2013 Project Abstract
For the Period Ending June 30, 2016

PROJECT TITLE: Harnessing Soudan Mine Microbes: Bioremediation, Bioenergy and Biocontrol.
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FUNDING SOURCE: Environment and Natural Resources Trust Fund)
LEGAL CITATION: [M.L. 2013, Chp. 52, Sec. 2, Subd. 03f]

APPROPRIATION AMOUNT: $838,000

Overall Project Outcomes and Results
The Soudan Iron Mine in Minnesota provides direct access to microbes with special adaptations that can be harnessed for biotechnology. We conducted research to harness these microbes to approach some of the most critical environmental challenges in Minnesota:

Metal Bioremediation: Our goal was to explore the native fungi living in the mine. These fungi live in extremely harsh and variable chemical conditions, including high metal concentrations in water. Because the Soudan Iron Mine fungi have adapted to the conditions in the mine, they might possess properties that we can use for cleaning up metal-contaminated water. When we use plants or microorganisms (like fungi) to remove metals from water, it is termed bioremediation. We investigated mine fungi that thrive in heavily contaminated waters with metals such as copper, cobalt, zinc, nickel, and mercury. We isolated 1014 different strains of fungi representing 140 different taxa, including novel species. We screened 60 fungal isolates and discovered that several species accumulate metals within their living biomass. These findings confirmed that: (1) many Soudan Iron Mine fungal isolates have promising metal removal characteristics in solid and liquid growth conditions; (2) the amount of metal removed from water was similar between natural and lab specimens; and (3) metal binding can be reversed in some cases. These results can be used to develop a suite of bioremediation strategies using fungi as passive sorbent materials or in living self-regenerating bioreactor.

Electrosynthesis Project: We characterized and developed methods for understanding two bacterial isolates from the Soudan Mine: *Marinobacter subterrani* and *Desulfuromonas soudanensis*. *D. soudanensis* is capable of producing electricity and dissolving rust in high salt concentrations, making it a very unusual organism. We sequenced and characterized its genome to better understand how electricity production works at high salt concentrations, a process that could be important for future applications in microbial bioremediation and desalination. We are currently in the process of developing a genetic system in *D. soudanensis* to further our understanding of how it generates electricity in high salt conditions. *M. subterrani* is a model for the study of metal precipitation, a process that, if better understood, could allow us to feed electricity directly into bacteria. These bacteria could then be engineered to produce desired products using electricity. Given the complexities of this biological process, we are still an early stage of understanding the fundamental pathway that enables metal precipitation. Our students working on these two projects have presented their work at national and international meetings and have produced 2 peer reviewed scientific manuscripts on their work as well.

White Nose Bat Syndrome Biological Control: White Nose Syndrome is a devastating bat disease causing catastrophic economic and biodiversity losses throughout the US. Our primary goal was to identify microbes that inhibit the fungal pathogen, *Pseudogymnoascus destructans* that could eventually be developed as a treatment in caves and mines. As part of this biocontrol strategy, we collected and screened new microbes from the

Soudan Mine. In total, 32/121 fungal strains and 60/262 bacterial isolates inhibited growth of *P. destructans*. Analysis of active strains provided us with a picture of which types of inhibitory microbes may be found in various mine locations, which may help future screening and discovery efforts. With this library of nearly 100 antifungal strains, we are poised to move forward into phase II, which will involve testing the ability of each active strain to inhibit *P. destructans* on specific substrates both in the lab and in the environment. An additional outcome is that a subset of at least 50 strains had activity against human pathogens and these will be further explored in a separate project.

Project Results Use and Dissemination

Information, discoveries, approaches and questions from our project have been used and disseminated in a number of different ways: Presentations about individual projects have been given to school groups, college students, local community groups and at professional scientific conferences (see examples, below). Several components of this project were completed and shared as peer-reviewed scientific manuscripts. Some of the fundamental scientific discoveries have been used to further develop and expand new ideas that were not a part of the original research plan. These new ideas and hypotheses have been incorporated into new grant proposals, resulting in successful new funding at both the state and federal levels (including several new LCCMR proposals that build directly on initial research accomplished in this period as Phase II projects). Some additional uses include the screening of these new, diverse microbial libraries against other targets, including human infectious disease pathogens. For example, several of the bacterial strains that showed no activity against the fungal bat pathogen did exhibit inhibition of human yeast pathogens. These strains will be further studied to purify and identify the active components for potential development as human therapeutics.

- "Will Soudan Mine research save our bats?" The Timberjay, 07/16/2014
- Bonis, B.M, Gralnick, J.A. 2015. Marinobacter subterrani, a genetically tractable neutrophilic Fe(II)-oxidizing strain isolated from the Soudan Iron Mine. Front. Microbiol. 6:719.
- Big Picture Science Radio Interview: http://radio.seti.org/blog/2014/12/big-picture-science-shocking-ideas-jeff-gralnick-microbe-power-plants/ (Gralnick)
- Rusman, Y, Held, B, Blanchette, RA, Wittlin, S, Salomon, CE. Soudanones A-H: Antifungal isochromanones from a *Cadophora* sp. fungus isolated from the Soudan Iron Mine. *Journal of Natural Products*. 2015, *78* (6), 1456–1460.
- Badalementi, J.P., Z.M. Summers, C.H. Chan, J.A. Gralnick and D.R. Bond. 2016. Isolation and genomic characterization of '*Desulfuromonas soudanensis* WTL', a metal- and electrode-respiring bacterium from anoxic deep subsurface brine. Front Microbiol. 7:913. doi:10.3389/fmicb.2016.00913
- Badalamenti, JP, Erickson, JD, Salomon, CE. Complete genome of *Streptomyces albus* SM254, a potent antagonist of the White Nose Bat Syndrome pathogen *Pseudogymnoascus destructans*. *Genome Announcements*, 20164(2):e00290-16. doi:10.1128/ genomeA.00290-16.

Date of Status Update Report: 10/14/2016

Final Report

Date of Work Plan Approval: July 2013

Project Completion Date: July 30, 2016

PROJECT TITLE: Harnessing Soudan Mine Microbes: Bioremediation, Bioenergy and Biocontrol.

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

Total ENRTF Project Budget:	**ENRTF Appropriation:**	**$838,000**
	Amount Spent:	**$838,000**
	Balance:	**0**

Legal Citation: M.L. 2013, Chp. 52, Sec. 2, Subd. 03f

Appropriation Language: $838,000 the first year is from the trust fund to the Board of Regents of the University of Minnesota to continue the characterization of unique microbes discovered in the Soudan Underground Mine State Park that have potential applications for metal remediation in water resources, microbial electrofuels, and biocontrol of whitenose bat syndrome. This appropriation is available until June 30, 2016, by which time the project must be completed and final products delivered.

I. PROJECT TITLE: Harnessing Soudan Mine Microbes: Bioremediation, Bioenergy and Biocontrol.

II. PROJECT STATEMENT: The Soudan Iron Mine in northern Minnesota is the state's oldest and deepest iron mine. The mine was active from 1882 until 1962 when it was closed and developed into Soudan Mine Underground State Park. Although iron is no longer being extracted, the mine continues to provide valuable resources to the state of Minnesota, including access to fascinating microbial communities that may not exist anywhere else on the planet. The water seeping into the mine from exploratory holes drilled by the miners is highly unusual: It is extremely salty (2-3 times saltier than seawater), high in dissolved iron and other metals, and completely anoxic (without dissolved oxygen gas) until it mixes with the air in the tunnels. The Soudan Iron Mine provides a window into this unique subterranean world and direct access to microbes with special adaptations that can be harnessed for biotechnology.

This proposal builds on the success and findings of our current research program (funded by ENRTF 2010-2013) focused on characterizing microbes that live in this extreme environment and their unique metabolic capabilities. We propose to apply knowledge gained from our initial research project and harness these microbes to approach some of the most critical environmental challenges in Minnesota:

1. **Removing metals from mine waters with microbes.** The park currently spends upwards of $200k/year to remove metals from mine effluent. Bacteria and fungi are found thriving in areas of the mine that are heavily contaminated with copper, cobalt and other metals. These microbes are adapted to Soudan conditions, and could be developed for removal of metals from mine waters to meet water quality requirements. We propose to identify efficient metal binding bacteria and fungi with the goal of incorporating them into a bio-filter to treat the contaminated mine water on-site (bioremediation). This technology could be utilized by Soudan Park as well as other mines and contaminated environments.

2. **Microbes and electrofuels**. Bacteria have the ability to eat and breathe iron. The iron-breathing bacteria can be used to generate electricity, an aspect that both Gralnick and Bond have studied for over 10 years. Using electrodes, we can grow both kinds of bacteria, depending on how we poise the electrode (negative for iron breathers and positive for iron eaters). The Soudan Iron Mine is a unique environment that has novel populations of both kinds of bacteria. Iron breathing bacteria can be harnessed to generate electricity, while iron-eating bacteria will help create biofuels in a process called 'electrosynthesis.' We are enriching and culturing novel bacteria from the mine with our current ENRTF. In this new proposal we will test the best bacteria for our desired applications in electricity and electrofuels.

3. **Inhibition of the White Nose Bat Syndrome fungus (Latin name: *Geomyces destructans*).** Since 2006, the fungal disease White Nose Syndrome (WNS), has decimated bat populations in the Northeastern US, incurring devastating economic and biodiversity losses. Although WNS has not yet reached Minnesota, the Soudan Mine serves as the largest hibernaculum in the upper Midwest and is threatened by the rapid westward spread of this deadly fungal disease (confirmed in Iowa and Ontario, Canada). We have tested microbial strains from the Soudan Mine against a panel of 10 different pathogenic bacteria and fungi, and found that ~40% of isolates inhibit the growth of fungi. We propose to identify strains (existing strain library and new isolates) that could potentially inhibit the WNS fungus. Our approach involves cultivating microbes from bats and bat roosts and testing them for inhibitory activity against at least three species of *Geomyces*. Because these anti-fungal isolates are already adapted to living in the extreme environment of the mine, they may be good candidates for potentially controlling or preventing WNS ("Biocontrol").

Some of the research being done in this project has potential intellectual property value and therefore Intellectual Property / Patent Strategies will be coordinated by the University of Minnesota Office of Technology Commercialization

Please note that, as part of the patent protection process, an Intellectual Property Disclosure will be filed with the Office of Technology Commercialization at the University of Minnesota on the technology proposed to be developed through this project. As a result, some information pertaining to this project is confidential at this time. This work plan omits confidential information and provides lesser detail than it otherwise might.

III. PROJECT STATUS UPDATES:

Project Status as of *(January 2014)*: Our initial studies have been focused on culturing and characterizing many different microbes from the Soudan Mine for each of our project areas. For the metal binding studies, the Blanchette and Toner groups are collaborating to optimize methods to measure fungal biomass and develop small scale bioreactors for the first set of metal uptake experiments. The Blanchette group has also successfully isolated several hundred fungal samples from all levels of the mine, including areas highly contaminated with metals. The Gralnick lab is generating a large transposon library in a Soudan Mine *Marinobacter* strain to help elucidate core metabolic pathways and identify genes involved in iron oxidation. The Bond lab is searching for bacteria capable of using metals as an energy source by culturing bacteria on electrodes in the absence of oxygen. The genomes of two species of *Geoalkalibacter* were also sequenced and annotated to identify conserved genes and metabolic strategies. The Salomon lab has been isolating and purifying bacteria from water, rock and bat swab samples to identify potential strains that inhibit the growth of the white nose bat pathogen *Pseudogymnoascus destructans* (formerly known as *Geomyces destructans*). Bioassays for testing inhibitory activities of both bacterial and fungal isolates are being tested, developed and optimized. Previously identified anti-fungal compounds isolated from Soudan microbes will also be tested against several pathogenic fungi related to *Pseudogymnoascus* and *Geomyces* species.

Project Status as of *(July 2014)***:** We have continued to isolate and characterize bacteria and fungi associated with each activity of the project. More than 50 bacterial and 170 fungal strains have been isolated in pure culture. The metal uptake experiments are continuing to be optimized, and the fungal collection will be subjected to screening to identify the best candidates for the metal binding system. For the electrosynthesis components of the project, we successfully isolated a novel bacterium from Soudan following enrichment on electrodes, and leveraged state-of-the-art long read DNA sequencing technology to assemble its complete, circular genome with remarkably high accuracy. This isolate is the first from Soudan with a complete, finished-quality genome sequence. These results validated our ongoing approach towards capturing novel microbial isolates using electrodes as bait, and provided direction in designing future experiments for understanding the genomic composition of mixed microbial communities enriched from Soudan. For the White Nose Bat Syndrome component of the project, we have assembled a diverse library of potential bacterial and fungal antagonists and are finalizing the assay system. We are also characterizing two species of *Pseudogymnoascus* fungi isolated from the Soudan Mine as potential proxy species for more efficient screening compared to the slow growing Pd pathogen.

Project Status as of *(Jan 2015)***:** We are actively characterizing the bacterial and fungal isolates identified for each of the three major areas of our project. The Toner and Blanchette labs have been collaborating on both fungal sorption experiments and plate growth experiments to measure the capacity and specificity of metal absorption by various fungal species. Additional fungi are also being characterized for metal tolerance related to growth rate. For the electrosynthesis project, we have characterized the physiology and metabolism of a *Marinobacter* strain through a variety of laboratory methods. This work has told us what the organism likes to eat, what it can breathe, what temperature it prefers, how much salt it likes in its environment and what pH it prefers. We have also recently completed a genome sequence for the organism and have identified several candidate genes that could encode proteins involved in iron oxidation and potentially extracellular electron transfer. We also continued experiments aimed at understanding the basic physiology of '*Ca*. D.

soudanensis', a novel halophilic isolate from Level 27 at Soudan. We discovered that this isolate can reduce a form of iron(III) oxide, called schwertmannite, approximately 2-3 times faster than more commonly tested iron(III) oxide forms, despite the fact that both iron forms provide a similar amount of energy to the cells. Growth experiments were completed to characterize the optimum salt tolerance and temperature and the data are providing clues about the natural ecological habitat of this species in the subterranean brines. For the White Nose Bat Syndrome project, we are developing a new bioassay to help us (and other researchers) screen for inhibitors of the fungal pathogen, *Pseudogymnoascus destructans*. Once the assay has been optimized, we will test known inhibitors of the fungus and then begin to screen our extensive library of Soudan microbes. We are also sequencing DNA fragments from the bacterial and fungal library to help us characterize the taxonomic diversity of cultivable microbes that we have isolated thus far.

Project Status as of *(July 2015)***:** For the metal bioremediation project, progress was made in several areas. Fungal collections have continued and more than 1000 strains have been isolated representing at least 140 different taxa. Two species of fungi were identified with excellent tolerance to extremely high levels of metals, and four of the previously identified best performers were tested in situ in the Soudan Mine for metal binding capacity. In the electrosynthesis project, microbes enriched on poised electrodes were sequenced using PacBio long read sequencing. This data allowed the assembly of the complete, circular genome of the proposed new strain '*Ca.* Desulfuromonas biwabikus DDH964'. To the best of our knowledge, this genome represents the first ever complete genome recovered from a mixed community metagenome. For the WNS project, we have characterized and compared several strains of non-pathogenic *Pseudogymnoascus* fungi collected from the mine. Screening of the Soudan bacterial and fungal collections are ongoing, and several promising strains that completely inhibit *P. destructans* growth have been identified.

Project Status as of *(Jan 2016)***:**

Metal Bioremediation: Screening for metal tolerant fungal isolates was continued, and nearly 25% of strains were able to grow when inoculated onto media containing high levels of metals (1000 times higher than Soudan water). Additional experiments testing metal sorption capacity and in situ fungal growth experiments indicate that the fungi selected have potential for development into bioreactors in the Soudan mine. For many metals, the concentrations sorbed to the fungal surface are orders of magnitude greater than the concentrations in mine waters. Our findings suggest that the reactivity of the fungal biomass in situ is suitable for bioreactors.

Electrosynthesis Project: We further explored how metal-transforming bacteria from Soudan function in the context of the larger microbial community. To this end, we mined metagenomic sequences from three boreholes on Level 27 and discovered that metal-reducing bacteria are extremely rare, underscoring our electrode enrichment approach. We also continued experiments focused on elucidating the biochemical mechanism of iron oxidation in another Soudan isolate, *Marinobacter subterrani.*

White Nose Bat Syndrome Biological control: We are continuing to characterize the bacterial and fungal taxonomic diversity of the culturable isolates from the Soudan Mine using both DNA sequencing and phenotypic observation techniques. The screening experiments against P. destructans so far indicate that approximately 60 strains of bacteria and fungi inhibit growth. We are beginning the next stage of testing to determine which of these inhibitors will be able to grow and survive on roost substrates and/or bat wing materials.

Project Status as of *(July 2016)***:**

Metal Bioremediation: New experiments were conducted to compare the metal tolerance of and sorption capacities of fungal strains on solid versus liquid substrates. The results are important because they will help determine which isolates will be best adapted to different growth and remediation conditions. For example, some of our isolates perform metal removal best when growing on a solid substrate. In contrast, some of our fungal isolates are strong metal sorbents, but do not grow well in the presence of the metal. For these isolates, a remediation strategy would involve growing large amount of biomass (metal free) and preparing that biomass as a reagent sorbent (e.g. in large batch reactors).

