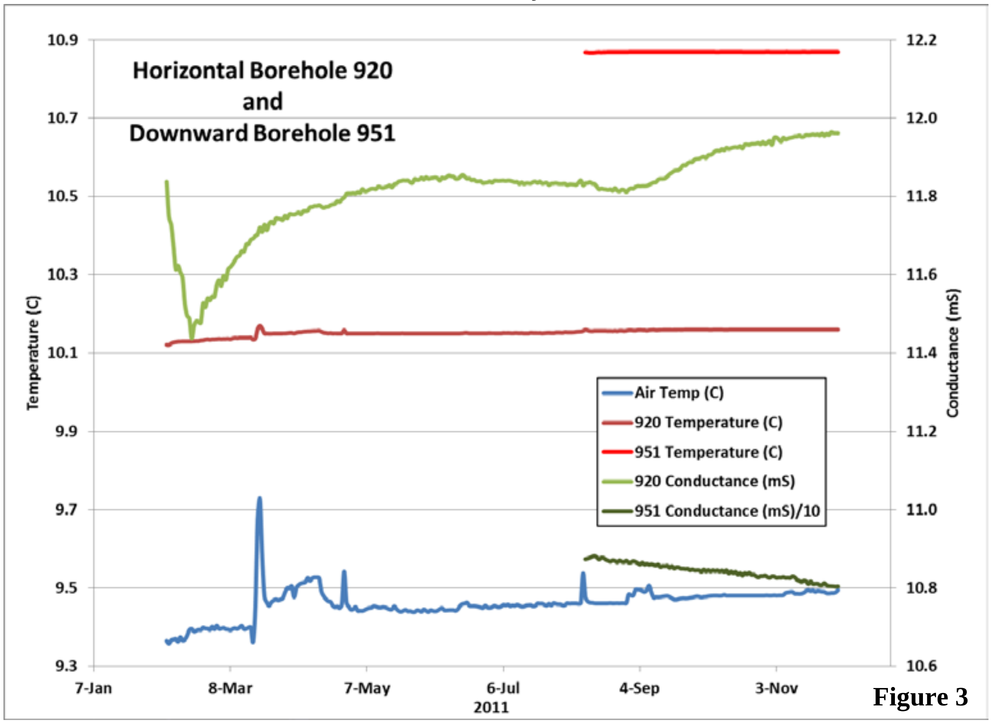
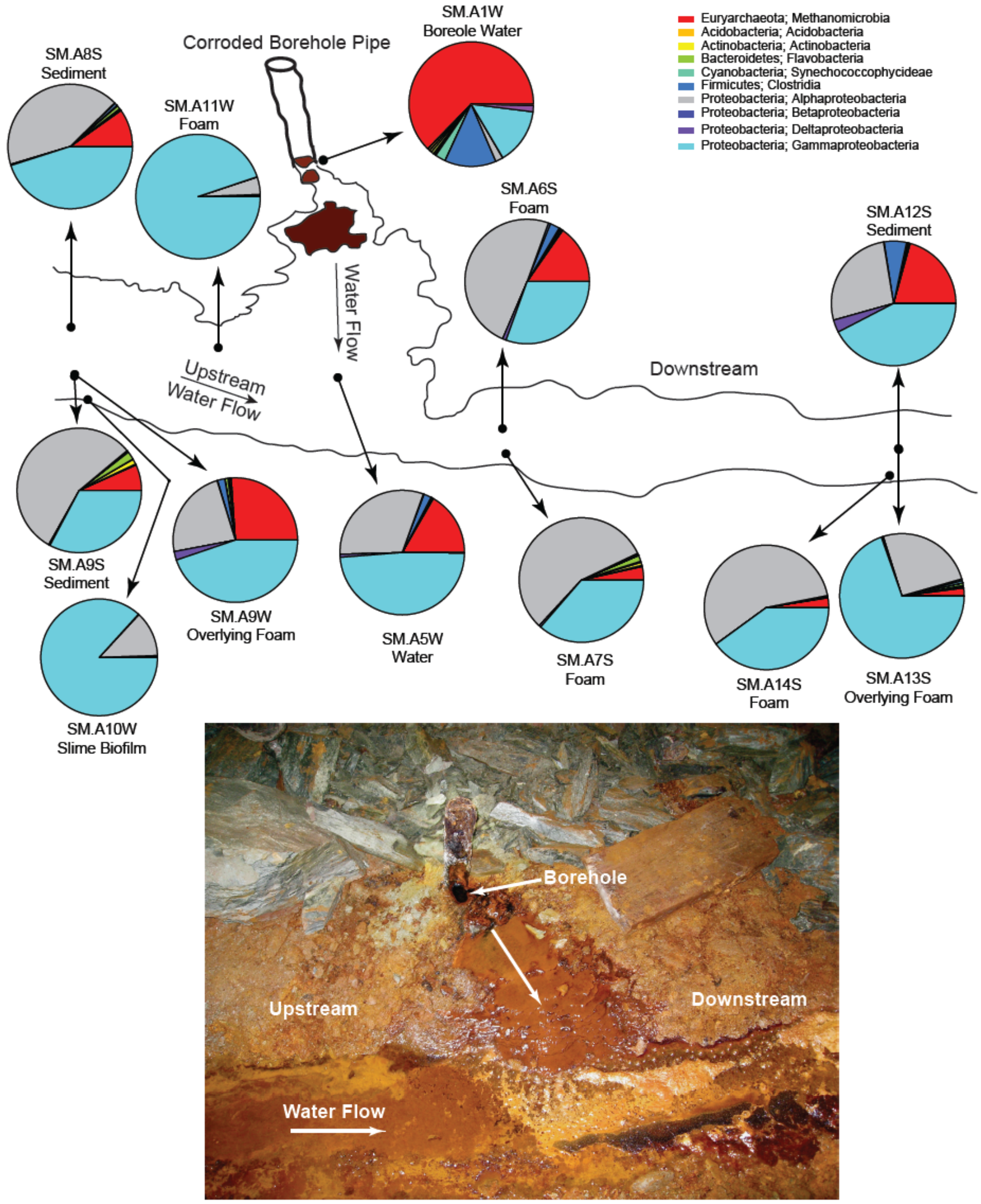


Figure 3

LCCMR, and augmented by the Census of Deep Life program, to describe the microbial community composition in Soudan Mine brines along gradients of increasing oxygen. This work will round out our molecular analysis of the bacterial and archaeal components from the level 27 boreholes (from the West side). We have leveraged new funds from the Census of Deep Life program and from the University of Michigan to complete this dataset. The first portion of the dataset is publically available through the Census of Deep Life program: http://vamps.mbl.edu/portals/deep_carbon/cdl.php (note that you must register on the website to access data).

The major conclusions for the microbial community analysis are summarized in the composite figure below for diamond drill hole (DDH) #951. Water and mat/sediment samples were collected from the outlet of the DDH along the flow path up to ~ 3 m. The anoxic brine emanating from the DDH spring has a strong signature of Archaea in the Methanomicrobia group (designated by the color red in the pie charts below). In less than 3 feet of flow path, the microbial community was observed to shift to Bacteria in the Gammaproteobacteria group. This microbial community shift is accompanied by strong changes in geochemical properties as oxygen is introduced to the anoxic brine groundwater (shown in next figure).



