M.L. 2016, Chp. 186, Sec. 2, Subd. 04o Project Abstract
For the Period Ending June 30, 2019

PROJECT TITLE: Reducing salt and metal removal costs with microbes
PROJECT MANAGER: Daniel R. Bond
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2016, Chp. 186, Sec. 2, Subd. 04o

APPROPRIATION AMOUNT: $596,000
AMOUNT SPENT: $ 589,037
AMOUNT REMAINING: $6,963

Sound bite of Project Outcomes and Results

New bacteria and fungi were discovered residing in the Soudan Iron Mine and other contaminated areas of Minnesota that demonstrate an ability to self-power salt-removing reactors or bind toxic metals in passive filtration devices.

Overall Project Outcome and Results

Many Minnesota waters are contaminated with salts and metals. Removing these contaminants can be more difficult than removal of compounds such as oils or pharmaceuticals which can be destroyed by bacteria or heat treatment. Metals and salts must be physically bound, or made to pass through a specific membrane to clean the water, making such treatments expensive and energy intensive. However, technologies have been proposed that use microorganisms as the power source to drive salts across membranes, or as binding agents to remove metals, significantly reducing the cost and complexity of treatment. Before such technologies can even be imagined at scale, naturally-occurring microorganisms that are tolerant of harsh conditions and able to power removal of salts from water must be made available. A key goal of this project was to discover such organisms, subject them to the stresses of life under the conditions, and understand what could limit implementation of these remediation strategies. After surveying a number of contaminated sites in Minnesota, we focused specifically on the power-generating abilities of bacteria related to the genus *Geobacter*, the salt-tolerance abilities of bacteria related to the genus *Marinobacter*, and the metal transforming abilities of fungal *Armilleria* and *Periconia* genera. We verified that these organisms can grow in high salt conditions, power model salt-removal reactors, and in some cases remove multiple metals from solution. In the case of salt removal, we showed that many of the operating conditions proposed, such as cycling of the cell voltage or operation at low redox potential, can be harmful to cells and will need to be addressed before the technology can be successful, as will issues related to high calcium content of some Minnesota waters. In contrast, because the use of fungi for metal removal does not require as much equipment or electrochemical control, scaling of this approach using organisms obtained via this project is deemed much more feasible.

Project Results Use and Dissemination

Our primary scientific dissemination activities are manuscripts crediting this project, two of which are under revision or submission and not available online at the time of this report. We presented our results at the 2nd Geobiology Society Conference in Banff, Canada, in June 2019 in the poster section titled as "Remediation of Metals by Mn-Oxidizing Fungi in Minnesota Soudan Iron Mine". Other examples of local exposure include also the Mycological Society of America 2019 Annual Meeting in Minneapolis, MN, in August 2019. In October, the research results will be presented at the Society for Mining Engineers conference in Minneapolis, MN.

As part of this project we conducted outreach activities to show the potential offered by bacteria powering salt-removal devices. Some examples of outreach during this project include: three 'Market Science' events, bringing demonstration devices to farmer's markets in the Twin Cities area, three events as part of the Bell Museum's 3rd and 4th-grade science camps where students constructed microbial powered devices and meet scientists in our laboratory, assisting two local Lego League teams who were incorporating microbial power into their demonstration projects and providing materials for their devices (one group progressed to the State competition), hosting a short workshop training graduate students in construction of microbial electrochemical devices, participation in the MN clean water summit and the American Society for Microbiology science outreach series. Our other stated goal was to facilitate group meetings with other collaborators and interested parties so this work could expand or continue. Due to these collaborations, work initiated in this project in terms of searching for new organisms from metal-impacted environments will be able to continue in a 5-year NSF-funded project to be based in the Soudan Mine, fulfilling a key goal described in our Long Term Strategy. We have also applied for new support from other state-based programs (such as MNDrive) to support the scale-up of new technologies for bioremediation.

We will continue to share these results, including demonstration experiments about bioremediation strategy use the type 1 bioreactor to general audiences on August 22nd, 2019 at the Minnesota State Fair with Market Science. Further, these type 1 bioreactors will now be regularly prepared for Sound Underground Mine State Park science tours of the mine. Mine tour guides will demonstrate these at the mine, and the cultures have been shared for observation under a microscope in collaboration with State Park staff.

Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2016 Work Plan Final Report

Date of Report: 8/14/2019

Final Report

Date of Work Plan Approval:

Project Completion Date: June 30, 2019

PROJECT TITLE: Reducing salt and metal removal costs with microbes

Project Manager: Daniel R. Bond

Organization: University of Minnesota Twin Cities

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Location: Statewide

Total ENRTF Project Budget:	**ENRTF Appropriation:**	**$596,000**
	Amount Spent:	**$589,037**
	Balance:	**$6,963**

Legal Citation: M.L. 2016, Chp. 186, Sec. 2, Subd. 04o

Appropriation Language: $596,000 the second year is from the trust fund to the Board of Regents of the University of Minnesota to continue to research the potential of recently discovered microbes from Soudan Iron Mine in northern Minnesota for removal of salts and metals from ground and surface water resources. This appropriation is subject to Minnesota Statutes, section 116P.10. This appropriation is available until June 30, 2019, by which time the project must be completed and final products delivered.