Electrosynthesis Project: We have isolated, characterized, developed a robust genetic system and a next-generation mutagenesis methodology in *Marinobacter subterrani*, isolated from the Soudan Iron Mine. *M. subterrani* is a model bacterium to study neutrophilic iron oxidation – a process that has several biotechnological applications in addition to being a biological process lacking a robust understanding. The organism has potential applications in bioremediation (through the production of highly reactive biogenic iron oxide minerals) and in electrosynthesis (though its ability to oxidize iron extracellularly). Future work with the organism will help us better understand how it lives in high salt, low nutrient conditions in the Soudan Iron Mine and through comparative genomics and genetics, we hope to better understand genes important for life in the deep subsurface.

White Nose Bat Syndrome Biological Control:
We have completed the phenotypic screening of the full collection of bacteria and fungi collected from the Soudan Iron Mine to date. It total, 32/121 fungal strains and 60/262 bacterial isolates inhibited the growth of *P. destructans* using plug assays or overlay assays, respectively. DNA sequence analysis of the active strains has provided us with a clearer picture of which types of inhibitory microbes are found on various substrate types, which may help future screening and discovery efforts. We have begun scaling up larger cultures of each of the active strains and making extracts to test and purify. The goals of this portion of the work are to identify the anti-fungal compounds produced and consider their mechanisms of action. Analysis of initial scaled up cultures indicate that some strains produce extractable anti-fungal metabolites and we will begin the process of purification and identification. We are now poised to move forward into phase II of this project which will involve testing the ability of each active strain to inhibit *P. destructans* on specific substrates both in the lab *in situ* (in cave and mine environments).

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: Removal of metals from mine waters with microbes
Description: The park currently spends approximately $200k/year to remove metals from mine effluent. Fungi are found thriving in areas of the mine that are heavily contaminated with copper (Cu), cobalt (Co) and other metals (e.g. mercury, Hg). These microbes are adapted to Soudan conditions, and could be developed for removal of metals from mine waters to meet water quality requirements. We propose to identify efficient metal binding fungi with the goal of incorporating them into bio-filters or bioreactors to treat the contaminated mine water on-site (bioremediation). This technology could be utilized by Soudan Park as well as other mines and contaminated environments. *This project will be focused on isolating and characterizing fungi from the most contaminated areas of the mine and testing the best candidates for further development into a biofilter.*

Summary Budget Information for Activity 1:

ENRTF Budget: $ 249,172
Amount Spent: $ 249,172
Balance: $ 0

Activity Completion Date:

Outcome	Completion Date	Budget
1. Complete culturing and characterization of fungi from high copper and cobalt areas of the mine	July 2014	$83,057
2. Screen up to 20 cultures for metal binding capacity as function of pH	July 2015	$83,057
3. Describe the metal binding capacity for most promising isolates as function of metal loading, ionic strength, and temperature.	Jan 2016	$83,057

Activity Status as of *(January 2014)*:

(1) Researcher hired: The budget for the Toner group included 3 years of support for a Ph.D. student to develop metal uptake screening methods and conduct laboratory experiments with fungi isolated from the Soudan site. Recruiting of a Ph.D. student for fall 2013 was not successful. To keep the project on the proposed timeline, a Research Scientist, Michael Ottman, was hired. Mr. Ottman is now established in the Toner group and has begun method development for the metal uptake experiments.

(2) Fungal isolates: In collaboration with co-PI Professor Robert Blanchette and Research Scientist Dr. Benjamin Held, several Minnesota fungal strains with high mycelium biomass were selected for method development. These non-hazardous (edible) strains will be used while isolates from the Soudan Mine are grown. The fungal strains are:

- *Pleurotus eryngii* (king trumpet mushroom)
- *Pleurotus ostereatus* (oyster mushroom)
- *Hericium erinaceus* (lion's mane mushroom)
- *Lentinus Edodes* (shiitake mushroom)

Figure 1. Wood associated with high concentrations of copper was sampled and culturing has provided unusual fungi that have the ability to grow in extreme toxic environments.

Fungal isolate sources and growth conditions

Each species of fungus was cultured in spent barley grains and wheat bran, and then added, mixed and propagated into sealed, sterile plastic bags containing elm sawdust. Barely grain was sterilized in an autoclave and inoculated with plugs taken from actively growing fungi on petri plates. After 2 weeks of growth the grains were used as an inoculum for mycobags that were prepared by hydrating hardwood sawdust and sterilized. The fungi in the bags were left to grow for several weeks. Subsamples of the integrated substrate / mycelium were taken in the sterile environment of a laminar flow hood (Thermo Scientific). A sterile metal spatula was used to cut into the mycelial mass to collect subsamples. Subsamples were placed on watch glasses, weighed and dried for 24 hours at 100° C. Dry weights were measured and percent moisture calculated. The samples were then placed into acid washed 50 mL Falcon tubes (BD Biosciences) and stored at room temperature in the dark until needed for metal uptake experiments. All laboratory glassware and utensils were sterilized by ethanol and UV treatment.

(3) Metal uptake experiments: In terms of method development, the first task is to develop processes for handling and measuring fungal (mycelia) biomass. The primary challenge is that the fungal mycelia incorporate themselves into the "feed stock" or the solid growth media (described above). The amount of

biomass present is difficult to measure but is a necessary normalizing factor for comparing the efficiency of different fungal strains for metal uptake. A gradual combustion approach, while measuring carbon dioxide gas, for quantifying biomass mass may work for this purpose. The Blanchette group has provided feed stock materials as controls for the method development.
At this time, the dry weight of mycelia plus feed stock for the test strains is being measured, and physical methods for increasing the porosity of the fungal biomass are being explored. Small bioreactors with suspensions of fungal mycelia (plus residual feed stock) are being set up in the laboratory. If suspensions are not reproducible due to large or variable particle sizes, a flow-through system will be devised.

The first experiments will mimic metal uptake in Soudan mine waters from Level 12 West and Level 13 West where the drainage waters have transitions metals, Cu and Co, with near-neutral pH.

(4) Extensive sampling of wood and soil was carried out at the Soudan mine on levels 7, 8, 9, 10, 11, 12, 15, 17, 18, 21, 23, 25 and 27. A total of 168 samples were obtained. Abundant fungal growth was observed in many areas on the various wood timbers in the mine. Small segments of wood or soil particles were placed into 3 different types of media to obtain cultures of fungi. Malt extract agar, acidified malt extract agar and a selective media for Basidiomycetes were used. Fungi growing from the substrates were sub-cultured onto malt extract plates and grown for several days. Mycelia of the fungi were taken from the plates and used for DNA extraction. Several hundred isolates are currently being grown and will be identified by DNA sequencing. Following identification, isolates will be selected for metal ion sequestering testing. These strains will also be tested for inhibition of fungal growth in "Activity 3".

(5) A group of fungi that can tolerate high concentrations of metal ions obtained from our previous investigations are being grown on a variety of different solid state substrates to determine the best methods for producing large amounts of fungal biomass to be used for metal ion sequestering (Figure 1). In addition, a selected group of fast growing fungi (not from the mine) have been grown in spawn bags for mycelium production and to develop procedures for biofiltering water with high metal ions.

Induced coupled plasma analyses of 24 samples from different wood substrates obtained from different levels within the mine has shown that exceedingly high concentrations of aluminum, cobalt, copper, iron, nickel, lead and zinc are present in the wood . Fungal isolates obtained from these areas are candidates for metal ion biofilter medium.

Activity Status as of *(July 2014)***:**

(5) Fungi, isolated from 13 levels of the mine, are now in pure culture and being studied in detail. One hundred and seventy different isolates representing three phyla have been identified using DNA sequencing. The list of fungi includes many diverse species. Some fungi are similar to those that have been previously found in other mines and in association with bats. Other fungi found appear unique and have been rarely reported in other studies. Several isolates obtained do not match any known species and likely represent new taxa. Numerous wood destroying fungi have been isolated from mine timbers that are located throughout the different levels of the mine. This includes wooden timbers used for rail tracks, mine timbers holding up walls and historic wooden items used during mine operations. Many of the isolates have been obtained from levels with exceedingly high concentrations of metals. One fungus was found to produce an extensive network of fungal strands called rhizomorphs that allowed it to move in the mine from one mine timber to another through areas of toxic metal ions. This fungus has been identified as *Armillaria sinapina*. The Sudan mine appears to have many unique taxa that have become adapted to the conditions of the mine and represent microorganisms that are not usually found in forest and agricultural landscapes. Screening and

selection of these isolates is underway to identify strains that tolerate and sequester high concentrations of meta l ions.

(6) Metal uptake experiments: The primary research effort in period 2 was finalizing the metal uptake method. Michael Ottman has developed a method to homogenize the fungal biomass (including feed stock) into a powdered reagent that can be used for metal uptake experiments. This method has now been tested for a Shiitake and a Soudan "surface rust" fungi, both grown on barley, for a multi-metal experiment copper (Cu), nickel (Ni), zinc (Zn), cadmium (Cd), and cobalt (Co). The initial experiments revealed that the barley feed stock has low affinity for metals, but can be a source of Cu to solution. As an example, in one of the multi-metal experiments, Zn has the strongest affinity for the Soudan fungal biomass, up to 60% of the initial Zn concentration. Percent removal of Co and Ni were around 15% of initial metal. These percentages will be a function of initial metal concentration, pH, salinity, and fungal type and concentrations.

Figure 1. *Oligoporus* sp. growing on a mine timber on Level 21.

Figure 2. Fungi being grown in flasks with liquid media for heavy metal sequestration testing.

We are now ready to tailor the multi-metal experiments to the water conditions in different levels of the mine. The only outstanding issue is identifying a good method for normalizing the fungal reagent to the amount of dry biomass present. We are working with our collaborators to identify a good proxy observation. One technical issue we faced during this period was good separation of the fungal biomass from the reacted water: primarily due to slow filtration and filter clogging. Although we have some yet-to-be resolved questions regarding very small particles moving through our filters and being counted as "dissolved" metal, the method is now repeatable and ready to survey the Soudan fungi isolated by the Blanchette group.

(7) Personnel: Rebecca Sims was transferred to this project for the summer months to help Michael Ottman with laboratory work.

Activity Status as of *(January 2015)***:**

(1) Soudan Mine fungi: In Period 3, fungi from high metal areas of the Soudan Mine were isolated and studied. The Blanchette Group is characterizing the *Oligoporus* species (**Figure 1**) along with other possible new species by sequencing multiple gene regions which allows for a more accurate species designation. The *Oligoporus* fungus is proving to be a dominant decomposer of wood in the mine on many levels and causes a brown rot type of wood decay. Methods for growing these metal tolerant fungi are being tested. Specifically, fungal biomass is being grown in pure culture for screening and preliminary studies of their use as a heavy metal biofilter by the Toner Group. Screening other fungi for metal tolerance is continuing. Currently, the Toner and Blanchette Groups are working together to test growth media amended with high amounts of metals, amounts similar to those found in the mine, as a tool to screen all of the fungi isolated to determine which cultures have high metal tolerance and metal absorption capabilities.

(2) Metal uptake experiments: The primary research effort in Period 3 was measuring multi-metal uptake by fungal biomass grown, by the Blanchette group, in liquid media (**Figure 2**). As with the "powdered reagent"

approach developed in Period 2, a multi-metal sorption experiment was conducted with copper (Cu), nickel (Ni), zinc (Zn), and cobalt (Co). This change in experimental approach (from cultures grown on barley to liquid media) allows us to normalize metal sorption data by the dry biomass weight of the fungi and was essential for robust comparisons of metal uptake by different fungi.

The data are reported as milligrams of metal removed from solution per kilogram of dry fungal biomass (mg / kg) (**Table 1**) or percent of metal removed from water [100 x (initial metal concentration – final metal concentration)/initial metal concentration]. These experiments were conducted for four fungal strains isolated from the Soudan Mine:

(a) *Armallaria sinapina*
(b) *Philophora olivacea*
(c) *Cadophora sp.*
(d) *Exophila sp.*

The aqueous conditions—ionic strength (mmol / L), pH, and metal concentration—were chosen by matching the experiments to Soudan Mine conditions at the sump pumps.

Table 1.

	Final Aqueous Metal Concentration (umol/L)				Final Loading to Biomass (umol / kg)				Final Percentage of Metal Adsorbed (%)			
Fungi	**Cu**	**Ni**	**Zn**	**Co**	**Cu**	**Ni**	**Zn**	**Co**	**Cu**	**Ni**	**Zn**	**Co**
Exophiala sp.	0.87	0.93	1.21	0.38	3.05	0.72	3.48	0.17	69.9	34.0	65.7	23.4
Armilleria sinapina	0.24	0.31	0.44	0.06	4.00	1.63	4.72	0.64	91.9	77.6	87.4	87.0
Armilleria sinapina (rhizomorphs)	0.12	0.49	0.31	0.05	3.26	1.08	3.77	0.53	95.8	65.0	91.1	90.2
Phialophora olivacea	1.06	0.73	1.30	0.34	2.73	0.83	2.70	0.19	63.3	48.2	64.0	31.5
Cadophora sp.	1.39	0.97	1.18	0.32	1.84	0.53	2.85	0.21	52.0	30.9	66.5	34.8
Units: Milliliter (mL); microliter (uL); micromole (umol); millimole (mmol); gram (g); kilogram (kg)												
Experimental conditions: Initial reactor volume (100 mL); pH=6.95; ionic strength 157 mmol/L; fungal biomass 1 g/kg suspension												
Initial metal concentrations (umol/L): 2.90 (copper, Cu), 1.41 (nickel, Ni), 3.52 (zinc, Zn), and 0.50 (cobalt, Co)												

(3) Metal content of rhizomorphs: *Armallaria* rhizomorphs were collected from the Soudan Mine. The metal content of the rhizomorph exterior was measured by leaching the rhizomorphs for 7 minutes in 50 mL of 0.1 M hydrochloric acid (HCl). The rhizomorphs released 0.78 mmol Cu / kg, 9.29 mmol Ni / kg, 14.26 mmol Zn / kg, and 0.19 mmol Co / kg (**Figure 3**). The product of the leach experiment, called "cleaned rhizomorphs", was then subjected to a metal sorption experiment. The metal binding capacity of the cleaned rhizomorphs was very similar to the *Armallaria* and *Philophora* cultures grown in liquid media (**Figure 4**). A full digestion of the rhizomorphs will be conducted next. These experiments will help us understand how fungi are currently taking up metals in the mine.

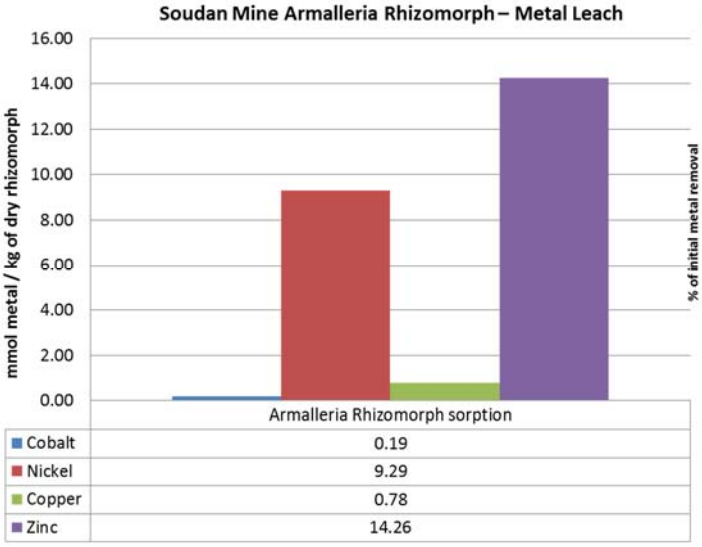


Figure 3. Metal content of *Armalleria* rhizomorphs collected from the Soudan Mine.

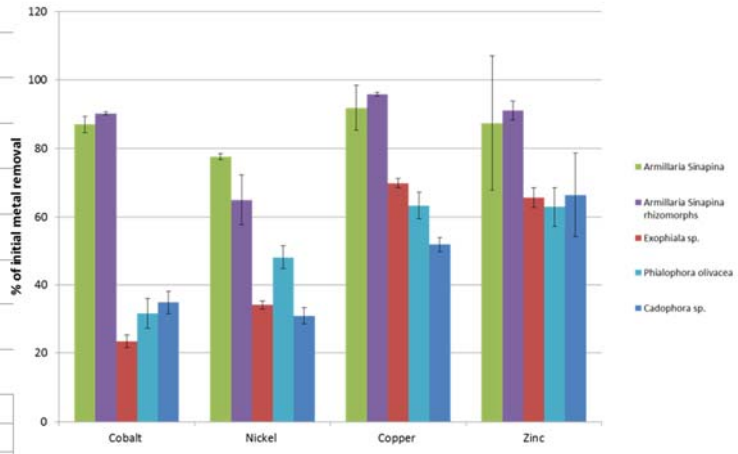


Figure 4. Percentage of original metal in water removed by fungal biomass. Metal uptake by "cleaned" *Armalleria* rhizomorphs (Arma rhizo, purple) is compared to *Armallaria* (Arma, green), *Philophora* (philo, turquois), *Cadophora* (cado, blue), *and Exophillia* (exo, red) cultures grown in liquid media. Error bars represent the standard deviation of replicates.