A Model Iron Oxidizing Bacterium from the Soudan Iron Mine:

Fe(II)-oxidizing bacteria act as important agents of environmental change and have significant influence on industry and infrastructure by contributing to the cycling of iron, sulfur, nitrogen, oxygen, and manganese. The microbial facilitated oxidation of Fe(II) to Fe(III) results in corrosion and biofouling of municipal water distribution systems as well as water-associated industrial structures. Despite a long and persistent scientific interest in the Fe(II)-oxidizing bacteria, little biochemical and genetic analysis has been conducted regarding this metabolism due to the particular requirements for their cultivation. Here we demonstrate light-independent, microaerophilic Fe(II)-oxidation at neutral pH by *Marinobacter* sp. strain JG233, isolated from the Soudan Iron Mine in Northern Minnesota. Unlike many of the current model organisms for microaerophilic Fe(II)-oxidation, JG233 grows heterotrophically to high cell densities on a variety of carbon sources, and is genetically tractable. Genetic tractability was demonstrated through the targeted deletion of the flagellin gene cluster, resulting in a motility deficient strain. Growth occurred at temperatures ranging from 2-37°C, with an optimum temperature of 30°C, and NaCl concentrations from 1-10%, with an optimum of 5%. JG233 tolerates a pH range of 5-10 with an optimum of 6, and is able to grow anaerobically respiring nitrate to nitrite. The 16S rDNA sequence of JG233 shares a 99.02% sequence identity with *Marinobacter guinea*, and a 98.8% sequence identity to *Marinobacter adhaerens* strain HP15. JG233 may utilize an uncharacterized mechanism of Fe(II)-oxidation, as the draft genome of JG233 does not contain homologs to proteins shown to be involved either in the oxidation or reduction of iron in other systems. *Marinobacter* strain JG233 represents a distinct model for the study of microaerophilic Fe(II)-oxidation at neutral pH as it is genetically tractable and capable of heterotrophic growth; traits previously unavailable in microaerophilic Fe(II)-oxidation models. Benjamin Bonis, a PhD student in the Gralnick Lab is currently working on a manuscript outlining the information presented here.

<u>Deliverable 2 - Mineralogy</u>

1) Findings from our LCCMR research activities were presented at the following venues since the last reporting period:

   Briscoe, L.J., Alexander, S.C., Alexander, E.C., Jr., Berquo, T.D., Fakra, S.F., Michel, F.M., Moskowitz, B.M., and Toner, B.M. Geochemical and biological gradients in the Soudan Underground Mine. Minnesota Ground Water Association (MGWA), St. Paul, MN (2013) [poster]

   Sheik, C.S., L. Briscoe, J. Gralnick, B.M. Toner, and G.J. Dick. Deep brine waters host low diversity microbial communities at Soudan Iron Mine. Deep Carbon Observatory, Deep Life Workshop, Portland, OR (2013) [talk]

2) Toner has one manuscript in preparation for peer review:

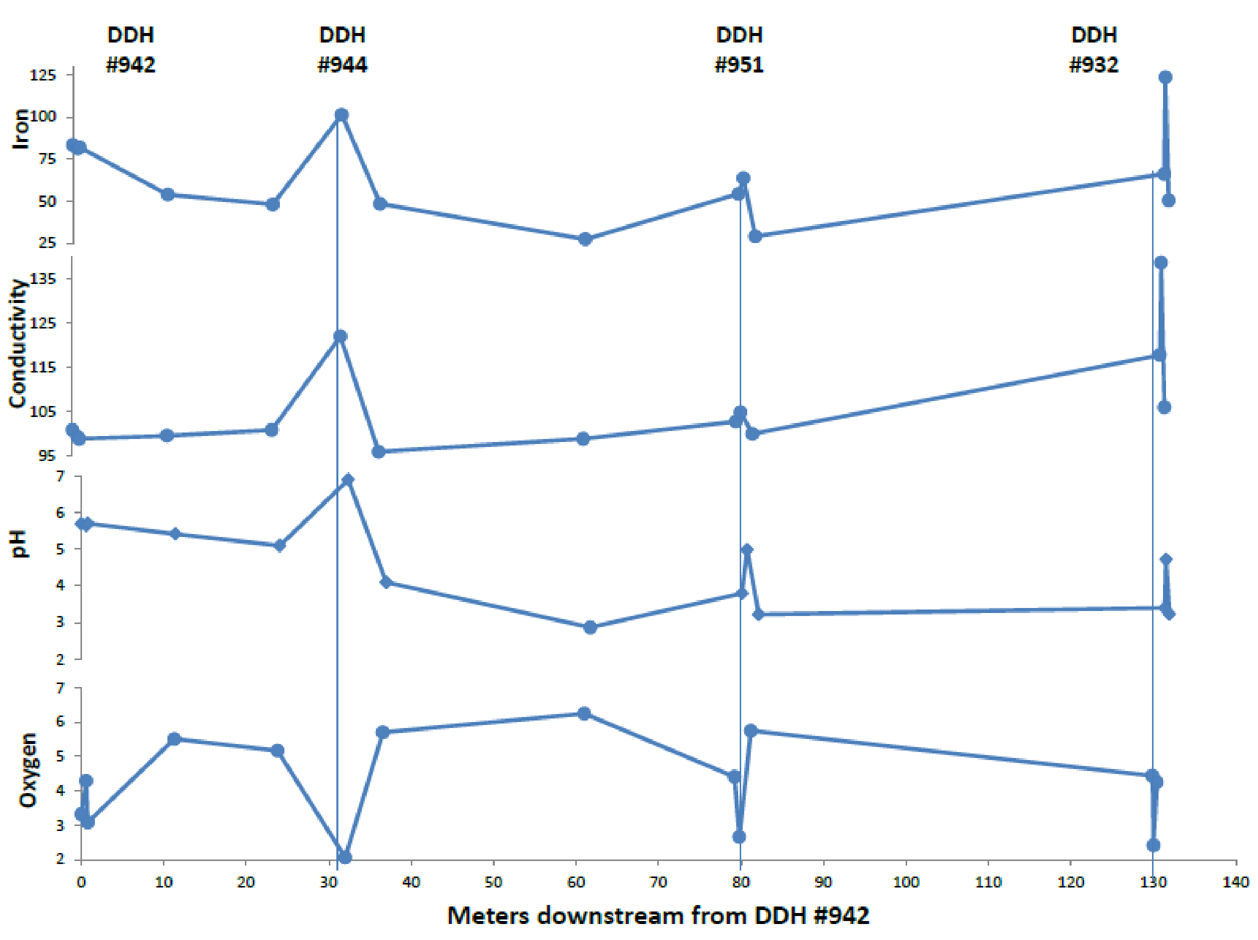
   Toner, B.M., Sheik, C.A., Bonis, B., Briscoe, L.J., Peterson, D., Alexander, S., Alexander, E.C. Jr., Dick, G.J., Gralnick, J.A. Microbial community dynamics in anaerobic, fractured-rock brines in response to oxidation-reduction potential gradients. In preparation.

3) Ph.D. student Briscoe has completed sample collection for her dissertation and will be working on publishing the results over the next couple of years. Briscoe has one manuscript in preparation for peer review:

   Briscoe, L.J., Alexander, S.C., Alexander, E.C., Jr., Berquo, T.D., Fakra, S.F., Michel, F.M., Moskowitz, B.M., and Toner, B.M. Iron mineral formation in microbial mats formed from continental shield brines. In preparation.