I. PROJECT TITLE: Reducing salt and metal removal costs with microbes

II. PROJECT STATEMENT:

The removal of metals and salts from water remains a serious and costly issue affecting industries as diverse as mining, oil/gas recovery, and production of domestic drinking water. **Our research aims to enable new technologies that can use microorganisms for desalination and metal recovery, with a focus on passive or self-powered processes.** The Soudan Iron Mine contains exploratory boreholes where water saltier than seawater and high in heavy metals contains unique organisms able to thrive under extreme conditions. Our previous research revealed that some of these microbes can generate electricity, while others can remove metals from contaminated waters though direct precipitation and sorption. These discoveries could power many biological water remediation technologies. The first, termed 'microbial desalination cells', harnesses bacteria to create a 'push' of charged electrons to help 'pull' charged salts across membranes. Conditions in these devices can also aid collection and recovery of heavy metals such as Cu and Co by binding to electrodes. The second technology, 'metal removal reactors', relies upon fungi thriving in areas heavily contaminated with metals such as copper, cobalt, iron and manganese, that precipitate metal-containing solids and create high surface-area sorption sites. If maintained properly, bioreactors containing these fungi can efficiently attenuate the metal contaminants within the system, effectively removing them from water. Together, we propose these biological agents can drive bacterial-powered salt removal reactors that discharge into fungal bio-filters to treat contaminated effluents in areas of high salt or metal contamination. **These microorganisms will be able to address applications specific to the chemistry of Minnesota, where sulfates, chlorides, metals and other salts can be present in stormwater and industrial effluents.**

Our overall goal is to deliver microbes from extreme environments that are highly salt- and metal-resistant, yet still able to catalyze electrical reactions. The molecular basis for their high performance will be investigated, so we can track these organisms in functioning reactors and discover genetic traits that make an inoculum useful throughout the industry. These bacteria will be used to power model microbial desalination reactors under harsh conditions to reduce the overall cost of salt removal from waters. This project will also show how novel fungi discovered in the most contaminated areas of mines can remove metals that would normally clog desalination membranes and create environmental toxicity issues

III. OVERALL PROJECT STATUS UPDATES:

Project Status as of *January 1, 2017***:** Our first objectives for both projects required multiple expeditions to the Soudan mine, to seed enrichments of novel bacteria and fungi. As detailed in each project's section, many cultures were obtained and key goals were met.

Project Status as of *July 1, 2017***:** In both projects, isolation of new salt- and metal-tolerant organisms was achieved, and experiments under both model desalination and metal-binding conditions were conducted. In anticipation of slower growth by many of the bacterial isolates, experiments with new model bacteria were initiated to troubleshoot and supplement data from Soudan bacteria.

Project Status as of *January 1, 2018***:** As part of our genetic screening in bacteria, a new gene was discovered that is part of the pathway allowing electrons to escape bacteria under difficult or extreme

conditions, one we were seeking because we hypothesized it produced the 'strongest' electrons in devices powered by bacteria. We also discovered a method for constructing new bacterial strains that are designed explicitly for taking electrons up from electrodes, which will accelerate our research into directly reducing nitrates or metals. In our fungal project, we have transitioned to the next phase of the work by testing fungal isolates under real-world salt and metal conditions, based on Soudan mine water analysis. These more detailed experiments reveal strains with an ability to both grow and remove metals under such conditions.

Project Status as of *July 1, 2018:* Progress continues in identifying the basis for salt tolerance in new strains isolated from the Soudan mine, and a full comparative genomic analysis was completed allowing us to identify genes in Soudan isolates linked to salt tolerance. In addition, new fungal strains with improved growth in the presence of cobalt were identified.

Project Status as of *January 1, 2019:* We successfully identified strains able to overcome inhibition by conditions experienced in high metal or highly oxidizing conditions, and showed that this could often be traced to changes in a single gene, creating strains with new abilities in our electrode reactors. We completed metal analysis of fungal materials incubated in simulated mine brines, showing successful recovery in both Mn and Co contaminated systems.

Overall Project Outcomes and Results

Overall, this project produced and characterized new biological agents able to tolerate the high salt or metal concentrations typical of both mining sites and proposed water treatment devices. These included anaerobic electricity-producing strains, aerobic biodegradation strains, and metal-binding fungal strains. We discovered the mechanism that supports production of the strongest voltages in the most commonly used electricity-producing strain, which showed that along with an ability to tolerate toxic conditions (salts or metals), organisms need adaptations that were previously unrecognized. The Soudan Mine, as a protected habitat with multiple zones containing high levels of salts or metals, remains a rich source of useful biocatalysts.