Activity Status as of *(July 2015)***:**

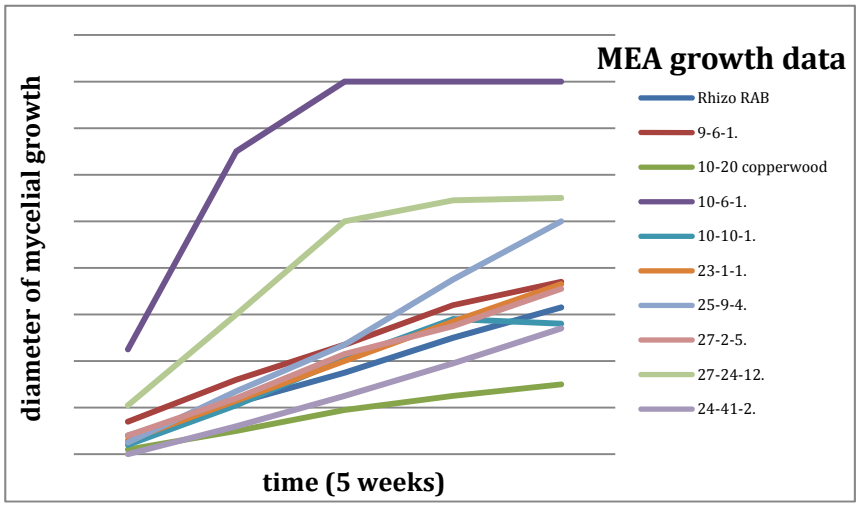
Research on the characterization of fungi from the Soudan Mine has continued and a large collection of fungi in pure culture is available for further testing. Additional sampling was also completed in June from many different levels of the mine. An unusually large area of fungal growth was found on level 15. This aggressive wood decay fungus tolerated the harsh conditions of the mine colonizing wood timbers and also expanded over large areas of rock. It is rare to find such prolific fungal growth covering an area this large in such a high metal environment. We are currently culturing this fungus and will complete rDNA sequencing to identify and characterize it further. Due to its extensive growth characteristics, it may prove to be a good candidate for further testing for metal absorption and tolerance. Several other areas were also found on different levels with unusual blooms of fungal growth. One of these areas is where *Armillaria sinapina* has grown on the surface of water with high metals. Our studies

Armillaria sinapina **with new growth occurring at the surface in a pool of water. Notice the older fungal strands at bottom covered in iron and other metals.**

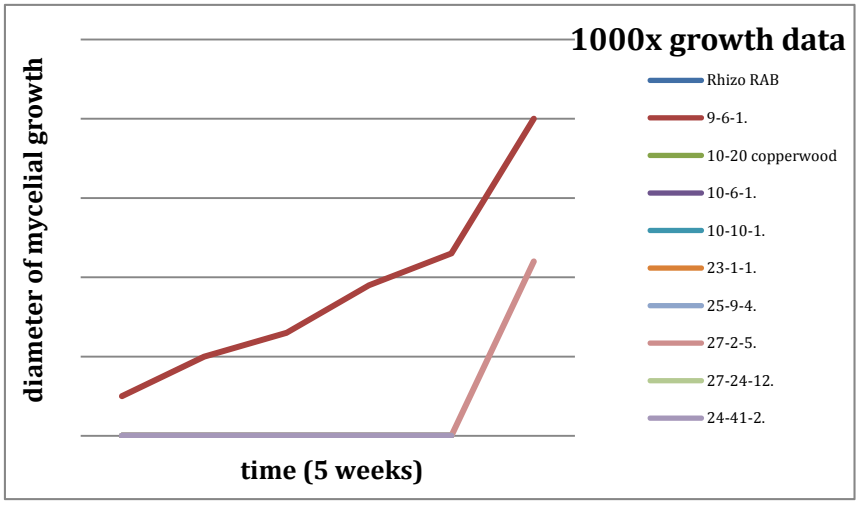
have shown that this fungus can accumulate very high concentrations of heavy metals enabling it to survive in these harsh conditions. Processing of these and other samples continues as we isolate and sequence fungal DNA from all the collections. Pure cultures of selected fungi obtained from the mine are now being used in a number of experiments. Selection of fungal cultures from the Blanchette group collection is also continuing for testing with the Toner group for heavy metal absorption studies. Thus far over 1000 fungal isolations have been made from samples from the mine, and approximately 140 different fungal taxa identified. Some of these taxa also represent new species and taxonomic studies are being done by sequencing of multiple gene regions to further characterize these isolates.

(1) Personnel hired: Jeffry Sorenson, a graduate student of Dr. Toner's, was hired as a research assistant to aid Michael Ottman in experiments utilizing the pH stat system. Reina Desrouleaux, a Carlton College undergraduate, was awarded funding through the North Star STEM Alliance program to work on the Soudan project. She will be assisting Michael and Jeffry, as well as performing metal sorption experiments designed for rapid screening of fungi using buffered solutions.

(2) Plate growth experiments: To measure metal tolerance of Soudan fungi, growth experiments were performed in collaboration with the Blanchette lab. Fungal isolates from the mine were grown in agar containing 500x and 1000x the metal concentrations being used for the sorption characterization experiments. Ten fungi have been characterized in this way; we have isolated just two, as of yet unidentified, fungi (strains *SM13-9-6-1* and *SM13-24-41-2*) that were able to grown in the extreme (millimolar) levels of metals in the 1000x media. Results from these experiments will inform our next characterization experiments and aid in choosing fungi to use in living bioreactors. Additional plate growth experiments will be performed by Ms. Desrouleaux this summer. Below are graphical data from the plate growth experiments.



Growth data from plate experiments: At left are the growth curves for the no-metal media (malt extract agar) for all of the fungi tested in the first round of experiments. SM13-10-6-1's growth flattens out in the middle because it grew to the edge of the plate.

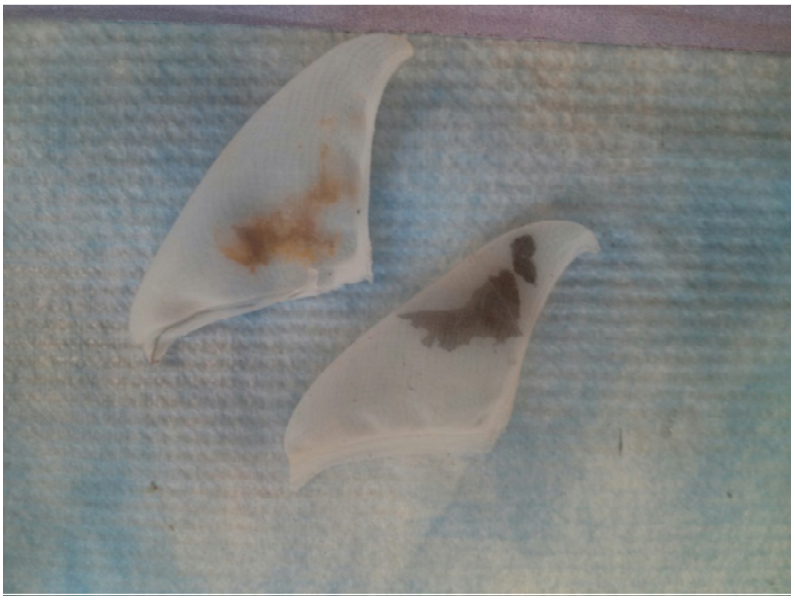


At left are the growth curves for the 1000x metal concentration for all of the fungi tested in the first round of experiments. Note that SM13-9-6-1 and SM13-27-2-5 are the only cultures that showed any growth after 5 weeks; therefore theirs are the only curves that reach above zero.

(3) Metal uptake experiments: Ms. Desrouleaux is developing and performing a series of buffered experiments wherein metals are added to Falcon tubes containing samples of Soudan fungi in brine, and sorption is quantified. pH will vary across a range seen in the mine; the data will be similar to that of the previously performed pH stat metal uptake experiments albeit obtained more quickly. Work on these experiments has already begun, but data are pending.

(4) Sorption capacity experiments: Sorption experiments performed on the previously described pH stat system are being carried out by Mr. Sorenson. These experiments are testing the stability of the fungi as biosorbents, as well as their metal loading capacity. Experiments probing the biosorbent potential answer the question of how pH changes the amounts of metal that reside on the fungal surface. Metal loading capacity experiments measure how much metal the fungi can actually absorb before their surfaces reach their capacity. Older experiments have shown which fungi sorb the most metal given the average conditions in the mine; these new experiments will detail which 'top performers' from the last experiments will be the best candidates for bioreactors.

(5) Litter bag experiments: In June of 2015, Mr. Ottman travelled to the mine with the Blanchette lab and performed a series of 'litter bag' experiments in which plastic mesh bags containing fungal mycelium (the 'roots' of the fungus) were placed into pools and sumps in the mine, removed 12 hours later, and then treated with 0.1 M HCl to remove the metals from the surface of the fungi. The four fungi that had sorbed the most metals from the previous pH stat experiments were selected for these litter bag experiments. The litter bag experiments are one step closer to an actual bioreactor experiment, and test the effects the low water temperatures have on metal sorption, as well as evaluating sorption characteristics of the fungi in the context of the water chemistry of the mine. Samples have been submitted to the UMN Earth Sciences ICP-MS facility and results are pending. Below is a photo of some of the litter bags containing the mycelia.



Activity Status as of *(Jan 2016)***:**

1) Personnel: Both Jeffry Sorenson (graduate student at the University of Minnesota) and Reina Desrouleaux (undergraduate at Carlton College) concluded their periods of summer research on the project in August of 2015. Mr. Sorenson was successful in carrying out sorption isotherm experiments on a number of fungal strains. Ms. Desrouleaux performed rapid buffered assays on metal sorption behavior, as well as acquiring time on a Scanning Electron Microscope (SEM) to observe surface phenomena of some of the samples she was responsible for running. Michael Ottman was a 20 hr/wk technician on the project.

2) Manuscript preparation: A collaborative scientific manuscript for peer reviewed publication is being prepared by Mr. Michael Ottman (Toner lab) and Dr. Ben Held (Blanchette lab). The manuscript will combine genetic characterization (Blanchette lab) with metal sorption capacities (Toner lab) for novel and known fungal species isolated from the Soudan Mine.

3) New experimental results:
   a) Further characterization of fungi isolated from samples obtained in June 2015 has continued and is adding to the very diverse fungal assemblage found in the Soudan mine. 43 additional taxa have been screened for heavy metal tolerance by growing cultures on media with 1000 times the concentration of heavy metals that occur in water in the mine environment (Fig. 1). Five weeks after inoculation the results show one particular species (SM14-25-13-1) grew nearly three times the amount as compared to many others, however 9 other isolates also showed substantial growth, indicating a tolerance to the heavy metals in the media (Fig. 2). These isolates could be used as candidates for further study and may indicate different mechanisms for metal tolerance by evidence of growth on concentrated heavy metal media.

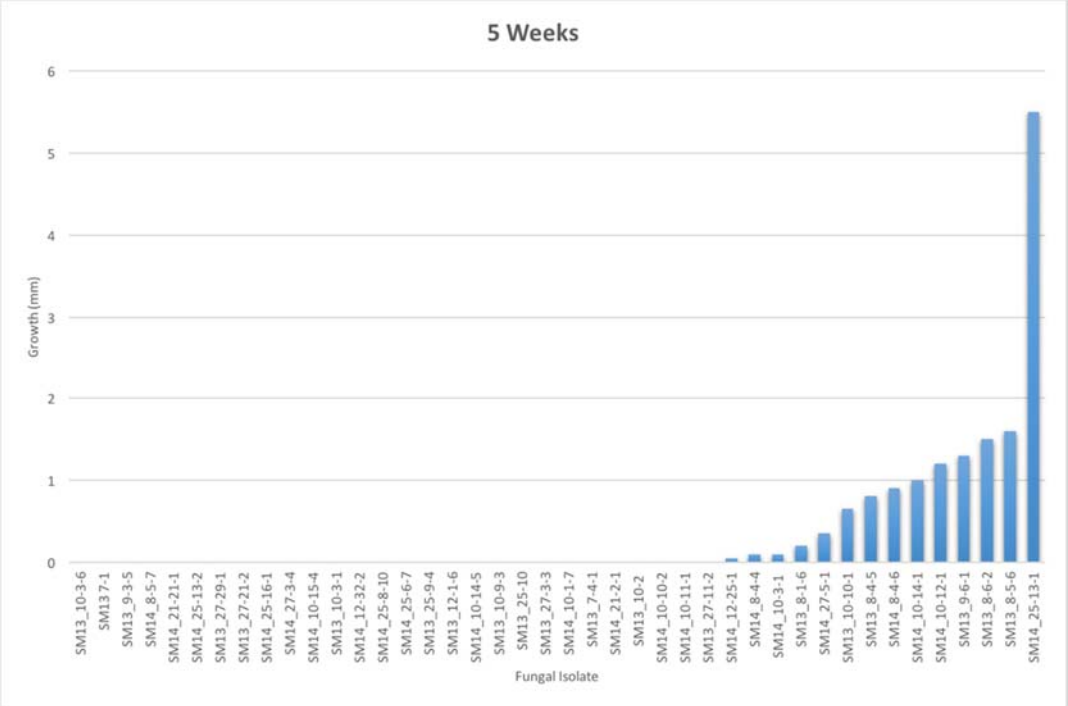


Figure 1. Bar graph showing the growth in mm over a 5 week period of 42 fungal isolates obtained from the Soudan mine. Those isolates showing 0.5mm growth were considered not tolerant.

b) ***Metal sorption capacity experiments*** – Example results from metal sorption capacity experiments are displayed below (Figure 3). The experiments were designed to probe the amount of metal that each fungi can absorb from the water and immobilize it on the surface of their cell walls. The data describes the amount of metal that will be removed from water by a fungus relative to the amount of metal in the water. These values are normalized on a weight basis, so that we can predict the amount of biomass needed to clean up a given waste stream.

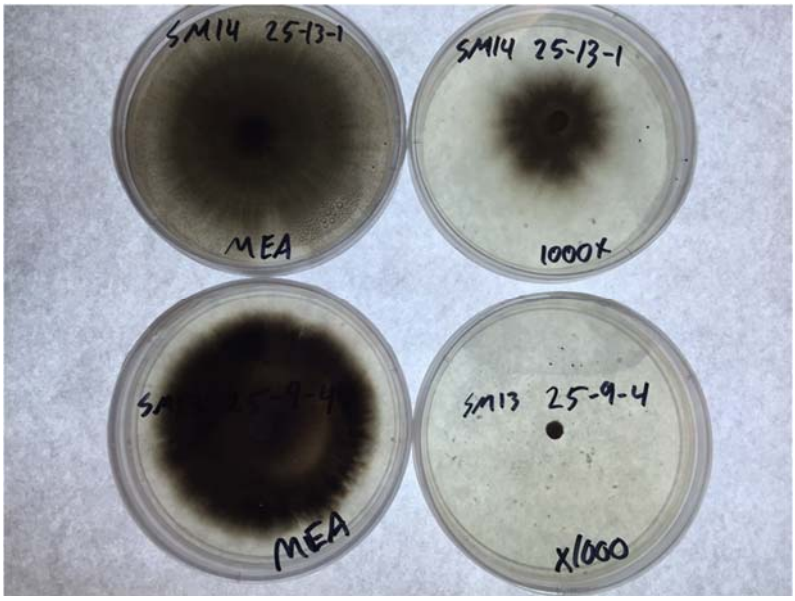


Figure 2. Several examples of fungi tested on heavy metal media. Cultures on the left are grown on normal media and cultures on the right are grown on media amended with heavy metals. Top right; growth on media amended with heavy metals showing tolerance. Bottom right; absence of growth on heavy metal media.

The species used for these experiments were in part selected by previous experiments performed in the study, as were the metals selected for these multi metal experiments. In all, three species of fungi were tested under the same pH (7) and ionic strength (156 mmol/L) that were used in the rest of the experiments conducted, but the metal concentrations added varied from a quarter to five times the initial concentrations used in previous experiments. The fungi represented by the red squares, *Armilleria sinapina,* is known to have a high affinity for metal uptake in soil and aquatic environments.

c) ***Litter bag experiments*** **-** These experiments sought to validate our laboratory results in a field setting. Four fungal species were selected for the litter bag experiments in which fungal biomass was placed into contaminated water at the Soudan Mine in plastic mesh bags containing fungal mycelium. The litter bags were placed into pools and sumps in the mine, removed 12 hours later, and then treated with 0.1 M HCl to remove the metals from the surface of the fungi. The graph below (Figure 4) shows the results from a litter bag containing *Armilleria*.