4) Toner and Briscoe conducted field work in the mine May 20-22, 2013, prior to the end of the grant. The goal of that final field work was synoptic sampling of the Level 27 flow-path. We collected co-located water and mat/sediment samples for geochemical and microbiological analysis. This sampling effort represented the best practices gained through the course of the grant period. The photos below show four of the main sampling sites visited during the field-work:

Most of the water and mat samples were analyzed prior to the end of the grant. A summary of selected parameters measured for the water along the Level 27 flow path (dissolved iron, conductivity, pH, and dissolved oxygen) are displayed below. At ~ 30 m downstream, diamond drill hole (DDH) #944 joins the flow path and a jump in dissolved iron is accompanied by increases in conductivity and pH. Along with the reduction in dissolved oxygen, these are signatures indicative of the fractured-rock brine: high iron, high salts, near-neutral pH, and very low oxygen (as shown in the next figure):



Dissolved iron (mg/L), conductivity (mS/cm), pH, and dissolved oxygen (mg/L) are displayed for the Level 27 diamond drill hole (DDH) springs and the flow path connecting them. Water flow is from left (0 m) to right (130 m) on the x-axis. Increases in iron, conductivity, and pH are indicative of a new DDH spring joining the flow path. Dissolved oxygen is very low in the brines flowing from the DDH springs.

<u>Deliverable 3 – Geochemistry</u>

The current results suggest that the salinity at each individual borehole is a mixture of fresher waters, originating ultimately from the surface, and deeper, older, continental shield brines. We have collected a suite of chemical and isotopic samples to help define the inter mixing of these waters. Results to date include tritium analysis of water from both horizontal and downward drill holes. Since there is no detectable tritium we now know that the even the fresh waters mixing with the deeper brines have been underground more than 50 years (no detectable tritium). We have conducted Carbon-14 age (^{14}C) dating of the dissolved bicarbonate from horizontal hole DDH 962 and on the methane gas from horizontal hole DDH 920 and downward hole DDH 942 and DDH 944. The apparent ^{14}C age of dissolved bicarbonate in DDH 962 is 15,000 years old or water that was last at the surface 15,000 years ago. As the entire Soudan Mine area was buried under a continental ice sheet at that time there is little likelihood of ground water recharge at that time. A more likely scenario builds on the Figure 2 flow model of mixing waters. The deep briny waters are very old with no measureable ^{14}C. These deep waters are mixing with some fraction of waters that are a few centuries old based on lack of tritium. DDH 962 has a measured fraction of modern ^{14}C of

0.165. This implies, based on the mixing model, that 10 to 20% of the water is younger, fresher waters moving down from the surface mixing with 80 to 90% very old, briny waters. The methane analyses provided complementary results where horizontal hole DDH 920 has a fraction of modern ^{14}C of 0.074 implying that something less than 10% of the carbon in the methane is from a younger surface source with the balance being very old carbon sources. The two downward bore holes, DDH 944 and DDH 942; have no measurable ^{14}C signifying carbon sources that have been isolated from the surface for more than 40,000 years. In actuality the water and carbon have likely been underground for much longer than 40,000 years but this is the limit of resolution for the ^{14}C technique.

Analysis of the chloride isotopic content of the downward borehole shows almost no detectable Chlorine 36 indicating ages greater than more than a million years for the chloride. The Alexander group is pursuing a collaboration with other researchers to apply additional isotopic techniques to further constrain the ages of the ground waters in the Soudan Mine. These additional isotopic techniques are well beyond the scope and budget of the current grant, but will be the focus of future efforts.

RESULT 2: Innovative Applications – Novel Compound Discovery, Bioenergy and Bioremediation.

Description: In this section we will determine the feasibility of utilizing microorganisms isolated from the Soudan Iron Mine in three specific commercial areas. Our priority here is to identify potential commercial assets deriving from microbes cultivated from the Soudan Mine.

Summary Budget Information for Result 2: **ENRTF Budget: $ 145,682**
Amount Spent: $ 140,821
Balance: $ 4,861

Deliverable	**Completion Date**	**Budget**
1. Screen novel actinobacterial isolates for production of anticancer and antimicrobial drugs	June 2013	$83,250
2. Test pure cultures and enrichments for the use of iron oxidizing bacteria in microbial fuel cells	June 2013	$42,445
3. Isolate and characterize bacteria that promote oxidation / retention of copper and cobalt	June 2013	$42,445

Result Completion Date: June 2013

Result Status as of *(January 2011)*:

Deliverable 1:
Members of the Salomon lab have made four research trips to the Soudan Mine since August 2010 to collect samples and data. Most collections were made on the 27th and 10th levels of the mine and include water samples from boreholes, water and sediments from trenches throughout the drifts and swabs from rock surfaces. The samples were cultivated on several solid medias to enrich for a variety of actinobacteria, and more than 30 unique bacterial isolates have been identified. DNA sequencing analysis of the strains suggests that these bacteria are highly

diverse and represent species from at least four different genera of actinobacteria. Each isolate has been tested for activity against a panel of eight human pathogens (drug-resistant bacteria and fungi) and almost 40% exhibit potent antifungal activity. Several of the strains produce compounds that disrupt established biofilms of MRSA (methicillin-resistant *Staphylococcus aureus*), the drug-resistant bacterium responsible for a growing number of hospital- and community acquired infections. Current studies are focused on isolating additional unique strains from the environmental samples, analyzing the phylogenetic diversity of cultured and uncultured actinobacteria from various locations throughout the mine and purifying and identifying the natural products responsible for the biological activities.

<u>Deliverables 2 and 3:</u>
Though these aspects of the project were not scheduled to begin until year 2 of the project, we have some preliminary experiments using electrochemical bioreactors to enrich for bacteria able to harvest electrons from a cathodic electrode.

Result Status as of *(July 2011)*:

<u>Deliverable 1:</u>
Members of the Salomon lab have focused on continued cultivation of actinobacteria from the brines on the 27th level of the mine. Genetic and chemical characterization of the cultured isolates has allowed us to group these bacteria into at least 22 distinct groups, several of which certainly represent new species and possibly new genera. Several of these strains have exhibited potent inhibitory activity against drug resistant pathogens including MRSA (methicillin resistant *Staphylococcus aureus*), VRE (vancomycin resistant *Enterococcus*) and *Cryptococcus neoformans* (an opportunistic fungal pathogen). We are currently scaling up two of the most active strains to identify the bioactive metabolites. Additionally, we are developing an assay in collaboration with David Blehert at the USGS in Wisconsin to test some of our strains against the causative agent of White nose bat syndrome, *Geomyces destructans*. Because we cannot grow this pathogen in the state of Minnesota (to reduce the risk of spreading the disease), we will be sending our strains to Dr. Blehert's lab for testing.

<u>Deliverables 2 and 3:</u>

Dr. Zarath Summers, who earned her PhD last spring from the University of Massachusetts – Amherst, was hired to work on the project under the supervision of Profs. Gralnick and Bond. Dr. Summers is trained in anaerobic microbial physiology and electrochemistry. She will carry out the experiments outlined in this section of the proposal. Special anaerobic degassing station has been constructed and samples from Aug. 2012 are currently being used to culture electrode-oxidizing microbes. Microscopy and electrochemical data is available, and we are currently working on a manuscript that we expect to publish in the next 1-2 months.