Our experiments with small-scale self-powered devices designed to remove salts suggest that, while they offer energy savings, they remain as complex as other membrane-based desalination or reverse osmosis devices. As all of these technologies are membrane-based, they are susceptible to scale build-up and mineral precipitation, and unless the energy costs are the majority of operating costs, microbial strategies are not recommended at an industrial scale. Protective wetlands constructed with conductive materials, designed to enhance removal of organic contaminants via action of these bacteria, appear to be the most useful application. Similarly, our data suggests that passive approaches such as encouraging fungal growth or use of fungal biomass as a passive filter remains a low-tech and reliable technology.

We successfully recovered several species of bacteria and fungi from the Soudan Underground Mine that oxide manganese and show potential for bioremediation of metals in salty, metal-rich waters containing Mn, Co, Cu, and Ni. We identified fungi species F1 (*Periconia sp.*) as the isolate with the greatest potential for removing metals from water in a constructed bioreator. Our results show that this fungus can quickly remove metals like Mn, Co, Ni, and Cu from simulated mine brines, most likely through adsorption and/or structural incorporation by biogenic Mn oxide minerals.

Based on these, two types of bioreactors built using fungi F1 demonstrate and allow us to optimize metal removal from the briny waters. The Type 1 design is a simple but safe bioreactor specifically designed to demonstrate the bioremediation strategy to general audience for outreach purposes. Type 2 is a passive through bioreactor using F1-mediated sponges as the main effective component. We completed preliminary experiments on the Type 2 bioreactor with simulated mine brines containing Mn and Co as the metals of interest. Our preliminary results suggest this reactor will remove Co from the briny waters. Based on laboratory flask experiments with strain F1 growing in multiple metals, this bioreactor shows high potential to remove other metals like Ni and Cu. These experiments and preliminary bioreactor studies show great promise that a more cost-effective technology using these fungi could be designed soon to treat metal contaminated waters in areas of high salt or high metals.

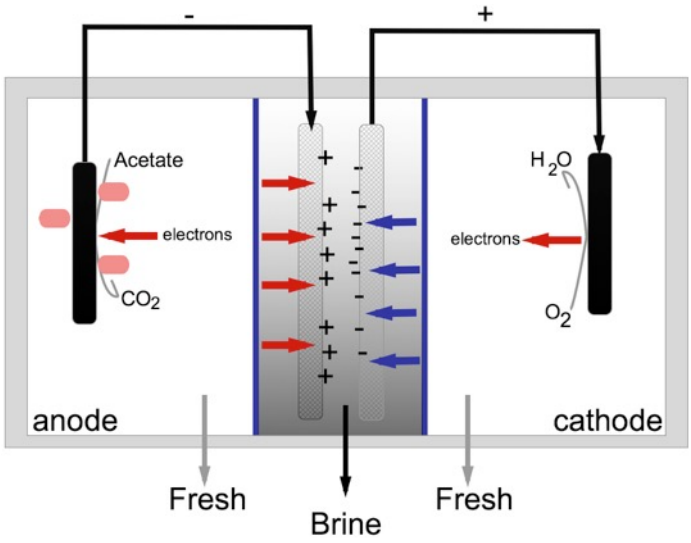
Final Project Abstract : Many Minnesota waters are contaminated with salts and metals. Removing these contaminants can be more difficult than removal of compounds such as oils or pharmaceuticals which can be destroyed by bacteria or heat treatment. Metals and salts must be physically bound, or made to pass through a specific membrane to clean the water, making such treatments expensive and energy intensive. However, technologies have been proposed that use microorganisms as the power source to drive salts across membranes, or as binding agents to remove metals, significantly reducing the cost and complexity of treatment. Before such technologies can even be imagined at scale, naturally-occurring microorganisms that are tolerant of harsh conditions and able to power removal of salts from water must be made available. A key goal of this project was to discover such organisms, subject them to the stresses of life under the conditions, and understand what could limit implementation of these remediation strategies. After surveying a number of contaminated sites in Minnesota, we focused specifically on the power-generating abilities of bacteria related to the genus *Geobacter*, the salt-tolerance abilities of bacteria related to the genus *Marinobacter*, and the metal transforming abilities of fungal *Armilleria* and *Periconia* genera. We verified that these organisms can grow in high salt conditions, power model salt-removal reactors, and in some cases remove multiple metals from solution. In the case of salt removal, we showed that many of the operating conditions proposed, such as cycling of the cell voltage or operation at low redox potential, can be harmful to cells and will need to be addressed before the technology can be successful, as will issues related to high calcium content of some Minnesota waters. In contrast, because the use of fungi for metal removal does not require as much equipment or electrochemical control, scaling of this approach using organisms obtained via this project is deemed much more feasible.