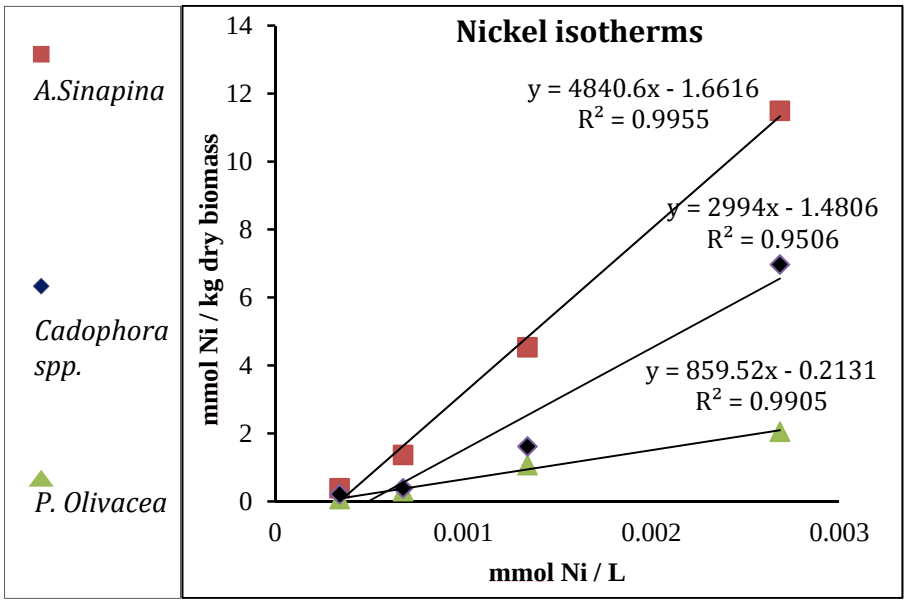


Figure 3. Each marker is representative of a different species of fungus. There are four sets of markers, each representing an experiment performed at a different concentration. The higher on the Y axes the marker sits, the more metal that was absorbed by that fungus at that concentration; another way of thinking about this is that the higher the rate of change for a given line, the more metal that fungi can absorb. For example, the fungus represented by the red squares sorbed more metal than the other two fungi shown by purple diamonds and green triangles

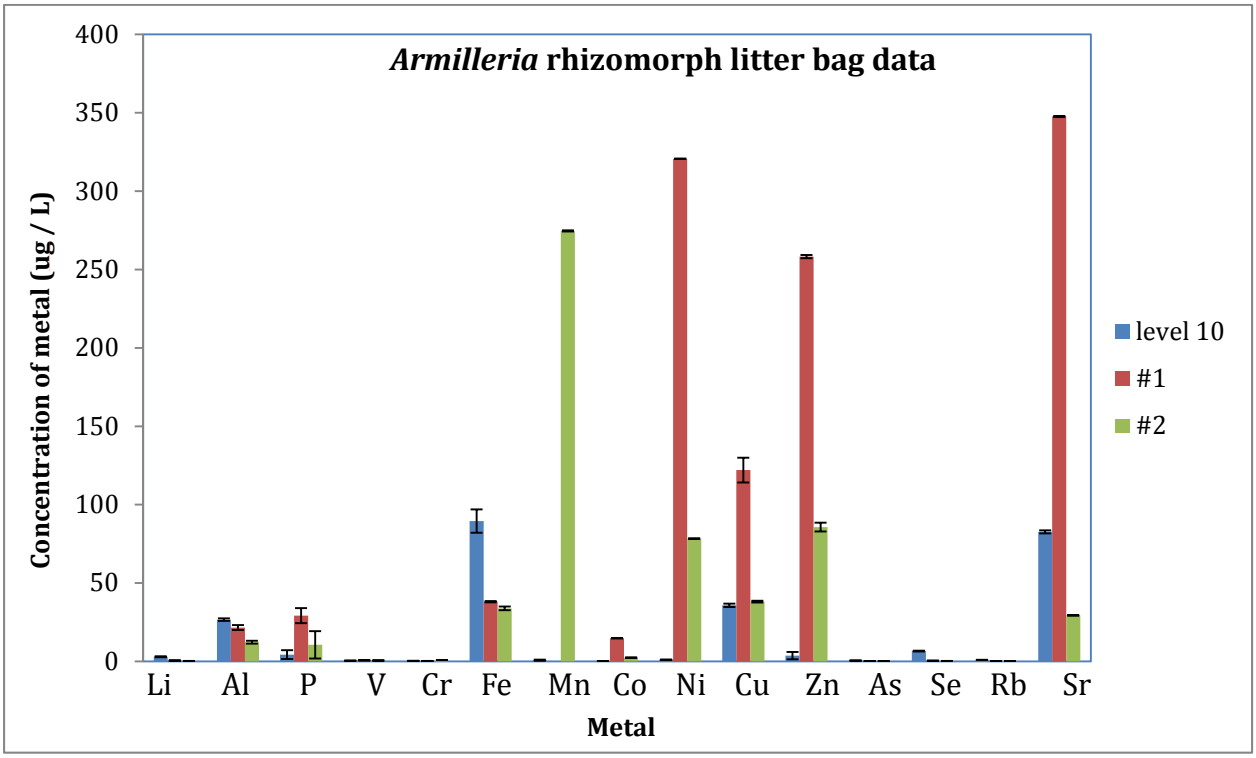


Figure 4. The blue bars show the concentrations of metals for a low-flow sump on Level 10 of the mine. The red and green bars show the concentrations of metals that were desorbed from the fungal surface over two successive acid washes (#1 and #2). Note that here, a greater range of metals were being analyzed for than in the other experiments.

These experiments indicate that the fungi we have selected have potential for bioreactors in the Soudan mine. For many metals, the concentrations sorbed to the fungal surface are orders of magnitude greater than the concentrations in mine waters. Our findings suggest that the reactivity of the fungal biomass in situ is suitable for bioreactors.

Activity Status as of *(July 2016)***:**

1) Personnel: Ph.D. student Colleen Hoffman and undergraduate student Aubree Dunshee designed and conducted an experiment to test the metal tolerance of Soudan Mine fungi in liquid media in collaboration with Mr. Michael Ottman and Drs. Robert Blanchette, Benjamin Held, and Cara Santelli.

2) Manuscript preparation: A collaborative scientific manuscript for peer reviewed publication is being prepared by Mr. Michael Ottman (formerly of the Toner lab) and Dr. Ben Held (Blanchette lab). The manuscript will combine genetic characterization (Blanchette lab) with metal sorption capacities (Toner lab) for novel and known fungal species isolated from the Soudan Mine. The metal tolerance in liquid media experiments from this period will be added to this manuscript.

The primary motivation for adding these new experiments is the possibility that metal tolerance on solid substrates (e.g. we have reported on metal tolerance during growth on petri dishes previously) could be quite different from liquid media. The results are important because they will help determine which isolates will be best adapted to different growth and remediation conditions. For example, some of our isolates perform metal removal best when growing on a solid substrate. These isolates would be developed for remediation scenarios using a trickle-filter approach in which the fungi grow on a solid substrate and the contaminated water is gravity fed over the fungi. In contrast, some of our fungal isolates are strong metal sorbents, but do not grow well in the presence of the metal. For these isolates, a remediation strategy would involve growing large amount of biomass (metal free) and preparing that biomass as a reagent sorbent (e.g. in large batch reactors). Finally, we would like to identify isolates that can grow in liquid media with metals present. Isolates with these characteristics could be used in a self-regenerating bioreactor for metal removal (much like sludge reactors in wastewater treatment).

3) Fungal growth experiments in liquid media: Three fungi species isolated by the Blanchette group from the Soudan mine were analyzed for growth in liquid media in the presence of a multi-metal spike (zinc, nickel, copper, and cobalt). The experiment is on-going, and its design included triplicate treatment flasks and one no-metal control flask per time point per fungi species (Fig.1).

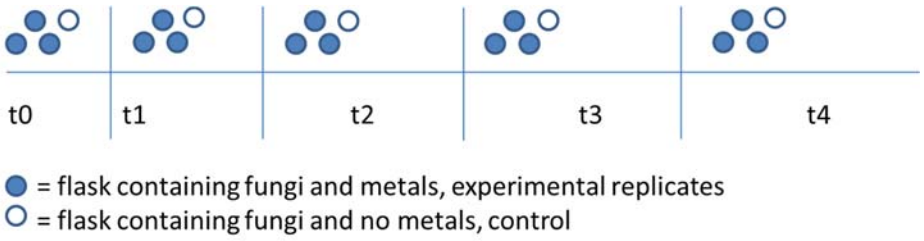


Figure 1. Schematic showing the experimental design for one fungal species. Replicate and control flasks are destructively sampled at each time point (t0 is the initial condition).

At each sampling time point, solids and liquids are separated by filtration (Fig.2). Several fractions are then retained for quantification of metals by inductively coupled plasma mass spectrometry (ICP-MS) In addition to total metal concentration in the liquid media and fungal biomass, a subsample of the liquid media is retained for ligand analysis (Dr. Christine Salomon's lab), and total biomass. The ICP-MS samples are acidified with 1% v/v trace grade nitric acid, and will be analyzed at a later date. Samples collected for ligand analysis were filtered and frozen at -20°C. Lastly, total biomass samples were filtered, dried at 40°C overnight, and weighted.

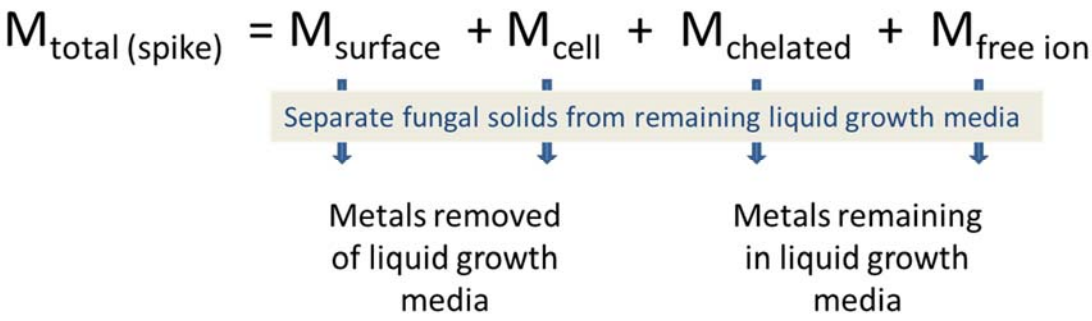


Figure 2. Schematic showing the partitioning of metals between liquid and solid phases during the experiment.

Final Report Summary:

The Sudan underground mine State Park is an extraordinary resource to the people of Minnesota. In addition to its historic mining history, the mine has an extraordinarily unique environment. The mine has high levels of heavy metals and lots of wood timbers used during the mining days. The wood timbers have served as a carbon source for microorganisms but the conditions of the mine, especially the high metal concentrations in certain areas, produced selection pressure allowing certain microbes to grow with tolerance to these metals. Our research has isolated into pure culture and identified over 1000 fungal strains from the mine. From these, there are over 140 different fungal taxa. Many of these organisms are different from those that grow above ground and sequencing of their DNA indicates that several are fungal species that have not been previously identified. Since these fungi can grow in an environment rich in metal ions, they are of great interest to study how they can tolerate these toxic compounds and elucidate the mechanisms they use to sequester the metals into non-toxic forms. These investigations have provided the organisms that are now being used for bioremediation studies and for the isolation and characterization of new compounds that may be useful in bioprocessing technology.

The objective for the Toner Group's portion of this LCCMR grant was to explore fungi isolated from the Soudan Underground Mine State Park for use in bioremediation of metals in mine effluent. One of our motivations for pursing this research was that the mine spends approximately $200k/year to remove metals from mine effluent. We know, from previous LCCMR support, that fungi are thriving in areas of the mine that are heavily contaminated with copper (Cu), cobalt (Co), zinc (Zn), nickel (Ni) and other metals (e.g. mercury, Hg). These fungi are adapted to mine conditions and represent a reservoir of potential bioremediation strategies. For this LCCMR, our main task was to screen these locally adapted fungal strains, isolated by our collaborators Professor Robert Blanchette and Dr. Benjamin Held, for metal uptake efficiency from mine waters.

Outlook

With the findings generated by this grant, we aim to match the capabilities of metal binding fungi with engineered approaches to treat the contaminated mine water on-site (bioremediation). The project focused on characterizing fungi isolated from the most contaminated areas of the mine, and identifying the best candidates for further development of bio-filter and bioreactor technologies.

Noteworthy Discoveries

During this LCCMR research project, we discovered that fungi living in the mine are accumulating metals within their living biomass. We also found that metal-binding by the naturally occurring rhizomorphs collected from the mine (*Armallaria* species) is reversible. What does reversible mean? In this case, it means that we could remove the metals with an acidic water rinse. Once "cleaned", the natural rhizomorphs retained metal binding capabilities similar to those of the *Armallaria* cultures we grew in the lab. These findings were important confirmations that: (1) the Soudan fungi do accumulate metals from the water in their environment; (2) the amount of metal removed from water was very similar between natural and lab specimens; and (3) metal binding can be reversible. Overall, we have evidence that the fungi and lab conditions are representative of the mine conditions. In addition, we find that rhizomorphs have the potential to be more than single-use biosorbents of metals.

Products

We are in the process of writing a manuscript for peer review that will detail the characteristics of Soudan fungi that produced the best metal-binding results for three different scenarios: (1) dried and ground fungal cultures grown on wheat and barley with no metal exposure during growth; (2) fungal cultures grown in liquid media without metals present during growth; (3) fungal cultures grown in liquid media with metals present during growth; and (4) fungal cultures grown on solid agar media in the presence of metals. These results are important because they will help us determine which isolates are best adapted to specific growth and remediation conditions. For example, some of our isolates perform metal removal best when growing on a solid substrate. These isolates would be developed for remediation scenarios using a trickle-filter approach in which the fungi grow on a solid substrate and the contaminated water is gravity-fed over the fungi. In contrast, some of our fungal isolates are strong metal sorbents, but do not grow well in the presence of the metal. For these isolates, a remediation strategy would involve growing large amount of biomass (metal free) and preparing that biomass as a reagent sorbent (e.g. in large batch reactors). Finally, isolates that can grow well in liquid media and in the presence of metals could be used in a self-regenerating bioreactor for metal removal (much like sludge reactors in wastewater treatment).

ACTIVITY 2: Microbes and electricity

Description: The Soudan Iron Mine is a unique environment that has novel populations of bacteria that can eat or breathe iron. Iron breathing bacteria can be harnessed to generate electricity, while iron-eating bacteria will help create biofuels in a process called 'electrosynthesis.' We have enriched and cultured novel bacteria from the mine with our previously funded ENRTF project. This proposal will explore three key elements that are necessary to realize the potential of electrosynthesis: Test and prove the use of isolated Soudan bacteria interfaced with electrodes, molecular characterization of electron transfer in novel mine bacteria and optimization of electron transfer by bacteria associated with electrodes.

Summary Budget Information for Activity 2: **ENRTF Budget: $ 317,646**

Amount Spent: $ 317,646
Balance: $ 0

Activity Completion Date:

Outcome	Completion Date	Budget
1. Test isolated Soudan bacteria interfaced with electrodes: cathode enrichments in the lab and in the mine	July 2014	$105,882
2. Illumina 16s rRNA sequencing and culturing of microbial communities on cathodes	July 2015	$105,882
3. Screen microbes for iron oxidizers	July 2016	$52,941
4. Initial molecular characterization of electron transfer in novel mine bacteria	July, 2016	$52,941

Activity Status as of *(January 2014)*:

(1) A key goal our portion of the project is to identify genes involved in the iron oxidation by the Soudan Iron Mine *Marinobacter* isolate strain JG233 that we have been studying for several years. The primary focus over the last six months has been to generate a massive transposon library in this organism to use a technique called 'tn-seq' to rapidly identify essential genes and determine the fitness contribution of every gene in the genome for a given growth condition. Transposon mutagenesis has not been performed, to our knowledge, in any *Marinobacter* isolate and we encountered some difficulties resulting in a modification to the transposon delivery methodology and to the actual vector itself. We now have a pool of 100,000 mutants and are in the process of sequencing transposon junctions to determine the coverage of the library. Two outgrowth experiments of this library were performed – one in minimal defined medium and one in rich medium. Results from these experiments will help us determine core central metabolic pathways and essential genes. We have also generated Transmission Electron Microscopy images of *Marinobacter* cells grown under iron oxidizing conditions (see representative image below). Cells appear to be accumulating iron oxide minerals on their surface. We are now undertaking a bioinformatic approach to identify putative redox-capable proteins that are predicted to be localized to the outer membrane.

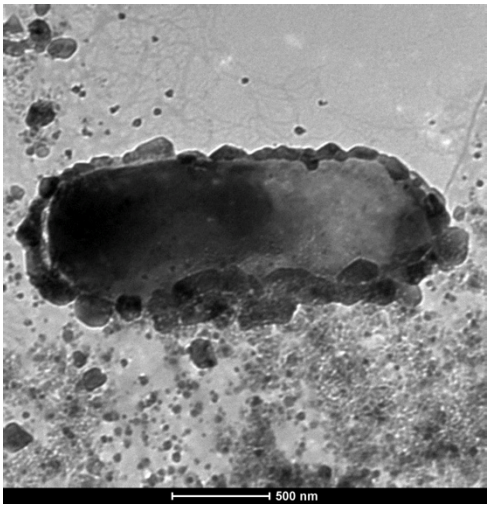


Figure 2. TEM image of *Marinobacter* sp. Strain JG233 cultured under iron oxidizing conditions with ferrous sulfide as electron donor and oxygen gas as electron acceptor.

(2) Researcher hired: A postdoctoral scientist, Jon Badalamenti, who has extensive experience in the cultivation of electricity-producing bacteria in saline environments, was hired for the project.

(3) Sequencing of isolates: In collaboration with researchers at the University of Arizona, where sequencing was performed, we obtained draft genome data for two new bacterial strains and assembled their genomes. These represented the first such genome sequences for metal-reducing bacteria from hypersaline environments. To begin our investigation of the conservation of genes and metabolic strategies within these kinds of bacteria, we assembled and annotated draft genomes of *Geoalkalibacter ferrihydriticus* DSM 17813^T and *Geoalkalibacter subterraneus* DSM 23483^T. Paired-end Illumina reads at ~100x coverage produced a 3,840,442-bp genome in 24 scaffolds for *G. ferrihydriticus*, while the *G. subterraneus* assembly was problematic due to a Gram-positive contaminant that appeared to utilize yeast extract in the standard medium. Upon re-isolation of *G. subterraneus*, PacBio sequencing at the Mayo Genomics Facility at ~150x coverage allowed assembly of a 3,729,032-bp genome in 5 contigs with no evidence of contamination. Annotation suggested functions in *G. ferrihydriticus* not previously described, including arsenate reduction and benzoate catabolism. Based on the genome of pure *G. subterraneus*, this strain is not auxotrophic for vitamins and amino acids and may have a more limited substrate range than reported for the contaminated culture.

This work allowed us to test our protocols for sequencing and annotation of genomes, and begin a database of genes possible important to growth under hypersaline conditions. Genomes from uncultivated halophilic members are needed to further expand upon this and to reveal shared adaptations driving metal reduction in saline environments.