Result Status as of *(January 2012)*:

<u>Deliverable 1:</u>

The Salomon lab has focused on isolating new strains of actinobacteria from more recent samples collected in the mine and culturing several of the most biologically active strains. One of the isolates, *Streptomyces* sp. CES254, was cultivated in liquid media, extracted and tested for activity. The extract inhibits the growth of several strains of pathogenic fungi, *Candida albicans* and *Cryptococcus neoformans* and was further fractionated to purify the active compounds. Several pure metabolites have been isolated and they are currently being tested to determine if they are the source of the antifungal activity. These samples are also being analyzed to elucidate the chemical structures and determine if they are new compounds. Two additional strains have been grown in scaled up liquid cultures and extracted, and these extracts are being tested and fractionated to identify the biologically active components.

We also began a collaboration with a fungal biologist, Professor Robert Blanchette, to identify and characterize the fungi found in the Soudan mine. Large numbers of fungi were cultured in the lab and are highly diverse, representing many different species and genera (Table 1). The fungi were identified by comparing the DNA sequences of these samples to those of known fungi in an online database (BLAST). Some of the fungi have relatively low DNA sequence similarities to species in the database (<95%), and these may represent new species or even members of new genera. Two unusual strains (*Cadophora* species) are currently being cultured in the lab and will be tested for activity and chemistry after 30 days of growth. Sixteen of the strains were isolated from high copper areas of the mine (samples from level 10) and we are exploring the possibility of developing a fungal biological filter to remove copper and other dangerous solubilized metals from mine effluent and other contaminated areas.

Table 1. Strains of fungi isolated from the Soudan Mine.

Sample ID	Source	Fungal ID (Best BLAST match)	Max id
10-1-1B	Rhizomorphs on wet wood	Postia fragilis	93%
10-1-2B	Rhizomorphs	Calocera cornea	84%
10-1-2	Rhizomorphs	Phialocephala dimorphospora	99%
10-1-5	Rhizomorphs	Nectria flavoviridis (Cosmospora vilior)	99%
10-1-6	Rhizomorphs	Cadophora melinii	100%
10-2-2M	Rhizomorphs underwater	Uncultured fungus	95%
10-2-3M	Rhizomorphs underwater	Cosmospora sp.	98%
10-3-4M	Copper log	Cosmospora sp.	99%
10-4-2B	wood near copper	Uncultured Cantharellales	92%
10-5-1M	copper log	Cadophora fastigiata strain 7R121-1	99%
10-5-2M	copper log	Cadophora fastigiata	99%
10-6-1M	Encrusted log	Phialophora sp.	99%
10-6-1B	Encrusted log	Calocera viscosa	87%
10-6-2B	Rhizomorphs	Uncultured Cantharellales	92%
10-7-1B	Rhizomorphs	Armillaria sinapina	100%
10-7-2M	Rhizomorphs	Hypocreales sp.	96%
23-1-2M	wood	Oidiodendron griseum	100%
23-2-1M	wood	Penidiella kurandae	88%
23-2-2M	wood	Meliniomyces sp.	98%
23-3-1M	Fruiting body	Hyphodontia radula voucher PDD:91616	99%
23-3-2M	Fruiting body	Cladophialophora sp. CBS 454.82	92%
23-4-1B	wood	Mortierella pulchella strain CBS 312.52	99%
23-5-1B	wood	Mortierella parvispora strain CBS 311.52	98%
23-6-2M	wood	Cladophialophora sp. CBS 454.82	93%
23-7-1M	wood	Oidiodendron griseum	100%
27W-3-2M	soil/wood	Fungal endophyte sp. ECD-2008	100%
27E-11-1B	Board at hole	Ascomycota sp. H27	95%

27E-12-1M	DDH 947, birch	Sistotrema coronilla	99%
27E-12-2M	DDH 947, birch	Fungal endophyte sp. ECD-2008	99%
27E-13-1M	DDH 948, across, on floor	Scytalidium lignicola	99%
27E-15-1M	DDH 948, rhizomorphs on large timbers	Nectria mariannaeae	99%
27E-17-1M	DDH 948, on water across from DDH	Sistotrema coronilla	99%
27E-23-1M	DDH 960, chipped wood on ground	Fungal sp. aurim1166 (Scytalidium sp?)	99%
27E-28-1M	DDH 966	Sagenomella sp. UAMH 9571	100%
27E-34-1M	Log on wall	Fungal endophyte sp. ECD-2008	99%
27E-34-2M	Log on wall	Fungal endophyte sp. ECD-2008	100%
27E-35-2B	White material	Fungal endophyte sp. ECD-2008	100%
27E-36-1B	X12 botton of stope - dripping water	Fungal endophyte sp. ECD-2008	99%
27E-36-2M	X12 botton of stope - dripping water	Fungal endophyte sp. ECD-2008	100%
27E-37-2M	Bottom of stope near cage	Mycosphaerellaceae sp. KH00300	87%
27E-39-1M	X12 flag, bottom of stope dripping water	Zygomycete sp. olrim272	99%
27E-46-2M	Bat	Nectria mariannaeae	98%
27E-50-1M brn	DDH 961, bat	Oidiodendron truncatum	100%
27E-50-1M ylw	DDH 961, bat	Lecanicillium sp. M289	99%
27E-50-3M grn	DDH 961, bat	Cosmospora vilior	99%
Water Samples			
10-8-1	Water	Aspergillus unguis	98%
10-8-2	Water	Cosmospora vilior	97%
27W4-1-1	DDH 944/964 foam/water	Exophiala xenobiotica strain CBS 118157	99%
27W4-2	DDH 944/964 foam/water	Uncultured Mycosphaerellaceae	95%
27W4-3	DDH 944/964 foam/water	Nectriaceae sp. BC4	99%
27W5-1	951 foam/water	Aspergillus unguis	100%
27W5-2	952 foam/water	Uncultured Mycosphaerellaceae	95%
27W5-3	953 foam/water	Exophiala xenobiotica	100%

Deliverables 2 and 3:

The Gralnick Lab has enriched for both iron oxidizing bacteria and iron reducing bacteria using samples from several sites on level 27 of the mine. Samples were mostly taken from sediment and sediment-water interfaces from boreholes on the west side. Cathodic and anodic bioreactors were inoculated with low oxygen or fully anaerobic for these enrichments (respectively). We also purchased a pure culture of the marine iron oxidizing zeta-proteobacteria *Mariprofundus ferroxidans* strain PV-1 from the Bigelow Stock Center. We have successfully grown this strain in the lab as a model for how to grow iron oxidizing bacteria and have begun preliminary experiments to grow this organism on a cathode electrode. We are in the process of organizing microbial community sequence analysis from the enrichments described above, and culturing representative bacteria from the enrichments. The individual isolates will be tested this summer by undergraduates for their ability to precipitate a variety of metals and also to test the role of biogenic iron oxides in their ability to sorb some of these metals.

Result Status as of *(July 2012)*:

Deliverable 1:

(Salomon Lab) Our recent efforts have been focused on culturing and testing fungi isolated from the Soudan Mine. Two species of *Cadophora* fungi were chosen for fermentation and extraction because we have recently identified several novel bioactive compounds from isolates of this genus collected in Antarctica. The extracts from the liquid cultures did not exhibit any activity, but when grown on solid rice media, the extracts showed significant inhibition of the gram positive bacteria MRSA (methicillin resistant *Staphylococcus aureus*) and VRE (vancomycin-resistant enterococcus), the fungi *Cryptococcus neoformans* and *Candida albicans* and HCT-116 cells (human colon tumor). This broad and potent activity suggests that either several different potent compounds are present in the extract or that there is a major active compound that is essentially toxic (non-selective). We have begun to purify this extract and are testing fractions to localize and identify the active compound(s).