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1:

Background: Microbe-powered desalination. Salt ions, from the sodium chloride in road salts to sulfates released from rocks and ores, can be the most difficult contaminants to remove from water. Energy must be invested to push salts across membranes in reverse osmosis desalination plants. Our team recently discovered microbes that have two unique skills: an ability to generate biological electricity, and the capacity to grow in extremely harsh environments. We will harness this rare combination in a new class of salt-removing reactors.

The 'microbial desalination cell' contains electrodes colonized by bacteria, who generate electricity inside the device by breaking down waste organic matter at the anode. The electrical flow created by bacteria helps drive salts across membranes, leaving a concentrated brine for collection or sale. Bacteria can reduce the power needed for this kind of water purification by 50%. With over 20,000 large-scale plants worldwide, and millions of on-site salt treatment plants operating at natural gas hydraulic fracturing and industrial sites, even minor improvements to desalination technology will have a significant impact on energy usage and water recovery.



Principle of a Microbial Capacitive Desalination Cell: Electrons produced by bacteria oxidizing organic matter drives formation of a negative charge on one electrode in the central chamber, while exposure to oxygen creates a positive charge on a second. Cations and anions bind in response, and electrode potentials are reversed for discharge into an increasingly saline brine. In each cycle, the water circulating in electrode chambers becomes progressively less saline and lower in waste organic matter, and the waste brine increases in salinity. The cycling creates dramatic changes in electrode voltage that have never been investigated for their effect on the bacteria driving this process.

While Microbial Desalination has been demonstrated in preliminary reactors using bacteria obtained from freshwater habitats, there have been no attempts to enrich or evolve electricity-producing communities able to tolerate extreme conditions. Our experiments will obtain bacteria from naturally saline and metal-contaminated sites, track the bacteria in these enrichments to identify strains consistently powering our devices, and sequence their genomes to determine genetic elements common to these strains.

This Activity is divided into three subprojects:

1. We will perform direct comparisons of naturally obtained communities to mixtures of pure cultures, and test the ability of recently isolated cultures to invade established biofilms. Initial experiments demonstrating the value of our enrichment (measured by increases in current), and identification of strains from our culture collection that could be included in a final inoculum will be complete within one year. Sequencing of communities, enrichment of new organisms for possible isolation, comparisons with strain mixtures, and cathodic experiments

are projected to be completed in years 2-3. An inoculum comprised of enriched, evolved, and/or isolated organisms for use in model microbial capacitive desalination cells will be assembled by year 3.

2. With the identification of bacteria and growth conditions that are able to provide higher current densities during voltage cycles and in the presence of harsh conditions, we will use genetic analysis to identify the basis for these adaptations. This will enable quick detection of new strains from other environments, and monitoring of community performance in future applications. We will aim to identify biological rate-limiting steps to reveal how future reactors can be constructed to harness the biological agents central to microbial desalination. Screening for genetic targets in each system will begin by year 2. For this objective, genes identified in these initial experiments will be deleted and verified to be involved in survival in desalination reactors by year 3.

3. For our final objective, we will construct model microbial capacitive desalination cells and inoculate them with our communities, for comparison with approaches practiced today which use organisms from freshwater or domestic sources. For these experiments, our primary objective will be reductions in start-up time, resistance to salt and metal shocks, and increases in current density compared to published data.

Summary Budget Information for Activity 1:

ENRTF Budget: $ 376,358
Amount Spent: $ 372,427
Balance: $ 3,931

Outcome	Completion Date
1. Develop enrichment strategies able to identify robust salt- metal- or voltage-adapted strains capable of powering microbial desalination cells under harsh conditions.	July 1, 2017
2. Identify genetic basis for high performance under desalination conditions in key strains, complete two full genetic screens by year 2.	July 1, 2018
3. Treat real-world salt- or metal-contaminated effluents using microbial desalination cells and our improved inoculum. Operate at least 3 desalination cells.	July 1, 2019

Activity Status as of *January 1, 2017***:** For outcome 1, we needed to enrich new bacteria from unexplored mine sites, which are proposed to have the ability to survive under conditions which would improve water treatment. Whereas our previous sampling excursions to Soudan examined microbial communities across multiple boreholes near the surface where access was easiest, we began this project by revisiting the borehole with the most unobstructed access, to gain samples at much greater depth: (borehole 951W11). Our prior microbial community analyses suggested that sampling depths below 1 m were needed to guarantee samples were collecting the organisms from a truly deep subsurface setting, where conditions were oxygen-free, high-salinity, and most novel. Using a new sampling approach, we collected brine and filtered biomass from well below 10 m depth—an order of magnitude deeper than has ever been collected at Soudan. Some samples were preserved for DNA analysis, and the remainder were transported back to the lab for enrichment experiments described in Outcome 1. As we obtain pure isolates, we will compare their DNA sequences to those found in directly filtered samples, to test if we are recovering bacteria representative of these locations. Preliminary sequencing reveals the predicted trend, that the community at this new depth contained primarily bacteria known to be oxygen-sensitive.