(4) Isolation of novel strains: As part of preliminary research for this project, samples from the Soudan Mine were incubated in our laboratory using an electrode as the sole means of growth. Basically, this involves providing a substrate such as acetate to the microbial population, but eliminating oxygen or other compounds which could support oxidation. If an electrode is included in the reactor, poised at an oxidizing potential, bacteria able to attach to this surface as if it were a metal in the mine environment could grow. Using this enrichment strategy, we have obtained cultures for study.

Preliminary phylogenetic analysis of these strains, to determine if they are related to commonly studied bacteria, has revealed at least one culture to be highly novel. However, it shows signs of containing multiple strains, thus a key goal of this portion of the project has been to purify the culture free of contaminants. As shown by work in #2, even cultures deposited in databases can be contaminated, as these bacteria can be difficult to cultivate in the laboratory. Dr. Badalamenti has developed techniques for using solid medium to grow these in isolation, and has subjected these enrichments to rounds of purification. We anticipate submitting DNA samples for preliminary sequencing by Spring. If the culture remains mixed, we will sequence the genome(s) of the total culture as a metagenomic project, to further investigate the presence of novel genes or species within this enrichment.

Activity Status as of *(July 2014)***:**

(1) *Iron oxidation in Marinobacter JG233*: Modified transposon used by Brutinel et al. from our lab resulted in low efficiency of transformation, even at large scale 1000's of colonies possible rather than 10,000's. After trying several variations on the mating protocol and several false positive results we decided to abandon the modified transposon in favor of the original transposon, lacking MmeI restriction sites near the inverted repeats. We hypothesized that the modification of the transposon affected its efficiency, which we had previously observed in our work with *Shewanella*, but the beginning efficiency was significantly higher than with JG233. MmeI is used to liberate a small amount of genomic DNA adjacent to the transposon, but other methods exist to accomplish a similar result. Through a combination of DNA sonication, size selection, C-tailing and PCR we expect to be able to generate our JG233 library. These individual steps are currently being

optimized and we have confirmed that the unmodified transposon can be used to generate ~ 50,000 random mutants in a straightforward manner.

To complement Tn-seq analysis in JG233 we have also initiated a PacBio genome sequencing run. Previously, we had generated a short read Illumina library of the JG233 genome that assembled into about 55 contigs. While the majority of the genome is represented, for accurate Tn-seq analysis, a complete genome is best. The Bond Lab has been working for the last 8 months with the sequencing core facility at the Mayo Clinic to use their PacBio sequencer. The JG233 run just completed and we have achieved approximately 250X coverage of the genome, with approximately 100X coverage in reads that are >10,000bp. We are in the process of assembling the genome right now.

(2) We achieved a major milestone this spring as we successfully isolated a novel, halophilic, metal-reducing bacterium from Soudan in pure culture. After having been enriched using electrodes as the sole means of growth in the laboratory, this isolate required optimizing growth conditions such as temperature and salt concentration in order to tease it into forming single colonies in a petri dish. Phylogenetic classification placed this bacterium as a member of the *Desulfuromonas* genus, with only 96% sequence identity to its closest relative. In order to understand its physiology, we sequenced its genome using the Pacific Biosciences (PacBio) single-molecule real-time sequencing platform, a revolutionary technology in microbial genomics for its ability to produce complete, finished quality genomes at a reasonable cost. Despite genome assembly using PacBio data being computationally intensive, we used the Amazon EC2 Compute Cloud to assemble a single 3,958,620-bp circular chromosome (Figure 1) with 99.9997% accuracy (less than three errors per 1,000,000 DNA bases) and a G+C content of 61%. Long read sequencing successfully resolved a tandem duplication of the ribosomal DNA operon, and this remarkably complete and accurate assembly allowed us to predict the chromosomal origin of replication, as well as to upload the genome sequence at finished quality to public databases. From the genome annotation, we discovered novel features including the ability to respire nitrate, sulfur compounds, and perhaps arsenate, as well as the potential to degrade aromatic compounds and widespread evidence for incorporation of foreign DNA from bacteriophage (Figure 1). Surprisingly, the genome predicted only 39 putative multiheme *c*-type cytochromes for extracellular respiration, a relatively low number when compared to the type strain for this bacterial family. Ongoing and future work is aimed at characterizing the isolate in terms of its growth optimum and substrate utilization, as well as designing a genetic system so that mutants can be screened and selected for further study.

Based on its genome sequence and phylogeny, along with the fact that it is the first bacterial isolate from Soudan for which we have a complete, closed genome, we propose an appropriate species designation for the isolate, naming it '*Candidatus* Desulfuromonas soudanensis WTL.'

(3) Isolation of novel strains: As part of ongoing efforts to survey for novelty among Soudan's microbes, we continued long-term enrichment experiments using electrodes as the sole means of growth in the laboratory. A sampling trip in February yielded electricity generation after a ~30-day incubation, and we have leveraged our experience from isolating '*Ca.* Desulfuromonas soudanensis' to design strategies for obtaining pure cultures from this successful electrode enrichment.

In parallel, we recovered ample mixed community DNA from electrodes and will use this material to characterize its metagenome, whereby large chunks of constituent genomes are assembled without first having to obtain isolates in pure culture. We will expand this approach to future electrode enrichments as well as for metagenomic characterization of Soudan mine microbial communities *in situ*.

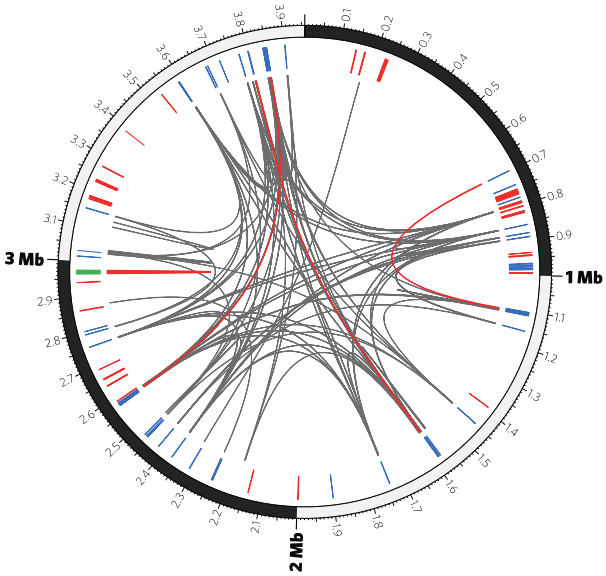


Figure 1. Circular representation of the complete genome of '*Ca.* Desulfuromonas soudanensis WTL,' where red bars indicate locations of putative multiheme *c*-type cytochromes, blue bars indicate transposons, and interior links show repeat sequences >500 bp.

Activity Status as of *(January 2015)***:**

(1) Getting to know a bacterium isolated from the Soudan Iron Mine: We continue to work with a novel bacterial isolate from the Soudan Iron Mine. We have characterized the physiology and metabolism of this *Marinobacter* strain through a variety of laboratory methods. This work has told us what the organism likes to eat, what it can breathe, what temperature it prefers, how much salt it likes in its environment and what pH it prefers. We have also recently completed a genome sequence for the organism and are working on a manuscript to describe our work.

Iron oxidation by *Marinobacter*

Based on the genome, we have identified several candidate genes that could encode proteins involved in iron oxidation. We are very interested in this process because transformation of Fe(II) to Fe(III) mediated by these bacteria should occur on the surface of the bacteria, requiring transfer of the electron from Fe(II) into the cell. Why do we care about this process? Understanding what proteins are involved in extracellular electron transfer may someday help engineer cells to use electricity to produce compounds for us (biofuels, for example). Along with a directed approach, we have been working on another genetic technique (Tn-seq) that can help us identify genes involved in iron oxidation. After many months of optimizing these experiments, we have samples being processed right now and expect to be analyzing candidate genes in the next few months.

(2) Characterization of '*Ca*. Desulfuromonas soudanensis WTL': We continued experiments aimed at understanding the basic physiology of '*Ca*. D. soudanensis', a novel halophilic isolate we recovered from Level 27 at Soudan. We discovered that this isolate can reduce a form of iron(III) oxide, called schwertmannite, approximately 2-3 times faster than more commonly tested iron(III) oxide forms, despite the fact that both iron forms provide a similar amount of energy to the cells. Surprisingly, growth experiments to test optimum salt tolerance and temperature showed that this isolate prefers salt concentrations approximately 6-fold lower than those typically occurring at DDH 944 (where the isolate was taken from), and an optimal temperature markedly higher than the relatively constant 11-15°C of Soudan brine. Taken together, these findings suggest that environmental conditions further down the boreholes on Level 27 are less salty and warmer than at the mouth. Thus, we postulate that the natural habitat of '*Ca*. D. soudanensis' lies at a much greater depth along DDH 944, and that naturally flowing brines contain not only

microbes which thrive near the borehole mouth, but organisms from the much deeper subsurface as well. We also continued to mine its genome (described in the July 2014 update) for novel capabilities, and we discovered an unmistakable gene cluster for breaking down malonate, an unusual 3-carbon substrate. We are currently confirming this activity with growth assays, but this finding is remarkable in that other close relatives are unable to metabolize malonate, indicating that this gene cluster was transferred horizontally from another microbe to '*Ca*. D. soudanensis.' Finally, we have deposited this strain in a publicly accessible culture collection (the German Collection of Microorganisms and Cell Cultures, DSMZ) under provisions specified in our sampling permit which stipulates that the Minnesota Department of Natural Resources retains ownership of this strain for any commercial purposes.

Metagenomic characterization of *in situ* and electrode-enriched Soudan microbes: Arguably the most transformational advance in microbiology in the last 10-15 years is the use of inexpensive DNA sequencing to assemble near-complete genomes of environmental microorganisms while bypassing the need to culture them in the laboratory. This approach is called whole genome shotgun metagenomics. As we described in the January 2014 update, enriching and isolating novel microbes from the environment is quite time- and labor-intensive, and the fact that most microbes are resistant to laboratory cultivation means that this approach misses >99% of the potential diversity in natural habitats. To complement our work with our isolate '*Ca*. D. soudanensis,' we applied shotgun metagenomics on both *in situ* microbial communities as well as those we enriched on electrodes. In October, Dr. Badalamenti led a group of investigators to Soudan to simultaneously collect and concentrate microbes from three boreholes on Level 27 using a technique called tangential flow filtration. Because Soudan brines contain such low cell counts (10^3-10^4 cells per milliliter), it was necessary to concentrate cells in the brine to a level allowing extraction of sufficient quantities of genomic DNA for sequencing. These samples are currently undergoing sequencing as part of the Deep Carbon Observatory's Census of Deep Life. In parallel, we recovered DNA from successful electrode enrichments and performed metagenomic sequencing. After assembling genomes from the cells attached to the electrode, we discovered that two Deltaproteobacteria (the same bacterial family to which '*Ca*. D. soudanensis' belongs) dominated the sample. While related, these two genomes differed from that of '*Ca*. D. soudanensis,' indicating that we enriched novel members of this bacterial lineage. Ongoing work is aimed at leveraging Pacific Biosciences long read sequencing (described in earlier status reports) to separate the two close relatives into two distinct genomes. We are in continued contact with PacBio scientists and bioinformaticians to design effective approaches towards this goal, as the application of long DNA sequencing in metagenomics is currently on the cutting edge.

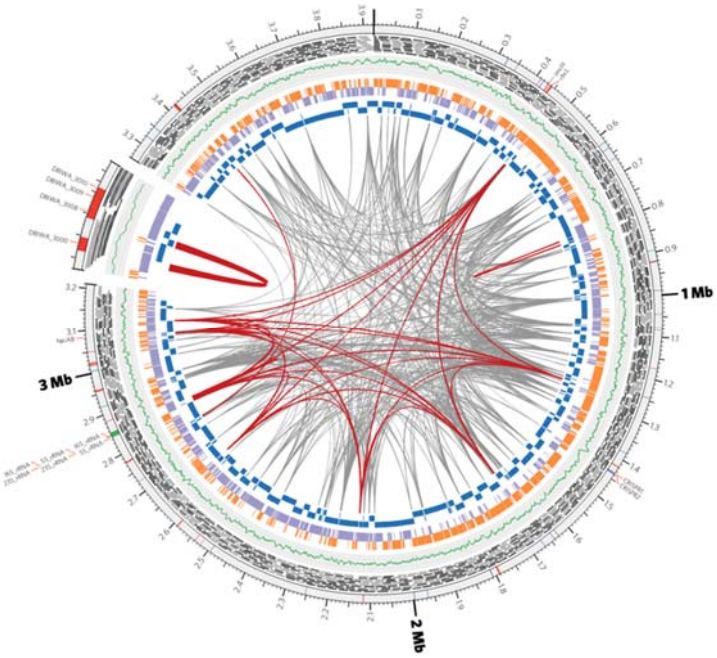


Figure 2. Complete, circular genome of '*Ca.* Desulfuromonas biwabikus DDH964' recovered from a mixed community of Soudan bacteria enriched on electrodes.

Activity Status as of *(July 2015)***:**

Recovery of a complete genome from a community of electrode-enriched metal reducers from Soudan. Despite technical challenges in sample preparation and DNA degradation, we successfully deployed PacBio long read sequencing to a mixed community sample of bacteria from Soudan that were enriched on poised electrodes. With just 270 Mpb of data, we were able to assemble the complete, circular genome of '*Ca.* Desulfuromonas

biwabikus DDH964,' named after the Ojibwe word for iron and the drill hole on Level 27 from which the sample was collected. To the best of our knowledge, this genome represents the first ever complete genome recovered from a metagenome and is a major breakthrough not only for this project but for the broader field of shotgun metagenomics and community analysis. This organism has never been isolated in the laboratory, and yet we now know its full genetic composition: an abundance of cytochromes for metal reduction, evidence for multiple past viral infections, and features of central metabolism that point to a habitat where low levels of oxygen may be present. With support from Pacific Biosciences, we performed additional sequencing of this sample and demonstrated complete or near-complete reconstructions of the 4 bacterial genomes present in the sample, a result otherwise impossible with traditional short read sequencing approaches. Armed with its genome sequence, efforts are underway to design conditions under which '*Ca.* D. biwabikus' can be isolated and further characterized for its potential en electrode-based desalination metal detoxification systems using Soudan Mine microbes.

Activity Status as of *(Jan 2016)***:**

Metagenomic characterization of *in situ* and electrode-enriched Soudan microbes: As mentioned in the January 2015 update we undertook metagenomic characterization of native Soudan mine brine microbial communities as part of the Deep Carbon Observatory's Census of Deep Life. This effort informs aspects of our electrode enrichment approaches by describing not only the structure of the microbial communities but the potential metabolic functions that they carry out. To our surprise, when we surveyed brines from drill holes 932, 944, and 951, genomic signatures corresponding to those we recovered after electrode enrichment were essentially absent, indicating that electrode-respiring microbes are rare in Soudan despite this environment being iron-rich. This finding underscores the efficacy of our approach towards using electrodes as "bait" to identify biotechnologically relevant microbes--literally pulling a needle from a haystack.

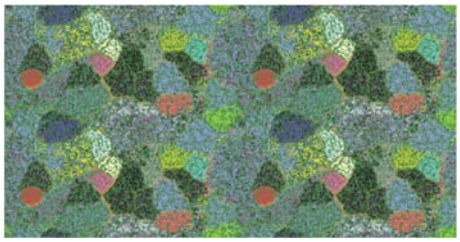


Figure 3. Binning metagenomic sequences by tetranucleotide frequency with ESOM. Colored patches represent potential bins.

By surveying the entire genomic landscape of these Soudan communities, we can use computational tools to assign bits and pieces of genetic material to individual organisms through a metagenomic approach called "binning." Figure 3 shows the results of a typical analysis called an emergent self-organizing map (ESOM). Every microbe has a characteristic genetic signature of DNA bases that allows sequences from the same organism to cluster together in 2-D space. We then pulled

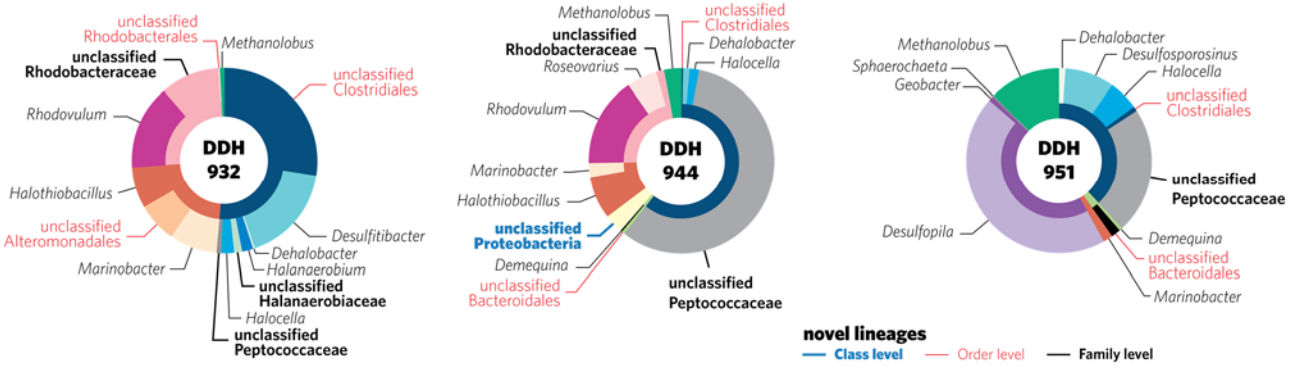


Figure 4. Microbial community structure of Soudan brines sampled from diamond drill holes 932, 944, and 951.

genomic fragments from individual bins and examined each bin's microbial identity as well as its genetic potential (Figure 4). The microbial communities are markedly different across the three boreholes sampled, and as mentioned above were essentially devoid of detectable electrode-respiring bacteria. However, we discovered that

the only archaeal population present in Soudan belong to the genus *Methanolobus*. Archaea are a domain of microorganisms that often dwell in extreme habitats and/or perform specialized metabolism such as methanogenesis. *Methanolobus*, unlike most other methanogens, requires methylated carbon sources, suggesting that other microbes present in Soudan are carrying out primary production (i.e. CO_2 fixation into cell material). We are investigating the genomic content of other bins to describe the metabolic relationships underpinning life in Soudan brines, and we intend to leverage this information to further enhance electrode enrichment experiments.