Deliverables 2 and 3:

From an abstract of a poster presentation by Dr. Z. Summers (Gralnick Lab) at the American Society for Microbiology General Meeting in San Francisco (June 2012):

The Soudan Underground Iron Mine is located in a 2.7 billion year old Banded Iron Formation that is over a half-mile underground in Northern Minnesota. At the lowest level of the mine we are investigating the microbes that live in the salty borehole water seeping out of the Banded Iron Formation. This calcium chloride rich brine from the Soudan Mine is highly unusual as it is almost three times saltier than seawater and is devoid of oxygen until hitting the atmosphere of the mine. Associated with many of these seeps are unique iron oxide structures, poorly characterized iron minerals, and thriving bacterial communities. These communities include iron oxidizing bacteria as well as iron reducing bacteria. Further investigation and characterization of these interesting microbes integrates tools and methods derived from diverse disciplines, such as novel electrochemical culturing devices, as well as identifying the community structure using both molecular biology and visualization tools. Working electrodes of three-electrode cells have been successfully used for the cultivation of the pure culture Fe(II) oxidizing zeta-proteobacterium, *Mariprofundus ferrooxydans*, and these same cells are being used for the enrichment of novel species from the Soudan Mine. The use of the poised electrode as a proxy for iron for the cultivation of iron reducing communities from the mine offers a constant source of terminal electron acceptor in a controlled setting. This system enables us to track in real-time the production of current as electrons flow through the biofilm. Initial 16S rRNA gene sequencing of electrode reducing enrichments reveled that the communities enriched from the anoxic borehole water are diverse, and many of the sequences were only distantly related to sequences deposited in public databases. This is an indication that many of the microbes enriched from the borehole water are novel species and/or genera. Scientific investigation of the Soudan Iron Mine and the microbes living there offers insight into not only microbial processes that may have occurred on Precambrian Earth with respect to the high ferrous iron and low oxygen regime, but these studies will also offer insight into the molecular basis for iron metabolism by resulting in the isolation and study of novel species capable of oxidizing or reducing iron.

Result Status as of *(January 2013)***:**

Deliverable 1

The Salomon Lab has been focusing on scaling up cultures of microbial isolates and purifying the biologically active compounds produced in culture. One bacterial isolate, CES254, was identified as a member of the *Streptomyces* genus and exhibited potent activity against several pathogenic yeasts (fungi). Purification of the active components led to the identification of a known lipopeptide known as iturin A (Figure 4). This compound is known to have antifungal and cytotoxic activities and is also known as a "biosurfactant". We are currently trying to identify other analogues of iturin A produced by the culture to determine if there are additional new compounds. We will also be testing this compound against a panel of *Geomyces* fungi to assess the potential for controlling or treating white nose bat syndrome.

We are also purifying compounds produced by a fungal species isolated from the high copper area of the mine on Level 10. This fungus was identified as a member of the genus *Cadophora*, and is commonly found in Arctic and Antarctic regions. We identified three novel compounds from a solid culture of the fungus in the isoflavonoid family of natural products. The structures of the compounds were similar to previously described molecules known to have anti-malarial activity. We therefore sent the new Cadophora compounds to the Swiss Tropical Research Institute for testing against the malarial parasite *Plasmodium falciparum*. Two of the compounds were found to have potent activity (150 nanograms/mL), and we are currently purifying more analogues for additional testing.

Deliverables 2 and 3

An excerpt from our publication that was recently accepted to the American Society for Microbiology journal *mBio*: "Electrochemical cultivation, supporting growth of bacteria with a constant supply of electron donors or acceptors, is a promising tool for studying lithotrophic species in the laboratory. Major pitfalls present in standard cultivation methods used for metal-oxidizing microbes can be avoided by the use of an electrode as the sole electron donor." This work represents results from our initial experimentation to develop cathode bioreactors to enrich iron-oxidizing bacteria from the Soudan Iron Mine. We chose to first work with a known obligate iron oxidizer, the marine bacterium *Mariprofundus ferrooxidans* strain PV-1. It was important to be able to maximize surface area of the cathode surfaces in the reactor while also maximizing oxygen delivery to the cells attached to the surface. These parameters are significantly different from the current bioreactor design in the Bond Lab, who collaborates with the Gralnick Lab on this project. We are currently in the process of enriching both cathode utilizing the new reactor design and anode-utilizing bacteria using the traditional reactor design.

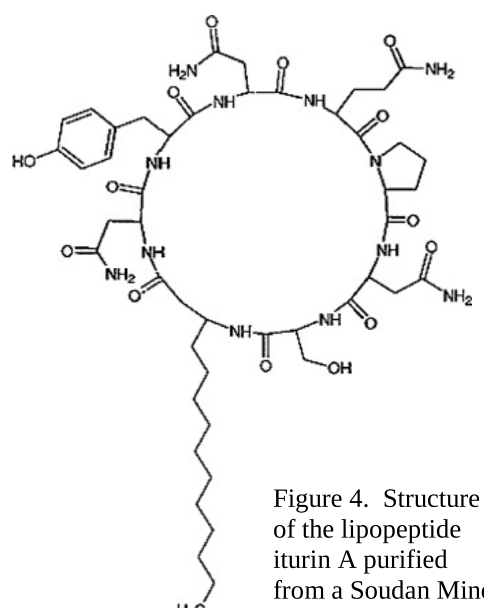


Figure 4. Structure of the lipopeptide iturin A purified from a Soudan Mine *Streptomyces* bacterial culture.

Final Report Summary:

The Salomon and Gralnick labs have cultured and characterized several microbial isolates from the Soudan Iron Mine that have interesting medical and/or biotechnological properties. The Salomon lab focused on bacteria from the genus *Streptomyces* and have recently begun working with fungal isolates as well (Deliverable 1). Many isolates produced compounds that showed activity against the yeast *Candida albicans* and four strains produced a compound active against *Cryptococcus neoformans*. One aspect of the work from the Salomon lab that is now being continued in a new project is to test these isolates for production of anti-fungal compounds that have specific activity against *Pseudogymnoascus destructans* (formerly known as *Geomyces destructans*), the causative agent of White Nose Syndrome. The Gralnick lab cultivated a variety of bacterial strains from the mine and screened them for the ability to catalyze iron oxidation (Deliverable 2). The most robust isolate was a strain of *Marinobacter* that the Gralnick lab has been studying in detail over the last ~ 2 years. The goal of the detailed studies is to understand the molecular mechanism of iron oxidation. The Gralnick lab sequenced the genome of the Soudan Mine *Marinobacter* isolate, developed a genetic system for this strain and has conducted many experiments toward the goal of understanding iron oxidation. A manuscript is currently in preparation describing the strain, its genome and the genetic system we developed.