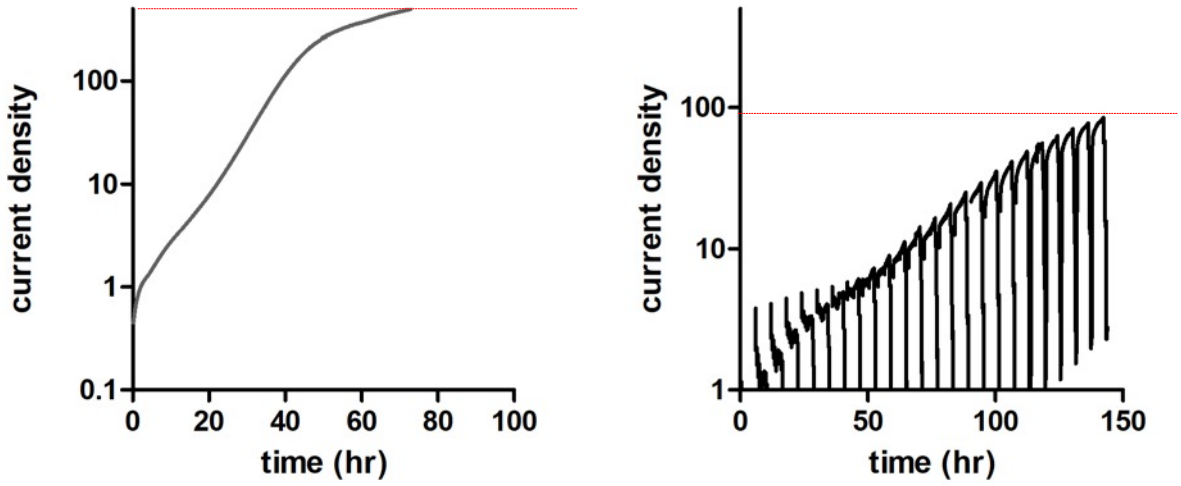
We also began to develop genetic methods which will be needed for Outcome 2, searching for the genetic basis of growth on electrodes under desalinating conditions. Plasmids and transposons are being mated into *Desulfuromonas soudanensis,* a strain recovered from the Soudan mine, as well as *Geobacter metallireducens,* as this organism possesses many similar traits to the Soudan isolate in terms of electricity production, and the ability to grow on both anodes and cathodes, yet it is not salt tolerant. For comparison, we are also beginning study of a strain recovered from an oil field that is highly salt tolerant, *Geoalkalibacter subterraneus.* The use of multiple strains will allow a comparison of electricity-related vs. salt-tolerance strategies, and more rapid testing of desalination-like reactors as the model *Geobacter metallireducens* grows much faster.

Activity Status as of *July 1, 2017***:** Slow growth in many enrichments delayed some outcomes, but within one year of inoculation, positive enrichments have now been obtained for multiple conditions. Additional culture conditions and enrichment strategies were also included during this time to increase the chances of positive growth. The fact that many of these cultures are very slow-growing relative to other organisms used for laboratory studies is encouraging, as this suggests cultures are adapted to the natural conditions of the deep subsurface where energy is limiting. Positive growth has been observed in enrichments using electrodes, iron, nitrate, and the organic compound fumarate (see below). Previous to this work, we only had one other positive enrichment, and never were able to obtain pure colonies directly from an anaerobic borehole mine sample. All cultures growing as isolated colonies are being tested for purity using DNA sequencing.

Example enrichments from Soudan Mine using electrodes (left), iron (center), and fumarate(right). The rise in electrical current at 50 days is a sign of microbial growth via electricity production; darkening of the iron indicates microbial conversion of the Fe(III) to reduced Fe(II) similar to what is seen in taconite; the isolated colonies on the plates inside an anaerobic chamber reveal possible pure isolates. These enrichments were a key goal for Outcome 1. Our target for reaching this goal will shift to later in this project due to the slow growth of some of these strains, but as they represent the first of their kind, we will aggressively pursue their isolation.

How will bacteria respond to desalination conditions? As part of Objective 2, we completed a new series of tests that simulate a poorly understood aspect of desalination conditions; the large voltage steps used to drive salts across membranes. For example, typical capacitive desalination systems

impose a potential across a membrane, which drives ion flux across the membrane. Then devices discharge this potential rapidly, to trap the ions in a specific chamber. When using bacteria to create this driving force, the organisms are subjected to rapid jumps in voltage. The effects of these transitions, and whether some organisms tolerate them better than others, are not studied. We built and operated a series of reactors under either constant potential ('control'), or variable potential ('desalination') conditions. In all cases, we found that the variable potential conditions severely impacted growth of the organisms on electrodes (see below). Based on these results, we must search for strains over the next activity periods to test hypotheses that bacteria can better survive these surges or changes in potential by storing the electrons themselves on electron-carrying proteins such as cytochromes, or that these periods can be modified to cause less harm to the organisms essential to the device.