Characterization of iron oxidation in *Marinobacter subterrani* :We have been studying a novel bacterial isolate from the Soudan Iron Mine capable of iron oxidation. We are interested in trying to understand iron oxidation for two reasons: first, it is a biological process that we know very little about and second, understanding how bacteria mediate iron oxidation could help us understand how to engineer organisms to utilize electricity through a process called 'electrosynthesis.' In 2015 we published a scientific manuscript describing this novel bacterial isolate as *Marinobacter subterrani* (http://journal.frontiersin.org/article/10.3389/fmicb.2015.00719/ abstract). We are now working to understand how iron oxidation works in *M. subterrani* as detailed below.

Bacterial oxidation of iron by *M. subterrani* may occur due to one of two mechanisms. The first involves the bacterium oxidizing iron for energy, much as we do when we eat food. The second possible mechanism is that the bacterium produces and releases a chemical that is able to chemically react with iron. *M. subterrani* produces several chemicals that are capable of reacting with iron, including reactive oxygen radicals and nitrite. The last six months of work has focused primarily on the contribution of these products to the observed iron oxidation by *M. subterrani*. Mutants in genes with possible importance to nitrite production have been made as a method of linking this observation to iron-oxidation. The potential of nitrite to oxidize iron in cell-free and cell-containing conditions has been assayed as well, with no evidence thus far suggesting a role in *M. subterrani* facilitated iron oxidation. Preliminary experiments to look further into reactive oxygen radicals have been started. We have also developed and refined a genetic technique to rapidly determine genetic information in *M. subterrani*. We are currently working on applying this technique to ask what genes and pathways are used by the organism to mediate iron oxidation. In parallel to these laboratory experiments, we are also trying to develop this methodology to better understand how *Marinobacter* is living in the salty brine of the Soudan Iron Mine.

Activity Status as of *(July 2016)***:**
During this portion of the project we continued our work with the newly described bacterial isolate from the Soudan Iron Mine, *Marinobacter subterrani*. This organism is capable of neutrophilic iron oxidation – a process that creates biogenic iron oxide as its waste product – and is of interest both from a basic biological standpoint of how these bacteria carry out the reaction and from an industrial standpoint for understanding how electrons can flow into living biological systems and the high reactivity of biogenic iron oxide minerals. Over these last six months we continued to develop a high-throughput genetic technique (called Tn-seq) that enable the construction and analysis of tens of thousands of mutants in parallel. We generated very large sequencing datasets that are used to interpret the results of this genetic technique and continue to refine the analysis to better understand it. After consulting with Professor Igor Libourel (UMN), we also spent time running the analytics in a different way, allowing us to generate slightly different data sets. We are currently working on refining these datasets and generating hypotheses to validate our prediction for the molecular mechanism of iron oxidation and what pathways are required by *M. subterrani* to survive conditions in the salty brine water from the mine.

We also initiated a collaborative project between two labs at Ohio State, a lab at University of Southern California and the Naval Research Laboratory to work on a comparative analysis of *Marinobacter* genomes from

a range of environments. The primary parameters we are comparing are deep subsurface vs marine, brine vs marine, iron oxidation and cathode oxidation. We are currently writing up these findings.

Final Report Summary:

Our work involves determining the components involved in bacterial iron oxidation and the means by which iron-oxidizing bacteria exploit this process. Oxidation is the process of removing electrons from a molecule; in this case, the electron is removed from iron and may be used by the organism to do work. These bacteria require and convert large quantities of iron, greatly influencing both solubility and reactivity, and as such are implicated in having deposited iron reserves, are utilized in the mining and refining processes, and are in part responsible for the corrosion of iron-bearing structures. Despite the economic and industrial advantages of a mechanistic understanding of iron-oxidizing bacteria and how they facilitate this process, the biochemistry and genetics of these organisms remain largely uncharacterized due to the particular requirements for their cultivation. Allowing us to expand beyond phenomenal use of these organisms to more directed and manipulatable utilization and control requires a better knowledge of the genetics, metabolism, and growth limits of the iron-oxidizing bacteria.

One possible mechanism of microbial-mediated iron oxidation is the bacterial production of soluble compounds capable of reacting with iron. *Marinobacter subterrani* strain JG233 produces at least two such reactive compounds, nitrite and superoxide. Nitrite generation can occur by several known mechanisms, as well as through mechanisms that remain undescribed. The potential role of the described mechanisms was investigated using mutants lacking genes important for the production of nitrite, while chemical assays were used to probe the role of reactive nitrogen species in general. The process by which microbes generate superoxide capable of reacting with iron remains enigmatic, thus making genetic studies impractical. Instead, enzymatic assays promoting the increase or decrease of superoxide under iron oxidizing conditions and the effect on iron-oxidation rates were used. Based on our results, it would seem unlikely that nitrite contributes significantly to iron oxidation by *Marinobacter subterrani*. Our results thus far suggest superoxide may play a role in *Marinobacter subterrani* mediated iron oxidation, but it has not yet been conclusively determined. Employing a technique that allows us to determine the importance of every gene in an organism under a defined growth condition, we were able to map the metabolism of *Marinobacter subterrani* when growing in rich and defined media. Given the harsh conditions of the mine, and the ability of *Marinobacter subterrani* to thrive there, knowledge of what contributes to survival and persistence is equally as important as understanding what is significant for growth. A modified version of this technique was developed, but instead of depending on growth to provide the resolution required to assign impact to genes, we instead used cell death; mimicking conditions in the mine from which *Marinobacter subterrani* was isolated. Results of the assay are being verified with mutants defective in the genes affecting persistence. By determining what genes contribute to the ability of cells to persist in this environment, we gain insight to the conditions the cells experience and how they cope with the difficulties of growing in the mine.

ACTIVITY 3: Biological control of White Nose Bat Syndrome

Description: Our approach for this proposal is focused on identifying inhibitory bacteria that live naturally in the Soudan Mine to utilize as biological control agents. Because these anti-fungal isolates are already adapted to living in the extreme environment of the mine, they may be good candidates for potentially controlling or preventing WNS ("Biocontrol"). We propose to survey the microbial population (existing strain library and new isolates) for strains that could potentially inhibit the WNS fungus. *Bacteria and fungi will be cultivated from hibernaculum areas and bat surfaces to identify "native" species pre-adapted to the mine environment. Isolates will be tested for the ability to inhibit the growth of multiple species of Geomyces fungi.*

Summary Budget Information for Activity 3: **ENRTF Budget: $ 271,182**

Amount Spent: $ 271,182
Balance: $ 0

Activity Completion Date:

Outcome	Completion Date	Budget
1. Sample collections from bat roosts, substrates and bats (near end of hibernation period)	July 2015	$45,197
2. Isolation and characterization of bacteria and fungi from bats and hibernaculum areas	July 2015	$45,197
3. Characterization of *Geomyces* species susceptibility and resistance profiles against clinical antifungal compounds.	July 2014	$45,197
4. Testing for presence of *Geomyces destructans* using RT-PCR	July 2016	$10,000
5. Testing of bacterial and fungal antagonists against library of *Geomyces* species	July 2016	$62,795
6. Fermentation and scale up of most promising antagonists.	July 2016	$62,796

Activity Status as of *(January 2014)*:

(1) Personnel: One research technician was hired (Mark Mulvahill) to assist with field collections, microbial isolations and development of the fungal bioassays to test for biocontrol agents against white nose bat syndrome. A postdoc, Dr. Yudi Rusman, will contribute part time effort towards identifying the active compounds produced by inhibitory strains. A graduate student, Josh Erickson, will also contribute part time effort towards testing some of the promising anti-fungal isolates already identified.

(2) Microbe isolations: The Salomon lab has been isolating and purifying bacteria and fungi from swabs of bat skin and other surfaces collected from the Soudan Mine. The bacterial isolates will be characterized by sequencing portions of the 16s rRNA genes and testing for antifungal activity against a panel of fungal strains including 4 isolates of *Geomyces* which are related to the WNS agent *Pseudogymnoascus destructans* (formerly known as *Geomyces destructans*).

(3) Fungal isolations: The Blanchette lab has been purifying and characterizing cultures of fungi from samples collected in November 2013. (see update (4) for Activity 1, above) These fungi will also be tested against the panel of *Geomyces* to identify the most inhibitory strains.

(4) Bioassay development: Methods are being developed and refined for testing both bacteria and fungi against *Geomyces* isolates. The slow growing nature and specific media preferences of the pathogenic fungi present a challenge for rapid testing of potential inhibitory isolates. We are testing a number of different methods to identify the best growth inhibition assays.

Activity Status as of *(July 2014)***:**

(1) Culture Collection of potential antagonists: We have focused our recent efforts on isolating and purifying bat-associated microbes. During the annual bat census with the DNR, we were able to obtain swab samples from approximately 25 hibernating bats and their associated substrates (roost areas). Each sample was diluted and plated onto several different solid media, and individual colonies of bacteria were isolated and purified over

the period of three months. A total of 53 pure isolates have been obtained so far, and additional slower growing organisms will continue to be isolated. An additional set of swabs from non-hibernating, roosting bats was obtained in July and these will be cultured in a similar manner. We anticipate having at least 100 isolates this year for testing against the Pd fungal pathogen.

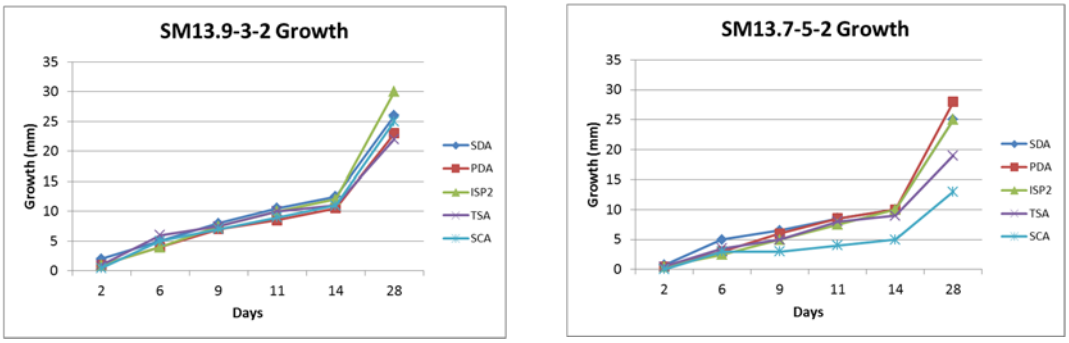


Figure 1. Comparison of growth rate of two strains of Pseudogymnoascus sp. fungi isolated from the Soudan Mine with five different media over four weeks (room temperature incubation). Growth is measured in mm from edge of initial mycelial plug.

(2) Screening and culturing of *Pseudogymnoascus* fungi: The fungus that causes white nose syndrome is of concern and its distribution in the mine is not known. Recently, this fungus had a name change from *Geomyces destructans* to *Pseudogymnoascus destructans*. We made a large number of isolations from areas of the mine where bats are located during our investigations and found several cultures that have been sequenced and identified as *Pseudogymnoascus.* We isolated this fungus from 5 levels in the Soudan mine. Initial identifications were made using sequences from the inter-transcribed spacer region of rDNA. The RNA polymerase II subunit (RPB2) RPB2 and translation elongation factor 1-alpha (TEF-1) gene regions were also sequenced and used for phylogenetic analysis. The analysis showed two different species are present within the mine, one isolated only from level 9 and the other species was isolated from levels 7, 10, 11 and 15. The *Pseudogymnoascus* isolates identified are not the same species as white nosed syndrome (*P. destructans*) but are very closely related. Similar isolates have been found in other mines and currently very little is known about the ecology of the closely related species in this complex and further studies are being completed.

We are characterizing the growth and susceptibility of these potential "proxy" species of *Pseudogymnoascus* with different media and various growth conditions. Initial studies suggest that they grow more rapidly than the Pd pathogen, with good visible growth after 5 days (figure 1) The next steps for characterizing the Soudan Pseudogymnoascus will involve testing their susceptibility to known anti-fungal compounds to compare the profile of Pd, which is published.

(3) Characterization of anti-fungal isolates: One of the *Streptomyces* strains obtained from the high copper area on level 10 was found to have potent anti-fungal activity and we isolated and identified two of the active metabolites as nocardamine and iturin. Due to our interest in the biosynthetic and metabolic capacities of this strain, we also obtained the full 7,167,846 base pair linear genome sequence using the Pacific Biosciences (PacBio) sequencing platform (see Activity 2 for details about this technology). The genome contains at least 16 gene clusters encoding secondary metabolites, and we are in the process of analyzing the data to predict what compounds could potentially be biosynthesized. We will also be testing this strain against the Pd pathogen as a potential antagonist.

Activity Status as of *(January 2015)***:**

1) Personnel: A new new postdoctoral researcher, Michael Wilson, was hired (starting January 2015) and two part time undergraduate students (Amanda Freiborg and Noora Hussain) were hired as technicians to work on this project.

2) Collection and Characterization of Bacteria and Fungi:
   a. We are continuing to isolate and characterize bacteria from samples collected during the past two field trips. Our current focus is on extracting DNA for genomic sequence analysis as well as biological testing of each strain. We will be testing each strain against a panel of bacterial and fungal pathogens to develop an activity "fingerprint" to help prioritize the most promising isolates and dereplicate potentially duplicate strains.
   b. Fungi have been isolated from 14 of the 16 levels of the Soudan mine from wood, "soil" and fungal material. To date we have identified approximately 170 different fungal taxa by isolating and sequencing DNA from these isolates. It appears the fungal assemblage in the mine is both unique and diverse. When comparing our isolates to those accessioned in The National Center for Biotechnology Information (NCBI) DNA database many of the taxa appeared to be specific to mine or cave environments and are not found commonly in other terrestrial ecosystems. The ecology of these of these organisms that live in these unusual environment is not well known and needs investigation. The diverse and growing list of fungi from the mine include many that do not match known species and appear to represent new taxa. One of these fungi, *Oligoporus* sp., has been found in many areas on six different levels of the Soudan mine and matches poorly with all other known species. We are further characterizing this fungus along with other possible new species by sequencing multiple gene regions which allows for a more accurate species designation. This fungus is proving to be a dominant decomposer of wood in the mine on many levels and causes a brown rot type of wood decay.

3) Development of *Pseudogymnoascus* bioassays:
We are still working on characterizing the potential proxy species of *Pseudogymnoascus* isolated from the Soudan Mine as part of a rapid screening system for testing possible antagonists. We have purchased a number of clinical anti-fungal compounds known to inhibit *P. destructans*, and will test these against the faster growing proxy species. We are exploring several different types of assays including contact-dependent and contact-independent methods.

Samples being taken from an area where suspected *Psuedogymnoascus sp*. are growing on a large area of the mine wall.

Activity Status as of *(July 2015)***:**

1.) Characterization of *Pseudogymnoascus* fungi in the Soudan Mine: Additional samples have been collected and are being processed where *Psuedogymnoascus* sp. have been found colonizing expansive areas on the walls of the mine. Sequencing four gene regions of *Psuedogymnoascus* species that have been isolated has been completed and the phylogeny of these isolates indicate two different species are present and these species are distinct from *P. destructans*.

2.) Biological control/inhibition of *Pseudogymnoascus destructans*: The Salomon lab has made significant progress in isolating bacteria from mine samples and screening all isolated Soudan microorganisms for their ability to stop *P. destrucans* growth. Since working with park staff to collect bat swabs and sediment samples from the mine in March 2015, we have isolated 114 Soudan bacteria and are working to isolate even more (Figure 1a). Some of these bacteria may be entirely new species, so we are currently focused on identifying our isolated bacteria and testing if any could be used to control *P. destructans*. Selection of fungal cultures

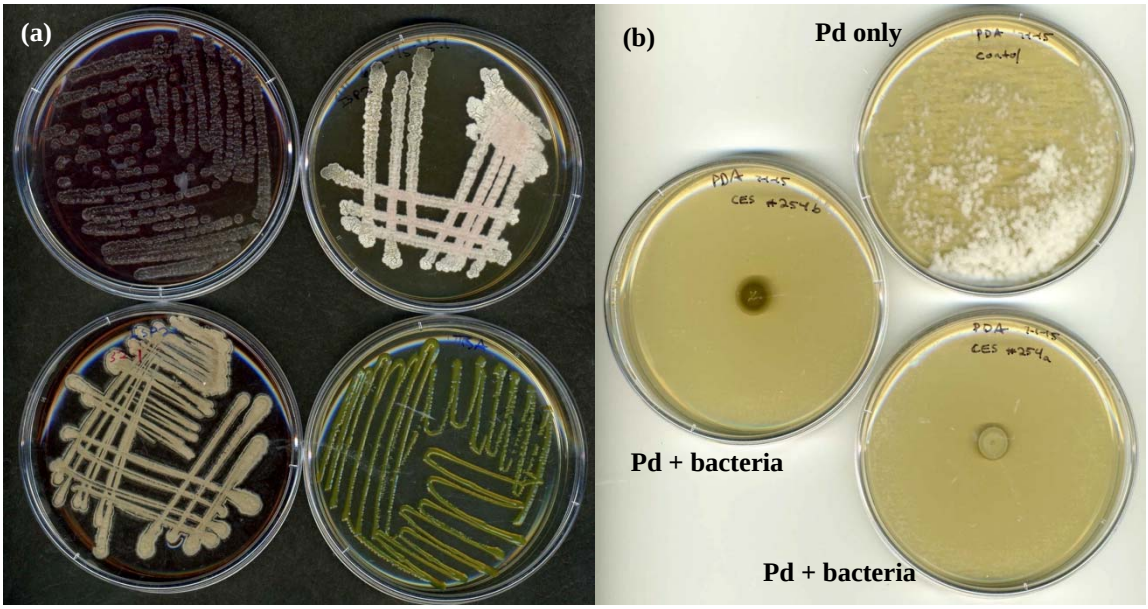


Figure 1 – (a) Examples of pure bacterial cultures that have been isolated from bats in Soudan mine. (b) Two bacterial antagonists that prevent *P. destructans* growth. No *P. destructans* grew on the plates with bacteria spots (3 weeks).

from the Blanchette group collection is also continuing for testing with the Salomon group working on *P. destructans* antagonism. Ninety five fungal cultures were selected and plated out for *P. destructans* antagonism studies.