The Gralnick lab, in collaboration with the Bond lab, developed electrochemical bioreactors that could be poised as cathodes to enrich iron-oxidizing bacteria from the Soudan Iron Mine. Several enrichments have been transferred multiple times and we continue to enrich and monitor the microbial population to determine how many different microorganisms are present and how the community is changing over time. These experiments were much more difficult than originally anticipated, which required us to begin working with a 'known' iron oxidizing bacterial strain, *Mariprofundus ferroxidans*, to refine the design of our cathode bioreactors. In parallel to this research we also have enriched for a high-salt electrode reducing microbe that represent a novel genus of bacteria based on its 16s rRNA gene sequence. We continue to characterize this isolate. Preliminary experiments for Deliverable 3 with several different isolates from the mine were unsuccessful in that redox transformation of cobalt or copper was not observed. Future experiments where isolates are selected to first be resistant and/or to utilize the redox properties of these metals might result in more success.

RESULT 3: Public Outreach and Education

Description: We will collaborate to develop training for DNR tour guides to describe the features, microorganisms, biogenic mineralogy and geochemistry within the mine and collaborate with staff to generate educational displays and to develop best practices for protecting this unique environment.

Summary Budget Information for Result 3: **ENRTF Budget: $ 10,000**
Amount Spent: $ 10,000
Balance: $ 0

Deliverable	**Completion Date**	**Budget**
1. DNR microbiology training module	June 2013	$ 500
2. Best practices assessment for level 27 ecosystems	June 2013	$ 500
3. Visitor Center interactive display	June 2013	$ 9,000

Result Completion Date: June 2013

Result Status as of *(January 2011)*:

This aspect of our project is scheduled for year 3.

Result Status as of ***(July 2011)*****:**

This aspect of our project is scheduled for year 3. We have identified contacts at the Science Museum of Minnesota who can provide advice for Result 3.

Result Status as of ***(January 2012)*****:**

No results, as this part of the project is scheduled for year 3, though we have included some outreach proposals with a NASA Astrobiology Institute proposal (Toner Co-Investigator) that is being organized at Princeton University.

Result Status as of ***(July 2012)*****:**

No results, but we are in the early stages of planning our strategy for the Visitor Center interactive display and continuing discussions with the Science Museum of Minnesota.

Result Status as of ***(January 2013)*****:**

We have been in the process of updating the visitor center at the Soudan Underground Mine State Park. We have created an interactive touch-screen display with which visitors can learn more about the research in the lower levels of the mine funded by our grant, and about life in the subsurface in general. Not only will the display inform visitors of all ages and scientific exposure about the various ongoing research projects, but it will also help to foster an interest in subsurface geobiological research.

Final Report Summary:

The interactive touch screen display for the Visitor's Center at the Soudan Underground Mine State Park has been purchased, the presentation designed and impmenented. We purchased the equipment (computer, 42 inch touch screen display, mounting bracket, security cables) and have finished the first presentation featuring work from this project. We decided to wait to install the unit until after the 2013 tour season to not disrupt the Visitor's Center operations, therefore the installation will take place before the mine reopens for visitors for the 2014 season (installation to occur in Jan or Feb 2014). Shown in the image to the right is the display monitor and computer (the small black square in the bottom right hand corner). Best practices have been

developed in collaboration with park manager Jim Essig through several discussions that occurred nearly every sample collection trip. The park will ensure that sample sites will continue to be preserved and remain accessible for research.

Below is the opening slide in the interactive touchscreen presentation developed in this objective.

V. TOTAL ENRTF PROJECT BUDGET:

Personnel: $ 400,856
- $0 – Jeffrey Gralnick (PI) 5% effort – Result 1.1, 2.2, 2.3, 3
- $0 – E. Calvin Alexander (Co-PI) – Result 1.3, 3
- $0 – Christine Salomon (Co-PI) – Result 2.1, 3
- $35,280 - Brandy Toner (Co-PI) 8% effort – Result 1.1, 1.2, 3
- $13,740 - Scott Alexander (Scientist) 8% effort – Result 1.3
- $68,250 - Post-doc (Center for Drug Design) 50% effort – Result 2.1
- $102,090 - Grad RA (Soil, Water, Climate) 50% effort – Result 1.2
- $111,606 - Grad RA (Microbiology) 50% effort - Result 1.1
- $69,890 - Grad RA (Microbial Engineering) 50% effort - Result 2.2, 2.3

Equipment/Tools/Supplies: $ 71,344

- $26,344 – Supplies for Microbiology PhD Student Molecular biology reagents required for this project (PCR reagents, DNA extraction kits, plasmid purification kits ($250 ea, ~12/year), enzymes, chemicals, microbiology consumables (agar, media), general lab supplies (tubes, tips, gloves etc.), cultivation supplies, sterile sampling supplies.
- $15,000 – Supplies for Soil, Water, Climate PhD Student supplies and consumables required for this project (chemicals / reagents, sample storage, sample preparation, general lab supplies - tips, tubes, pipettes, etc).
- $15,000 – Supplies for Post-doc (Center for Drug Design) chemicals and glassware for culturing microbes, DNA isolation and sequencing for strain identification, solvents for compound isolation, HPLC and MS time for compound identification and structural characterization, laboratory consumables.
- $15,000 – Supplies for Microbial Engineering MS Student Bioremediation experiments (heavy metal quantitation, pure and mixed culture screening for bioreduction, characterization of strains, laboratory consumables) and bioenergy experiments (electrode maintenance, new reactor design for Fe oxidizers, media preparation) and laboratory consumables for MS student.

Travel: $ 16,500
- $6,000 - In-state travel to and from the Twin Cities to Soudan Underground Mine State Park. $220 lodging per trip (2 hotel rooms) and $180 for food and gas for a total of $400 per trip ($2000 total per year).
- $10,500 - Toner and the SWC graduate student will attend beamtime (Argonne National Lab) to conduct mineralogical analyses three times yearly. Per year, we plan for two long trips (5 days of instrument time and 7 days of total travel) plus one shorter trip for micro-probe instrument time (2 days of instrument time and 3 days of total travel). This research requires travel by car from St. Paul to Argonne, IL (near Chicago, IL; car rental Enterprise $405 × 3; gasoline $127 × 3), and lodging at the Argonne Guest House (six nights per long trip, 3 nights per short trip $63.60/night/person). Total: $3500 per budget year. **Note: Minnesota does not have a facility like Advanced Photon Source at Argonne, which is unique in the United States. Work at the APS at Argonne is user-based (as it is a DOE National Lab), therefore travel to this facility is essential for the proposed work.**

Additional Budget Items: $ 56,300
- $1,500 - Printing (publication fees to publish scientific research)
- $6,000 - Soudan Mine Usage: Sampling trips (5 per year) will be scheduled in advance with the Park Manager during scheduled operation hours and during regularly scheduled shift schedules. Hoist trip charges are $30.74 per one way trip. A typical sampling trip will require 4 hoist operations ($122.96). An 8 hour sampling trip will require 8 hours of personnel time (Mine Hoist & Maintenance Lead at $33.36 per hour) for a total of $266.88. A single 8 hour sampling trip is estimated to cost $389.84 (approx $400 per trip or $2000 per year).
- $2,500 – Microscopy - Scanning electron microscopy ($37/hour) Light microscopy ($48/hour) - User fees at CBS Biological Imaging Facility, St. Paul - estimate 9-10 hours SEM and 9-10 hours Light per year.
- $6,300 – Sequencing - for phylogentic analysis of microbial communities - bacteria, fungi and archaea (AGAC Sequencing facility on UM campus $3.50 / reaction, estimate 600 reactions / year over 3 years)
- $30,000 – Chemical, isotopic and gas analysis - ICP/OES Measurements 135 @ $20 ($2700 total), Field Measurements 135 @ $12 ($2100 total), IC Measurements 135 @ $20 ($2700 total), ICP/MS Measurements 135 @ $20 ($2700 total), D and 18O isotope Measurements 105