Example data from 'constant potential desalination reactors (left) vs. 'variable potential' reactors (right), showing how potential surges result in less performance (lower current). A goal of this project is to identifying the cause of this damage and possible solutions to this effect of variable potential.

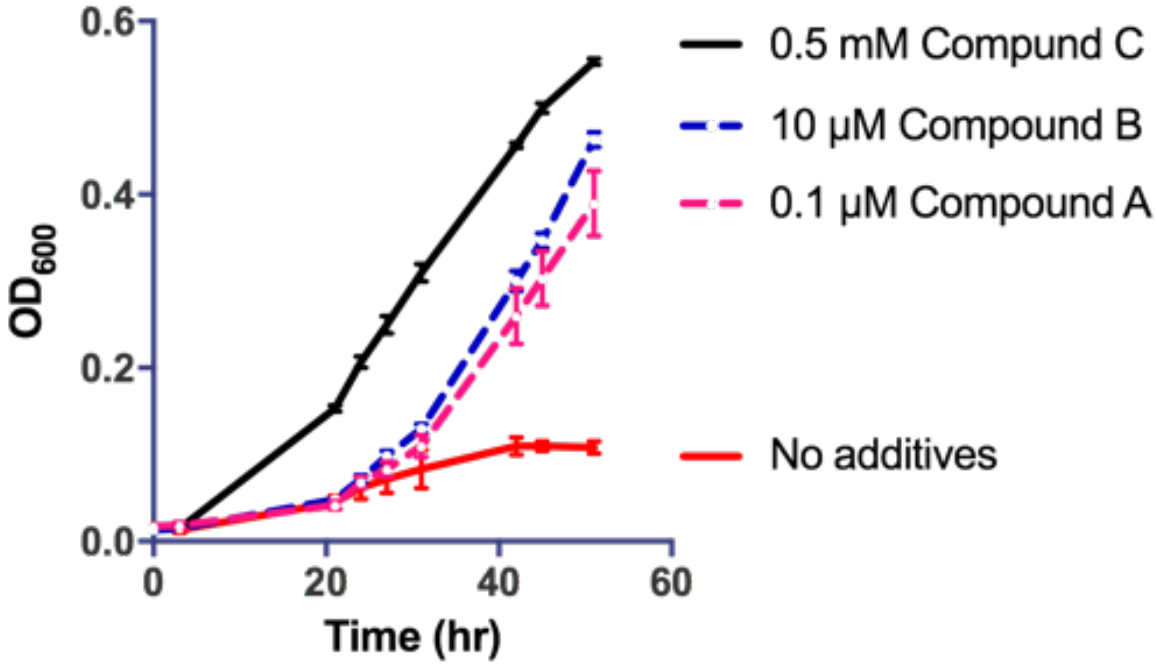
New genetic tools for discovering mechanisms: Another key goal in Objective 2 is a series of genetic tools that will accelerate screening of cultures under desalination conditions. We have successfully shown that plasmids developed by our group can be transferred to new strains such as *Geobacter metallireducens* for the first time, making possible the deletion of genes and the construction of mutant libraries. This example will allow us to screen more strains for genes involved in either the release or uptake of electrons from electrodes, and expands the range of contaminants we can study, as this strain is also able to act on aromatic contaminants and nitrate.

Activity Status as of *January 1, 2018***:** The central goal of Objective 2 remains identification of core components in bacterial pathways that enable microbial desalination, so we can choose or build strains to be used throughout this emerging industry. Work described in earlier reports as part of Objective 1,

aimed at enrichment and isolation of new strains, continues. Main findings of Objective 2 we are pleased to report include; discovery of conditions able to support growth of all our isolates (fresh, saltwater, and mine-sourced) in the presence of the water contaminant nitrate, and discovery of a new gene essential to conducting experiments regarding what supports electron flow out our cells to electrodes. We also began analysis of a large genetic dataset that compares the physiology of *Marinobacter* strains isolated from Soudan Mine brines with seawater isolates around the world, to more specifically identify components enabling growth and metabolism of cells in these harsh environments. This is being achieved by sequencing the Soudan isolates for comparison with those isolated by collaborators from electrodes in seawater, and comparing both the conserved gene set and genes essential during transposon mutagenesis. We will provide a more detailed report of this analysis in the next review.

We also consulted with one of the most prominent research groups in the field of microbial desalination, who actively treats fracking production waters and petroleum-contaminated sites with microbial desalination, regarding the need for better performing strains. Based on these consultations, we will be placing an additional focus in the coming periods on salt tolerance mechanisms and how this research could license or provide inoculum cultures to people operating larger reactors across the country.