Between our collection of Soudan bacteria and fungi (isolated by the Blanchette lab), we have eight promising bacterial and fungal strains that can significantly inhibit *P. destructans* growth in our lab experiments (Figure 1b). We are continuing to screen our Soudan mine collections, and we expect more biological control candidates to emerge in the coming months. These preliminary findings are encouraging, but much work remains to determine if these organisms can be deployed in safe and effective manner.

Activity Status as of ***(Jan 2016)*****:**

1) Fungal isolations and characterization: Approximately 140 fungal taxa have been isolated thus far from the Soudan mine. After isolation, DNA extraction and sequencing, each distinct taxa was identified and grown for the Salomon lab for further studies on biological control and inhibition studies of *P. destructans*. Characterization of the different fungal species that do not match described species is also continuing. This involves sequencing various gene regions and making phylogenetic comparisons to described species. In some cases, as with Ascomycetes, morphological descriptions can be made from hyphae and asexual spores that are normally abundantly produced. However, those species belonging to a different phylum, Basidiomycota, do not regularly produce asexual spores, so inducing a sporophore (or mushroom) which then produces spores

for morphological descriptions can be challenging. We are testing different methods to induce spore production in several of the unusual basidiomycete species isolated from the mine (figure 1). We have isolated at least 15 species of *Mortierella*, one of the most frequently isolated genera from the mine.

Figure 1. Spawn being grown to inoculate larger bags of substrate to induce fruiting bodies (needed for describing new taxa)of some undescribed basidiomycete species from the Soudan mine.

2) Characterization of non-pathogenic *Pseudogymnoascus* species from the Soudan Mine: We have fully characterized several non-pathogenic strains of *Pseudogymnoascus* fungi collected previously from the Soudan Mine. Our original goal was to utilize these faster growing and non-pathogenic strains to conduct all of our initial antagonism assays. However, the resistance and susceptibility profiles of these strains turned out to be highly different from those of an authentic culture of the pathogen (*P. destructans*) and are therefore less suitable as a "proxy" strain for testing. Since these species are very common throughout the Soudan Mine (and other caves and mines in other parts of the country), we decided to characterize their growth rates on different medias and at various temperatures as well as their ability to utilize individual carbon sources to better understand what they "eat" in the cave environment. Our results suggest that these non-pathogenic *Pseudogymnoascus* species are generalists, and able to utilize most sources of nutrients. This is in contrast to *P. destructans*, which was only able to consume a very small number of the same nutrients tested. This work is being written into a scientific manuscript and should be submitted for publication this Spring.

3) Testing of Soudan fungal and bacterial isolates against Pd: We are continuing to test pure cultures of microbes against Pd in various solid and liquid based assays. The solid media overlay assay is providing the most consistent results. Of 121 fungal isolates tested, eight display contact-independent inhibition, while 19 exhibit contact-dependent inhibition. Among the bacterial isolates tested, 36 strains inhibited Pd growth. We are now sequencing the 16s rRNA genes from these strains to identify them and determine any patterns of activity versus taxonomy. Next steps will also include testing their ability to grow on substrates common in hibernacula areas. We have also recently gained access to bat carcasses for initial *ex-vivo* experiments (antagonist growth and inhibition).

Amendment request (01/01/16)
The following changes were made to the budget:
Activity 2: The budget for supplies (microbiology, molecular biology, general lab) was increased by $6000 and the personnel costs were reduced by the same amount. These funds are needed to provide the supplies and equipment required for ongoing experiments.

Activity 3: The budgets for supplies (microbiology, molecular biology and general) was increased by $2000 due to additional costs in these areas for the project. The budget for sequencing was increased by $1770 due to the larger number of samples submitted for DNA sequencing analysis. The budget for printing was also increased by $1500 to provide funds for additional publication of open access manuscripts (for dissemination). All of these increases (total= $5270) were offset by decreases in travel expenses (reduced by $3000) and mine usage costs (reduced by $2270).

Activity Status as of *(July 2016)***:**

Fungal isolations and characterization: In addition to sequencing additional fungal isolates from the Soudan mine, a manuscript on the fungi found the Soudan mine is being written. This paper characterizes the fungal diversity (including over 140 different fungal taxa) in the mine by genetic sequencing across all levels in the mine and reveals many undescribed species that are present. Comparisons are also made between the fungal diversity between the Soudan mine and other underground mines and caves where similar research has been done.

Characterization of non-pathogenic *Pseudogymnoascus* species: We have continued to characterize and measure the differences between *P. destructans* and other non-pathogenic *Pseudogymnoascus* species found in the Soudan Iron Mine. Once we measured their growth rates on various nutrient sources, we utilized a visualization method to analyze the data known as a "heat map". This map (Figure 1) indicates the relative amount of growth on a given carbon source, and provides an easy way to compare the efficiency of nutrient usage between various strains for the 95 different conditions. This analysis suggests that Pd uses fewer kinds of nutrients that non-pathogenic members of the same species, although it's critical to note that the ecological relevance of many of the tested nutrients is unknown. We also determined that if Pd is given enough time to grow, it can utilize some of the nutrients available with high efficiency (comparable to non-pathogenic *Pseudogymnoascus* species).

Screening of bacterial and fungal isolates for inhibition of Pd: We have continued to isolate new strains of bacteria and fungi from the Soudan Mine, and

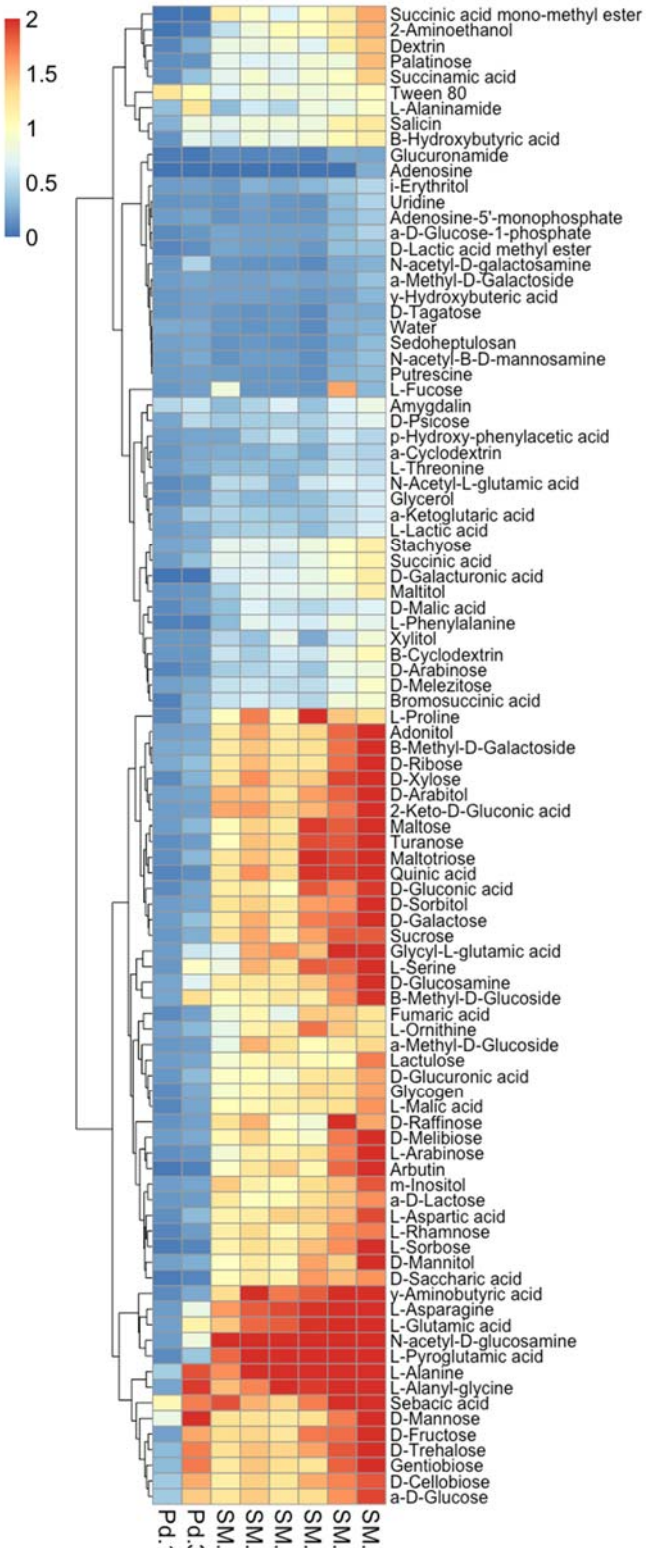


Figure 1. Heatmap of *Pseudogymnoascus* spp. substrate utilization at 15°C. Rows correspond to average growth (OD_{750}) in wells of Biolog FF phenotype microarrays, while columns correspond to different *Pseudogymnoascus* spp. SM *Pseudogymnoascus* isolates were evaluated after 7 days of growth, while *P. destructans* was evaluated after 14 and 30 days of growth.

continued to test each strain for inhibition of Pd. Bacteria were all tested using a solid media overlay assay and fungi were tested using a side by side plug assay. A total of 60 out of 262 pure bacterial isolates collected between 2009-2015 were found to be inhibitory. A total of 32 out of 121 fungal isolates exhibited inhibitory activity against Pd.

Phylogenetic characterization of active strains:
Diagnostic gene fragments were sequenced from inhibitory bacterial and fungal isolates to provide phylogenetic information. This information is useful because it allows us to identify patterns of activity among different kinds of microbes. For example, among fungi, the most common inhibitory genera were members of *Mortierella* and *Pseudogymnoascus* species. Among bacteria, *Streptomyces* and *Bacillus* species were the most common inhibitors. We also determined that the most active strains were isolated from bat swabs versus substrate samples, although this may also be indicative of the larger number of bat samples overall.

Additional activities of microbial strains isolated from the Soudan Mine:
In addition to using the microbial library isolated from the Soudan Mine to identify potential inhibitors of the bat pathogen P. destructans, we have also been screening these strains for activities against other human and agricultural infectious disease pathogens. Some strains have exhibited selective activity against human fungal pathogens such as Candida albicans and Cryptococcus neoformans, while others display inhibition of agricultural fungi such as *Alternaria*, *Verticillium* and *Pithium* plant pathogens. Surprisingly, there is very little overlap among strains that inhibit *P. destructans* versus these additional fungal pathogens. We will continue to test and prioritize the most potent strains for these additional targets and will begin scale up cultures, extractions and purifications of active components.

Final Report Summary:
Our primary goals for this project were to develop a library of bacterial and fungal species from the Soudan Iron Mine and identify the most promising strains for further development as a biological control agent for White Nose Bat Syndrome. A total of 1200 fungal strains representing 140 unique taxa and 262 bacterial strains were isolated during this project. All of the bacterial isolates and 121 representative fungal isolates were tested for their ability to inhibit the growth of *P. destructans* on solid media. Bacterial candidates were tested using an overlay assay and fungi were tested using a side by side plug assay. A similar percentage of tested microbial strains exhibited inhibitory activity (60/262 bacteria and 32/121 fungi). Among the bacterial inhibitors, most strains appeared to produce diffusible inhibitory substances. Comparisons of the active fungi suggests that some species may produce diffusible inhibitors while others may only be active during direct contact of mycelia with *P. destructans*. These differences will be important as we move forward with prioritizing various strains for specific purposes in biological control.

We have also determined the phylogenetic identity of the active isolates and have a better of understanding of which genera are most likely to be isolated from various surfaces (substrates, bat swabs, etc.) using our specific isolation methods. We also know which genera are more likely to be inhibitory, which may aid future endeavors to identify additional inhibitory strains. Additional accomplishments for this project include the development of robust liquid assays for testing of active strains and extracts. As we move into phase II of this project to refine the list of best inhibitors, it's critical that we have efficient methods for screening and testing fractions during purification. We have begun to scale up cultures of all active strains and will test extracts using this assay to identify the active components.

Studies of fungi closely related to *P. destructans* provided valuable information about their differences and similarities in nutrient use, antifungal drug susceptibility and resistance. We learned that free living, non-pathogenic *Pseudogymnoascus* fungi living in the Soudan Mine are very flexible in terms of what kinds of carbon they can utilize and can also grow at a range of temperatures. In contrast, P. destructans can utilize significantly

fewer nutrient sources, and takes a much longer amount of time to utilize. Another important difference between these strains is that P. destructans is more sensitive (susceptible) to clinical anti-fungal agents in comparison to free-living non pathogenic species in the same genus. These results suggest that *P. destructans* may not be able to compete effectively in the general environment when not associated with a bat host. However, additional studies need to be done to further elucidate the role and prevalence of *P. destructans* in complex microbial communities in the environment as a persistent reservoir of disease.

Overall, we have accomplished our goals of identifying potent inhibitors of P. destructans from a single hibernaculum. With 92 active strains, we will now move into the next phase of determining which strains can grow on cave/mine substrates and continue to exhibit inhibitory activity. This large number of active isolates will also allow us to consider developing combinations of multiple strains for different applications such as hibernaculum surfaces versus bats.

Overall Project Outcomes and Results

The Soudan Iron Mine in Minnesota provides direct access to microbes with special adaptations that can be harnessed for biotechnology. We conducted research to harness these microbes to approach some of the most critical environmental challenges in Minnesota:

Metal Bioremediation: Our goal was to explore fungi for bioremediation of metals in mine effluent. We know that fungi thrive in heavily contaminated areas of the mine containing copper, cobalt, zinc, nickel and mercury. We screened 60 fungi and discovered that several species accumulate metals within their living biomass. These findings confirmed that: (1) Soudan fungi remove metals from the water; (2) the amount of metal removed from water was similar between natural and lab specimens; and (3) metal binding can be reversed. These results provide essential data for development of these fungi as sorbent materials or living bioreactor filters.

Electrosynthesis Project: We characterized and developed methods for improving the bacterium *Marinobacter subterrani*, which we isolated from the Soudan Mine. *M. subterrani* is a model for the study of metal precipitation – a process that remains poorly understood. The organism has applications in bioremediation through production of useful iron oxides, and in improving electrodes to catalyze new reactions.

White Nose Bat Syndrome Biological Control:

White Nose Syndrome is a devastating bat disease causing catastrophic economic and biodiversity losses throughout the US. As part of a biocontrol strategy, we collected and screened new microbes from the Soudan Mine. It total, 32/121 fungal strains and 60/262 bacterial isolates inhibited growth of the White Nose pathogen *P. destructans*. Analysis of active strains provided us with a picture of which types of inhibitory microbes may be found in various mine locations, which may help future screening and discovery efforts. We are poised to move forward into phase II, which will involve testing the ability of each active strain to inhibit *P. destructans* on specific substrates both in the lab and in the environment.

V. 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 an interactive display at the Soudan Mine Visitor Center that will be developed as part of our current ENRTF project. We are also pursuing the

development of a satellite kiosk at the Minnesota Science Museum in St. Paul which will enable us to disseminate our research to an even greater audience.