@ $20 ($2100 total), C, S and Sr isotope Measurements 72 @ $100 ($7200 total), Radiometric Dating Measurements 75 @ $100 ($7500 total). Data logger ($1500) and Sensors ($1500) will also be purchased.
- $10,000 - Outreach development (display, content development, education, implementation, content updates)

TOTAL ENRTF PROJECT BUDGET: $*545,000*

Explanation of Capital Expenditures Greater Than $3,500: *None*

VI. PROJECT STRATEGY:

A. Project Partners:

James Essig (DNR, Park Manager – Soudan Underground Mine State Park) will help coordinate research trips to the mine, outreach activities on site, training modules for park staff and future commercialization possibilities.
Dr. Daniel Bond (University of Minnesota) is an Assistant Professor of Microbiology and member of the BioTechnology Institute. Dr. Bond is an expert in microbial fuel cell technology and metal reduction and will co-advise the student working on the bioenergy portion of the project in years 2 and 3.

B. Project Impact and Long-term Strategy:

This project seeks to explore a novel resource of Minnesota: the extreme microbes found at the bottom of the Soudan Iron Mine. Deep terrestrial brine environments have not been studied extensively anywhere and this particular brine environment might be unique on our planet, because it is located within an ancient iron deposit. The Soudan Underground State Park is uniquely equipped to facilitate studies of this environment, where experiments and samples can be processed in the physics laboratory located just minutes away from the sample sites. We will explore potential applications of microorganisms found in the mine for applications in bioenergy, bioremediation and production of novel antimicrobial and anticancer compounds. Because environments such as this have never been sampled for such applications, we believe the chances of success are high, positioning the University of Minnesota and the DNR for potential revenue-generating discoveries.

The basic science portion of this project will be used to generate a fundamental understanding of the microbiology, geochemistry and mineralogy of these brine ecosystems. This information will be critical to determine how best to protect and preserve these unique ecosystems, while still accessing them scientifically and displaying them to the public tours. It is our long-term goal to develop this site for additional research and outreach with funding from the National Science Foundation and NASA.

C. Other Funds Proposed to be Spent during the Project Period:

University of Minnesota is contributing the salaries of Profs. Gralnick, Alexander and Salomon towards this project (estimated $45,000). In kind contribution of Soudan Underground State Park staff time towards protection planning, display development and potential commercial application development (estimated $1000).

D. Spending History:

Preliminary studies have been conducted five different times during the past three years on site through the donation of staff time and hoist operation by the Soudan Underground State Park (estimated ~ $2,000). The University of Minnesota has contributed to preliminary studies by funding undergraduate summer students working in the labs of Profs. Gralnick and Alexander (estimated ~ $10,000). Profs. Gralnick, Salomon and Toner have used startup funds provided by the University of Minnesota to fund initial microbiological, mineralogical and novel compound studies (estimated ~ $25,000)

VII. DISSEMINATION:

- Publications to primary scientific journals will be submitted covering all aspects of this proposal. Strains of interest will be made available through the American Type Culture Collection (ATCC, with appropriate usage restrictions agreed to by the University of Minnesota, LCCMR and the DNR).

- Intellectual Property / Patent Strategies will be coordinated by the University of Minnesota Office of Technology Commercialization, LCCMR and the DNR.

- Results will also be communicated to the general public through the interactive display to be developed as a part of Result 3 in this proposal, serving the public of the State of Minnesota and visitors to the Soudan Underground Mine State Park.

Activities:

1) December 2010: Gralnick published a technical report in the Minnesota Ground Water Association newsletter entitled 'The Soudan Underground Mine: A window into the deep subsurface microbial biosphere.'

2) January 2011: Soudan Underground Mine State Park Director Jim Essig presented aspects of our project to a local Lion's Club in Northern Minnesota.

3) February 2011: Gralnick presented a seminar to the University of Minnesota's Undergraduate Microbiology Club entitled 'Microbes all the way down: Exploring the deep terrestrial biosphere at the Soudan Iron Mine.'

4) February 2011: The project was featured on Channel 2 WCCO (Twin Cities) for a ~ 5 minute segment showing footage from the mine and interviews with the team members. Gralnick has a digital copy of the feature, available on request.

5) June 2011: C. Salomon interviewed by MoBio, a company that specializes in DNA extraction kits related to her work in the Soudan Iron Mine and the challenges it presents for DNA isolation: http://www.mobio.com/blog/2011/02/06/microbial-warfare-in-the-underworld-searching-for-new-antibiotics/

6) August 2011: J. Gralnick interviewed on location at the Soudan Iron Mine by a reporter from Northland's NewsCenter (http://www.northlandsnewscenter.com/news/iron-range/Soudan-Mine-Researchers-Seek-Window-to-Past-127533473.html).

7) August 2011: Toner, B. M., Briscoe, L. J., Michel, F. M., Alexander, S., Alexander, C., and Gralnick, J. A., 2011. Iron Microbial Mat Formation from Deep Continental Brines. Talk at the *Goldschmidt International Geochemistry Conference*. Prague, Czech Republic.

8) October 2011: Briscoe, L. J., Alexander, E. C., Jr, Alexander, S. C., Berquo, T. S., Gralnick, J. A., Michel, F. M., Moskowitz, B., Salomon, C. E., and Toner, B. M., 2011. Iron mineral formation in microbial mats formed from shield brines along an oxidation-reduction gradient. Talk at the *Geological Society of America Meeting,* Minneapolis, MN.

9) November 2011: Gralnick, J.A. Presented a seminar to the Department of Ecology, Evolution & Behavior at the University of Minnesota entitled 'Ecological and Evolutionary Approaches to Engineering Bacteria that Generate Electricity' which featured preliminary work with isolates from the Soudan Iron Mine.

10) March 2012: Gralnick, J.A. Presented a talk at the TEDxUMN event entitled "Discovery in Unexpected Places" highlighting some of the work done in his lab related to this project. A video of the presentation can be found online: http://www.youtube.com/watch?v=wNXI8lJ8SyU

11) May 2012: Poster presentation by Z. Summers (Gralnick Lab) at the American Society for Microbiology General Meeting entitled "Taking the Geo Out of Geomicrobiology: Electrochemical Cultivation of an Obligate Fe(II) Oxidizer"

12) April 2013: A poster presentation by Ph.D. student L. Briscoe (Toner Lab): Briscoe, L.J., Alexander, S.C., Alexander, E.C., Jr., Berquo, T.D., Fakra, S.F., Michel, F.M., Moskowitz, B.M., and Toner, B.M. Geochemical and biological gradients in the Soudan Underground Mine. Minnesota Ground Water Association (MGWA), St. Paul, MN

13) May 2013: Poster presentation by B. Bonis (Gralnick Lab) at the American Society for Microbiology General Meeting entitled "Characterization of Microaerophilic Fe(II)-Oxidation Utilizing *Marinobacter* from the Soudan Iron Mine"

14) June 2013: Oral presentation at the Census for Deep Life workshop presented by Dr. Cody Sheik, a postdoc in the Dick Lab at U. Michigan, collaborators on our sequencing project: Sheik, C.S., L. Briscoe, J. Gralnick, B.M. Toner, and G.J. Dick. Deep brine waters host low diversity microbial communities at Soudan Iron Mine. Deep Carbon Observatory, Deep Life Workshop, Portland, OR

VIII. REPORTING REQUIREMENTS: Periodic work program progress reports will be submitted not later than _Jan / July 2011, Jan / July 2012 and Jan 2013. A final work program report and associated products will be submitted between June 30 and August 1, 2011 as requested by the LCCMR.