New methods for studying nitrate removal: Devices aimed at cleaning water often face the challenge of removing co-contaminants, such as nitrate or sulfate. For example, with an input of electrons, microbial treatment can transform nitrate into either ammonia or nitrogen gas, if the right bacteria are



Example data showing how new compounds can relieve inhibition of *Geobacter* growth with nitrate, possibly enabling more efficient nitrate removal from water. Compound A is nontoxic and effective at low concentrations.

provided. However, for decades most organisms that can grow with electrodes have shown an unexplained defect in the presence of nitrate, often growing poorly or showing toxicity. Without robust growth, genetic studies are impossible, electrode-based growth cannot be studied, and treatment is impossible. By screening a series of compounds for ones able to alleviate this problem, we found growth conditions that eliminate this toxicity in both freshwater and high-salinity strains, and have identified at least one environmentally friendly compound that could be easily added to reactors if needed. We are conducting final genetic experiments to confirm our hypothesis, but this finding has already allowed acceleration of genetic studies and robust growth of our model strains. Example data is shown above of the enhanced growth effects seen when we remove nitrate toxicity via this simple method (to be reported publicly in an upcoming manuscript), and this has led to a reliable high-efficiency genetic system that was a goal of our first reporting period. Experiments mating plasmids containing transposons into these *Geobacter* strains can now routinely achieve 2 – 4 x 10^3 mutants/ml, which is finally sufficient for genome-wide screening and other molecular studies.

New components in electron transfer pathways to electrodes: Devices using bacteria for power will operate more efficiently at higher voltages. We have been screening our cultures for the genes

required for growth under these conditions, and have new evidence of a system able to operate at lowest reported voltage yet. Referred to here as “cbcXL” system, mutants lacking these genes are defective only in the lowest potential, or lowest voltage region. Since most microbial fuel cells and desalination devices are operated with electrodes near these potentials, we believe this could be highly relevant to real-world conditions.

Activity Status as of *July 1, 2018: Understanding Life at High Salt – Comparison of Marinobacter genomes from the Soudan Mine to identify what traits make good desalination catalysts.* Bacteria of the genus *Marinobacter* are found ubiquitously in aquatic and terrestrial salinous environments. In work for this project, we identified many new strains isolated from the Soudan mine that grows in salinities as high as 20.5%, and can be acclimated to sodium chloride concentrations high as 35%. A noted recalcitrance to environmental shifts, coupled with broad utilization of carbon sources, has prompted our interest in *Marinobacter* species as industrially relevant hosts and biocatalysts.

After sequencing the genomes of our Soudan isolates, we used 71 quality genomes acquired from public and private collections, and conducted full genome comparison across phenotypic and phylogenetic groups, to compare what was conserved in genomes of highly salt-tolerant organisms, and discover was might be unique among Soudan isolates.

Initial analysis of the genomes resulted in the relatedness chart (Table 1) below, the first comprehensive evaluation of this microbial group. It shows an analysis of the relatedness of these different isolates based on crosswise genome comparisons. Analysis of individual genome similarities then produced Table 2, which identifies genes and pathways putatively involved in tolerance to salinity and other environmental conditions for *Marinobacter* isolates.