Status as of *(January 2014)*:

Status as of *(July 2014)***:**

Status as of *(January 2015)***:**
Lectures/seminars

1. Ely Naturalists, "Harnessing Soudan Mine Microbes: Bioremediation, Bioenergy and Biocontrol" July 2014 (Salomon)
2. Department of Biology, St. Scholastica University, "New approaches for infectious disease", October 2014 (Salomon)
3. The Scientist Webinar, "Perspectives on Natural Products and Drug Discovery" Nov 4, 2014 http://www.the-scientist.com/?articles.view/articleNo/41018/title/Perspectives-on-Natural-Products-and-Drug-Discovery/ (Salomon)
4. Presentation at the International Society for Subsurface Microbiology: 'Subsurface Microbiology at the Microbe-Electrode Interface' http://www.2014issm.com/support/issm2014_conference_program.pdf (Gralnick)

Articles

1. "Will Soudan Mine research save our bats?" The Timberjay, 07/16/2014
2. Big Picture Science Radio Interview: http://radio.seti.org/blog/2014/12/big-picture-science-shocking-ideas-jeff-gralnick-microbe-power-plants/ (Gralnick)

Status as of *(July 2015)***:**
Lectures/seminars

1. Deep Carbon Observatory Deep Life Meeting, "Metagenomes of native and electrode-enriched microbial communities from the Soudan Iron Mine" May 2015 (Badalamenti)
2. Pacific Biosicences User Group Meeting, "Deep sequencing in the deep subsurface: De novo metagenomic assembly to recover complete genomes from the Soudan Iron Mine" July 2015 (Badalamenti)
3. TEDx UMN "Nature's Microbial Toolbox: Hope for Bats and Human Health" http://tinyurl.com/SalomonTEDX2015 May 2015 (Salomon)

Status as of *(Jan 2016)***:**
Lectures/seminars

1. International Society for Microbial Electrochemistry and Technology (ISMET) Meeting, "Capturing complete genomes of novel halophilic metal reducers from the deep subsurface: Short and long read metagenomics of Soudan Mine communities enriched on electrodes" Tempe, AZ, October 2, 2015 (Badalamenti)
2. Carleton College Biology Department Seminar, Northfield, MN, "Unlocking genomic secrets of the unseen microbial majority" October 26, 2015 (Badalamenti)

Conference poster

1. Mycological Society of America Meeting, "Subterranean Fungal Diversity in a Minnesota Iron Mine", July 2015. (Held, Salomon and Blanchette)

Status as of *(July 2016)***:**
Publication

1. Badalementi, J.P., Z.M. Summers, C.H. Chan, J.A. Gralnick and D.R. Bond. 2016. Isolation and genomic characterization of '*Desulfuromonas soudanensis* WTL', a metal- and electrode-respiring bacterium from anoxic deep subsurface brine. Front Microbiol. 7:913. doi:10.3389/fmicb.2016.00913
2. Badalamenti, JP, Erickson, JD, Salomon, CE. Complete genome of *Streptomyces albus* SM254, a potent antagonist of the White Nose Bat Syndrome pathogen *Pseudogymnoascus destructans*. *Genome Announcements*, 20164(2):e00290-16. doi:10.1128/ genomeA.00290-16.

Presentations

1. Biogeochemistry and Redox Transformations of Iron, Telluride, CO (Gralnick)
2. Henrici Society for Microbiology, Minneapolis, MN (Gralnick)
3. Screening and identification of microbial antagonists for development of biological control agents for White Nose Syndrome, National White Nose Syndrome Workshop, Denver, CO (Salomon)

Final Report Summary

Information, discoveries, approaches and questions from our project have been used and disseminated in a number of different ways: Presentations about individual projects have been given to school groups, college students, local community groups and at professional scientific conferences. Several components of this project were completed and shared as peer-reviewed scientific manuscripts. Some of the fundamental scientific discoveries have been used to further develop and expand new ideas that were not a part of the original research plan. These new ideas and hypotheses have been incorporated into new grant proposals, resulting in successful new funding at both the state and federal levels (including several new LCCMR proposals that build directly on initial research accomplished in this period as Phase II projects). Some additional uses include the screening of these new, diverse microbial libraries against other targets, including human infectious disease pathogens. For example, several of the bacterial strains that showed no activity against the fungal bat pathogen did exhibit inhibition of human yeast pathogens. These strains will be further studied to purify and identify the active components for potential development as human therapeutics.

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget (3 years total):

Budget category	Amount	Explanation
Personnel:		
Brandy Toner , Activity 1	$7,916	2% FTE , requesting 1 week of summer salary per year for 3 years (74% salary, 26% fringe)
2 Graduate Research Students (Microbiology) Activity 2	~~$257,646~~ 251,646	50% FTE, (53% salary, 47% fringe)
1 Graduate Research Student (Soil,Water,Clim) Activity 1	$116,845	50% FTE, (48% salary, 52% fringe)
1 technician (Plant Path) Activity 3	$113,388	50% FTE (72% salary, 28% fringe)
1 undergraduate student worker (Plant Path.) Activity 3	$15,435	25% FTE (100% salary)
1 Postdoctoral Research Associate (CDD) Activity 3 (82% salary, 18% fringe)	$151,200	100% FTE (82% salary, 18% fringe)
Equipment/Tools/Supplies:		
Activity 1		
Microbiology supplies	$24,000	Sterile sampling equipment, collection materials, growth media, culture reagents, sample tubes, pipette tips, plasticware,

		consumables) for two scientists x 3 years.
General lab supplies	$24,000	glassware, vials, bottles, gloves, filters, filter cartridges for purified water ($1000 per year), pH electrodes for field and lab measurements (4 x $300, oxygen electrodes (2 x $200), chemical reagents and buffers. For two scientists x 3 years.
Analytical chemistry analysis expenses & services	$12,000	UMN Geochemistry core facility charges ($50-75 per sample for metal analysis for ~85 samples over 3 years)
Activity 2		
Microbiology supplies	~~$20,000~~ $22,000	Sterile sampling equipment, collection materials, growth media, culture reagents, sample tubes, pipette tips, plasticware, consumables) for two scientists x 3 years.
Molecular biology supplies	~~$20,000~~ $22,000	PCR reagents, enzymes, DNA extraction kits, plasmid and gel purification kits ($300 ea, ~12/yr), electrophoresis supplies (agarose, ladders, stains). For two scientists x 3 years
General lab supplies	~~$20,000~~ $22,000	glassware, vials, bottles, gloves, filters, filter cartridges for purified water ($1000 per year), chemical reagents and buffers, for two scientists x 3 years
Activity 3		
Microbiology supplies for sampling and lab assays	~~$12,000~~ $13000	Sterile sampling equipment, collection materials, growth media, culture reagents, sample tubes, pipette tips, plasticware, consumables
Molecular biology supplies	~~$8,000~~ $9,000	PCR reagents, enzymes, DNA extraction kits, cloning and gel purification kits ($300 ea, ~6/yr), electrophoresis supplies (agarose, ladders, stains).
General lab supplies	$10,000	glassware, vials, bottles, gloves, filters, chemical reagents and buffers
Sequencing for phylogenic analysis of microbial isolates	~~$6,300~~ $8,070	Sequencing fees (UMN core facility sample charge) for

		phylogenetic analysis of bacteria and fungi (all activities) $3.50-$7 per sample x ~500 samples over 3 years
Microscopy	$2,500	Microscope hourly charges at UMN Imaging Core Facility (scanning electron, light, confocal)($37-45 per hour, est 15 hrs/yr x 3 yrs)
Publication fees	~~$1,500~~ $3,000	Page and color charges for publishing scientific manuscripts, publication fees for open access journals
Travel: In-state round trip travel between St. Paul and Soudan Mine Park	~~$9,500~~ $6,500	Travel, room/board for 4-8 researchers. $250-500 lodging per trip, $250 for food/gas, for a total of $500-750 per trip x ~ 5 trips per year.
Soudan mine usage	~~$5,770~~ $3,500	Hoist trip charges ($31.74) x 4 per average trip (~$130). An 8 hour sampling trip will require 8 hours of park staff (Mine Hoist and maintenance personnel at $35 per hour for a total of $280) Total charges estimated to be $1923 per year x 3 years
TOTAL ENVIRONMENT AND NATURAL RESOURCES TRUST FUND REQUEST =	**$838,000**	

Explanation of Use of Classified Staff: n/a

Explanation of Capital Expenditures Greater Than $3,500: none

Number of Full-time Equivalent (FTE) funded with this ENRTF appropriation: 9.81

Number of Full-time Equivalent (FTE) estimated to be funded through contracts with this ENRTF appropriation: none

B. Other Funds:

Source of Funds	$ Amount Proposed	$ Amount Spent	Use of Other Funds
Non-state			
1 month of salaries + fringe for each investigator (Salomon, Gralnick, Toner, Bond and Blanchette) annually x 3 years	$161,691	$0	In-kind support of effort for PIs from each department (Center for Drug Design, Microbiology/Biotechnology

			Institute, Soil, Water, Climate and Plant Pathology)
State			
	$	$	
TOTAL OTHER FUNDS:	**$161,691**	**$0**	

Add or remove rows as needed

VII. PROJECT STRATEGY:

A. Project Partners: Additional partners include **Jim Essig** (DNR Park Manager of Soudan Mine State Park) who will help coordinate research activities and **Dr. David Blehert** (USGS, WI) who will provide advice and future assistance with testing microbial isolates against the WNS fungus *Geomyces destructans*.

B. Project Impact and Long-term Strategy:

Our long term goal is to develop practical and valuable applications using the unique microbes that we have identified in the Soudan Mine. The metabolic capabilities of these microbes have real potential for applied and novel biotechnologies. The proposed work will contribute essential information towards our understanding of the roles and capacities of fungi in bioremediation, the development of electrofuels, and the potential for biological control of the fungal pathogen involved in White Nose bat Syndrome. In addition to the expected knowledge and increased understanding in these areas, we also expect to make progress towards tangible products harnessing these biotechnologies such as a bio-filter to remove dissolved metals, electrofuels, and biological control strains of bacteria that inhibit *Geomyces destructans*.

C. Spending History:

Funding Source	**M.L. 2007 or FY08**	**M.L. 2008 or FY09**	**M.L. 2009 or FY10**	**M.L. 2010 or FY11**	**M.L. 2011 or FY12-13**
ENRTF				545,451 Subd. 3(f)	

VIII. ACQUISITION/RESTORATION LIST:

IX. MAP(S): graphic attached

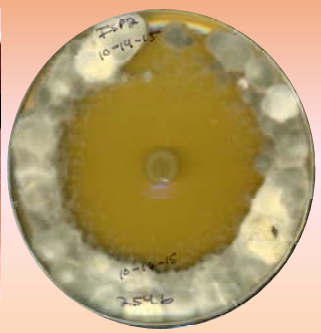
X. RESEARCH ADDENDUM: Due to the patent protection being sought for the technology being developed with this project, in consultation with LCCMR staff it was determined that ENRTF research project requirements for a research addendum and peer review could be satisfied through an internal University of Minnesota peer review process that meets standard University of Minnesota protocols for a patent seeking situation. As a result the research addendum produced for the project and potentially revised through the peer review process will remain confidential while patent protection is pending.

XI. REPORTING REQUIREMENTS:

Periodic work plan status update reports will be submitted not later than [January 2014], [July 2014], [January 2015], [July 2015], [Jan 2016] and [July 2016]. A final report and associated products will be submitted between Jan 30 and August 15, 2017 as requested by the LCCMR.

Harnessing Soudan Mine Microbes: Bioremediation, Bioenergy and Biocontrol

1. Biological control of white nose bat syndrome

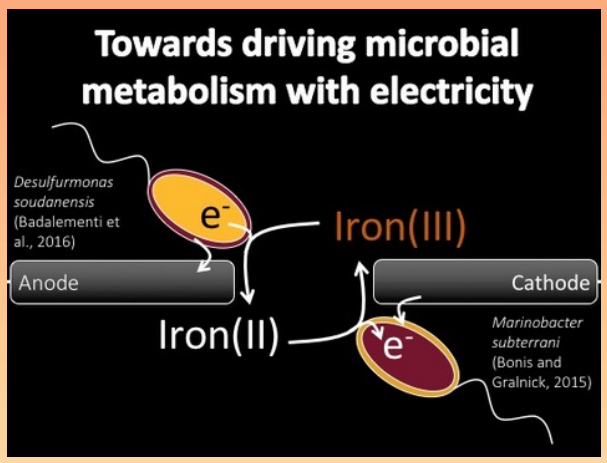


62 bacterial strains and 30 fungal isolates were identified from the Soudan Mine that inhibit the growth of the bat fungal pathogen *P. destructans*. We also developed plans for testing promising strains on relevant substrates and are poised to move into phase II testing of the best inhibitors.

2. Bioremediation of toxic metals

Fungal strains were isolated and identified growing in areas with high heavy metal accumulations (top). Sixty fungi were screened for growth media amended with heavy metal and several were found to tolerate high concentrations of heavy metal (bottom). These isolates were further tested in bioreactors to remove metals from mine waste water.

3. Bioenergy and microbial electrosynthesis



Bacteria isolated from the Soudan Iron Mine with the capacity to eat and breathe iron can also be used to generate and possibly use electricity.

Harnessing Soudan Mine Microbes: Bioremediation, Bioenergy and Biocontrol

Final Attachment A: Budget Detail for M.L. 2013 Environment and Natural Resources Trust Fund Projects

Project Title: *Harnessing Soudan Mine Microbes: Bioremediation, Bioenergy and Biocontrol*
Legal Citation: M.L. 2013, Chp. 52, Sec. 2, Subd. 03f
Project Manager: *Christine E. Salomon*
M.L. 2013 ENRTF Appropriation: $ *838,000*
Project Length and Completion Date: 3 years (completion and final report Dec 2016)
Date of Update: 02/25/16

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET (3 years total)	Activity 1 Budget	Amount Spent	Balance	Activity 2 Budget	Revised Activity 2	Amount Spent	Balance	Activity 3 Budget	Revised Activity 3	Amount Spent	Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	***Result 1. Removal of metals from***			***Microbes and electricity***				***Biological control of White Nose Bat***					
Personnel (Wages and Benefits) overall	189,172	198,348	-9,176	~~257,646~~	251,646	250,561	7,085	215,612	215,612	234,428	-18,816	662,430	-20,907
Brandy Toner, Co-PI, 2% effort (74% salary, 26% fringe) (Estimated $7916)		8,543											
TBN Grad RA (Soil, Water, Climate), 50% effort (48% salary, 52% fringe) (Est. $116,845)		124,184											
Robert Blanchette, Co-PI, 10% effort, no salary requested													
TBN technician (Plant Pathology), 50% effort (72% salary, 28% fringe) (est. $113,388)		61,442								61,442			
TBN undergraduate student worker (Plant Pathology), 25% effort (100% salary) (est.$ 15,435)		4,179								4,179			
Jeff Gralnick, Co-PI, 10% effort, no salary requested													
Daniel Bond, Co-PI, 10% effort, no salary requested													
(2) TBN Grad RA (Microbiology), 50% effort, (53% salary, 47% fringe) (Est. $257,646)						$250,561							
Christine Salomon, PI, 10% effort, no salary requested													
TBN, Postdoctoral associate, 100% effort, 82% salary, 18% fringe (Est. $151,200)										168,807			
Equipment/Tools/Supplies													
Activity 1: Microbiology supplies: Sterile sampling equipment, collection materials, growth media, culture reagents, sample tubes, pipette tips, plasticware, consumables) For two Grad RA scientists x 3 years	24,000	4826	19,174									24,000	19,174
Activity 1. General lab supplies: glassware, vials, bottles, gloves, filters, filter cartridges for purified water ($1000 per year), pH electrodes for field and lab measurements (4 x $300, oxygen electrodes (2 x $200), chemical reagents and buffers. For two Grad RA scientists x 3 years.	24,000	14134	9,866									24,000	9,866
Activity 1: Analytical chemistry analysis (Geochem core facility fee for service)	12,000	10129.2	1,871									12,000	1,871
Activity 2: Microbiology supplies: Sterile sampling equipment, collection materials, growth media, culture reagents, sample tubes, pipette tips, plasticware, consumables) for two scientists x 3 years.				~~20,000~~	22,000	21798	202					20,000	202
Activity 2. Molecular biology supplies: PCR reagents, enzymes, DNA extraction kits, plasmid and gel purification kits ($300 ea, ~12/yr), electrophoresis supplies (agarose, ladders, stains). For two scientists x 3 years				~~20,000~~	22,000	21798	202					20,000	202
Activity 2. General lab supplies: glassware, vials, bottles, gloves, filters, filter cartridges for purified water ($1000 per year), chemical reagents and buffers, for two scientists x 3 years				~~20,000~~	22,000	21798	202					20,000	202

Activity 3: Microbiology and assay supplies: Sterile sampling equipment, collection materials, growth media, culture reagents, sample tubes, pipette tips, plasticware, consumables								~~12,000~~	13,000	4826	8,174	12,000	8,174
Activity 3. Molecular biology supplies: PCR reagents, enzymes, DNA extraction kits, cloning and gel purification kits ($300 ea, ~6/yr), electrophoresis supplies (agarose, ladders, stains).								~~8,000~~	9,000	4826	4,174	8,000	4,174
Activity 3. General lab supplies: glassware, vials, bottles, gloves, filters, chemical reagents and buffers								10,000	10,000	34725	-24,725	10,000	-24,725
Other Direct Costs											0		
Sequencing fees (UMN core facility sample charge) for phylogenetic analysis of bacteria and fungi (all activities) $3.50-$7 per sample x ~500 samples over 3 years, all activities								~~6,300~~	8,070	12195	-4,125	6,300	-4,125
Microscope hourly charges at UMN Imaging Core Facility (scanning electron, light, confocal)($37-45 per hour, est 15 hrs/yr x 1.5 yrs) All activities								2,500	2,500	1712	788	2,500	788
Printing publication and color page fees to publish work in scientific manuscripts								~~1,500~~	3,000	2154	846	1,500	846
Travel expenses in Minnesota In-state round trip travel between St. Paul and Soudan Mine Park: room/board for 4-8 researchers, mileage, plus mine hoist charges, est 5-6 trips/yr (1-3 days each trip) for 3 years. Expense base on U of M employee expense reimbursement plan.								~~9,500~~	6,500	5741	759	9,500	759
Soudan mine usage costs Hoist trip charges ($31.74) x 4 per average trip (~$130). An 8 hour sampling trip will require 8 hours of park staff (Mine Hoist and maintenance personnel at $35 per hour for a total of $280) Total charges estimated to be $1923 per year x 3 years								~~5,770~~	3,500	0	3,500	5,770	3,500
COLUMN TOTAL	$249,172	$227,437	$21,735	$317,646	$317,646	$315,954	$1,692	$271,182	$271,182	$300,608	-$29,426	$838,000	$0