IX. RESEARCH PROJECTS:

216-G Map Attachment, Program Manager: J. Gralnick

Attachment A: Budget Detail for 2010 Projects - Budget per year by Result Cumulative Budget July 1, 2010-June 30, 2013

Project Title: Science and Innovation from the Soudan Underground Mine State Park #216-G

Project Manager Name: Jeffrey Gralnick

Trust Fund Appropriation: $545,000

2010 Trust Fund Budget	**Result 1 Budget:**	**6/30/2013**	**Balance** *(06/30/2013)*	**Result 2 Budget:**	*Amount Spent (06/30/2013)*	**Balance** *(06/30/2013)*	**Result 3 Budget:**	**Amount Spent** *(06/30/2013)*	**Balance** *(06/30/2013)*	**TOTAL BUDGET**	**TOTAL REMAINING**
	Basic Science - Microbiology, Mineralogy and Geochemistry			**Innovative Applications - Drug Discovery, Bioenergy and Bioremediation**			**Public Outreach and Education**				
BUDGET ITEM											
PERSONNEL: Wages											
Jeffrey Gralnick PI 5% effort no salary requested											
Brandy Toner Co-PI 8% effort	26,667	26,667	0							26,667	0
Scott Alexander Scientist 8% effort, Geog.	9,807	9,807	0							9,807	0
Lindsey Briscoe SWC	63,331	63,331	0							63,331	0
Z. Summers. / B. Bonis	91,802	91,802	0							91,802	0
Post-doc Assoc (Center for Drug Design) 50% effort, Yudi Rusman				57,000	52,139	4,861				57,000	4,861
Gralnick Post-Doc (Summers)				38,500	38,500	0				38,500	0
PERSONNEL: Fringe Benefits											
Brandy Toner Co-PI 32.3% fringe ($3546 retirement & $2832 health insurance)	4,075	2,170	1,905							4,075	1,905
Scott Alexander Scientist 32.7% fringe ($516 retirement & $1689 health insurance)Geog	3,933	3,933	0							3,933	0
Lindsey Briscoe	38,757	38,757	0							38,757	0
Z. Summers/B.Bonis	42,264	42,264	0							42,264	0
TBN Post-doc Assoc (Center for Drug Design) 19.75% fringe ($0 retirement & $6054 health insurance)				11,250	11,250	0				11,250	0
Post-doc fringe - Summers				8,932	8,932	0				8,932	0
Supplies											
Molecular biology reagents required for this project (PCR reagents, DNA extraction kits, plasmid purification kits ($250 ea., ~12/year), enzymes, chemicals, microbiology consumables (agar, media), general lab supplies (tubes, tips, gloves etc.), cultivation supplies, sterile sampling supplies - Micro PhD Student - Years 1-3	30,882	30,882	0							30,882	0
Laboratory supplies and consumables required for this project (chemicals / reagents, sample storage, sample preparation, general lab supplies - tips, tubes, pipets, etc) - SWC PhD Student - Years 1-3	15,000	15,000	0							15,000	0
Lab supplies for Postdoc (Center for Drug Design) - specificly required for this project: chemicals and glassware for culturing microbes, DNA isolation and sequencing for strain identification, solvents for compound isolation, HPLC and MS time for compound identification and structural characterization, laboratory consumables for Postdoc (50% time) - Years 1-3				15,000	15,000	0				15,000	0

Bioremediation experiments - Heavy metal quantitation, pure and mixed culture screening for bioreduction, characterization of strains, laboratory consumables. **Bioenergy experiments** - electrode maintenance new reactor design for Fe oxidizers, media preparation, laboratory consumables for MS student - Years 2-3				15,000	15,000	0				15,000	0
Other Direct Costs											
Microscopy - Scanning electron microscopy ($37/hour) Light microscopy ($48/hour) - User fees at CBS Biological Imaging Facility, St. Paul - estimate 9-10 hours SEM and 9-10 hours Light per year.	2,500	2,500	0							2,500	0
Sequencing for phylogentic analysis of microbial communities - bacteria, fungi and archaea (AGAC Sequencing facility on UM campus $3.50 / reaction, estimate 600 reactions / year over 3 years)	6,300	6,300	0							6,300	0
Chemical, isotopic and gas analysis - ICP/OES Measurements 135 @ $20 ($2700 total), Field Measurements 135 @ $12 ($2100 total), IC Measurements 135 @ $20 ($2700 total), ICP/MS Measurements 135 @ $20 ($2700 total), D and 18O isotope Measurements 105 @ $20 ($2100 total), C, S and Sr isotope Measurements 72 @ $100 ($7200 total), Radiometric Dating Measurements 75 @ $100 ($7500 total). Data logger ($1500) and Sensors ($1500) will also be purchased.	30,000	30,000	0							30,000	0
Soudan Mine Usage: Sampling trips (5 per year) will be scheduled in advance with the Park Manager during scheduled operation hours and during regularly scheduled shift schedules. Hoist trip charges are $30.74 per one way trip. A typical sampling trip will require 4 hoist operations ($122.96). An 8 hour sampling trip will require 8 hours of personnel time (Mine Hoist & Maintenance Lead at $33.36 per hour) for a total of $266.88. A single 8 hour sampling trip is estimated to cost $389.84 (approx $400 per trip or	6,000	6,000	0							6,000	0
Printing											
Publication fees (~ 3 total, $500/publication - page charges required to make scientific discoveries available to other scientists and the public)	1,500	1,500	0							1,500	0
Travel expenses in Minnesota											
Travel: In-state travel to and from the Twin Cities to Soudan Underground Mine State Park. $220 lodging per trip (2 hotel rooms) and $180 for food and gas for a total of $400 per trip ($2000 total per year).	6,000	6,000	0							6,000	0
Travel outside Minnesota											
Travel: Toner and the SWC graduate student will attend beamtime (Argonne National Lab) to conduct mineralogical analyses three times yearly. Per year, we plan for two long trips (5 days of instrument time and 7 days of total travel) plus one shorter trip for micro-probe instrument time (2 days of instrument time and 3 days of total travel). This research requires travel by car from St. Paul to Argonne, IL (near Chicago, IL; car rental Enterprise $405 × 3; gasoline $127 × 3), and lodging at the Argonne Guest House (six nights per long trip, 3 nights per short trip $63.60/night/person). Total: $3500 per budget year. **Note: Minnesota does not have a facility like Advanced Photon Source at Argonne, which is unique in the United States. Work at the APS at Argonne is user-based (as it is	10,500	9,142	1,358							10,500	1,358
Other											
Outreach development (display, content development, education, implementation, content updates)							10,000	10,000	0	10,000	0
COLUMN TOTAL	**$389,318**	**$386,055**	**$3,263**	**145,682**	**$140,821**	**$4,861**	**$10,000**	**$10,000**	**$0**	**$545,000**	**$8,124**