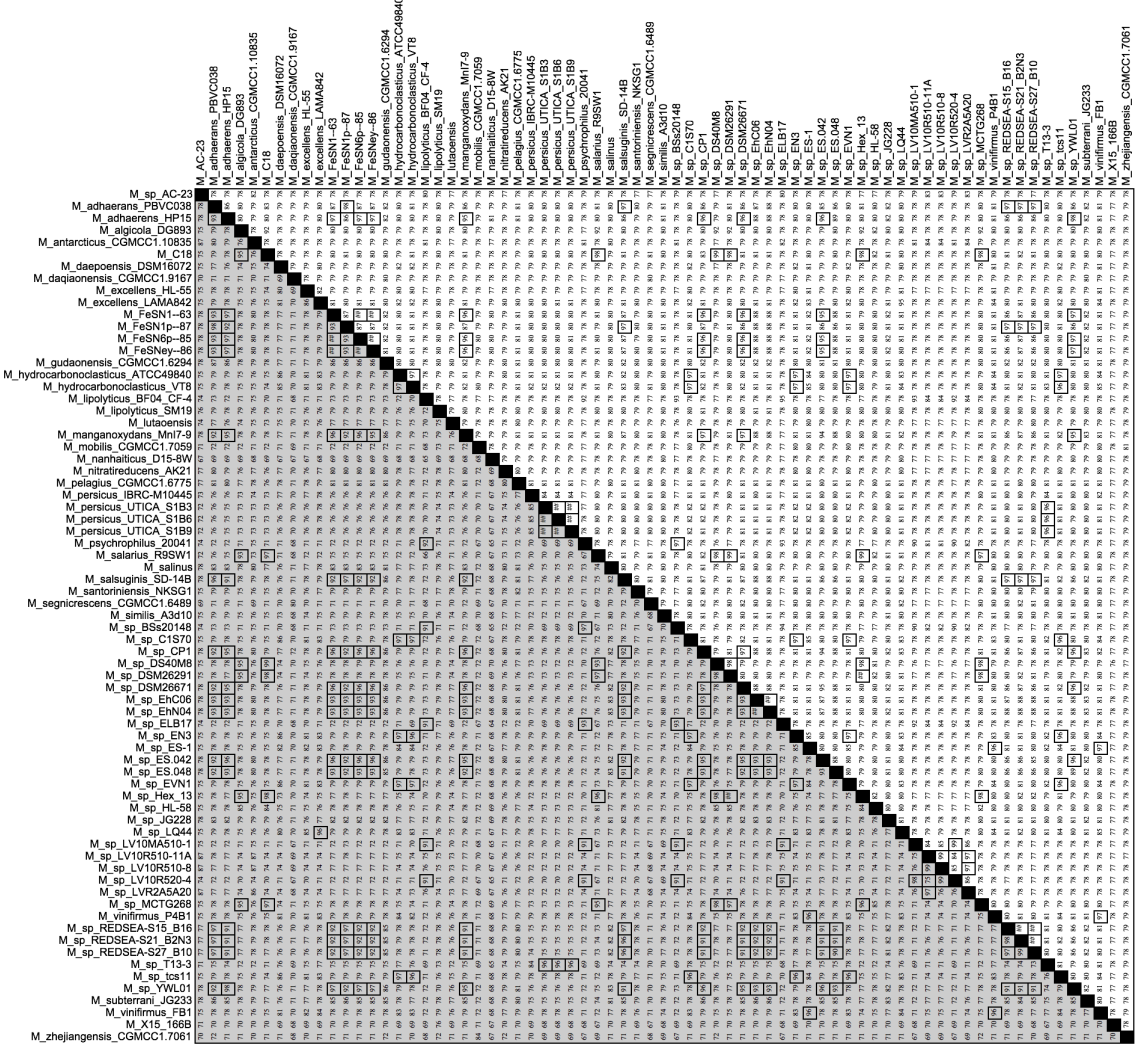


***Table 1:** Shaded boxes indicate Average Amino Acid Identity, while white boxes indicate Average Nucleotide Identity. Bordered values indicate values at, or above, the species threshold proposed by Rodriguz and Konstantinidis, 2014. This complete genome comparison allows us to show which Soudan isolates are unique from other seawater- or high-salt organisms.

Comparative Genomics

Genes defining tolerances to temperature, salinity, acidity, or biome of isolation were determined by inclusion in every member of one group to the full exclusion of the other group. For example, comparison of isolates found only at high or low maximum temperature tolerance yielded 105 genes; 89 found in high temperature isolates and 16 in the lower. Genes found in higher temperature isolates putatively encode products involved in membrane and cell wall maintenance. Of interest to this project, comparison of the high and low maximum salinity tolerance groups yielded 48 genes, of which 38 were found in the high salt tolerance group. These trended towards regulation and membrane transport. We are using these as a "signature" to aid isolation of additional salt-tolerant strains.

Table 2. Genes exclusive to *Marinobacter* groupings.

Maximum Temperature Tolerance

High temperature genes exclusive of Low temperature isolates:

1-acyl-sn-glycerol-3-phosphate acyltransferase
2,3-diketo-5-methylthio-1-phosphopentane phosphatase
23S rRNA (cytidine2498-2'-O)-methyltransferase
ABC transporter [3]
acetolactate synthase-1/2/3 large subunit
Activator of Hsp90 ATPase 1 family protein
Biopolymer transport protein ExbD [2]
C4-dicarboxylate ABC transporter
C4-dicarboxylate transport sensor histidine kinase DctB
cation transport regulator ChaB
Co/Zn/Cd efflux system
coprogen and ferric-rhodotorulic acid
cspV cold-shock DNA-binding protein family
cyclohexanone monooxygenase
cysteine synthase A
Dicarboxylate transport
diguanylate cyclase
dihydrolipoamide acetyltransferase
ectoine hydroxylase
exopolyphosphatase
exosortase
Exporter of O-antigen and teichoic acid
FhuF 2Fe-2S C-terminal domain-containing protein
glnD (Nitrogen regulation)
Glycosyltransferase
Glycosyltransferase involved in cell wall bisynthesis [3]
Glyoxylase, beta-lactamase superfamily II
GntR family transcriptional regulator
haloalkane dehalogenase
heat shock protein Hsp20
histidine kinase
hypothetical [17]
L-lactate dehydrogenase complex protein LldE
L-lactate dehydrogenase complex protein LldG
metal dependent phosphohydrolase
MFS transporter
mgsA methylglyoxal synthase
MoxR-like ATPase (chaperone)
MSHA biogenesis protein MshI, fimbria assembly
Na/Pi cotransporter
NAD(P)H dehydrogenase (quinone)
nitrogen regulatory protein P-II
O-antigen ligase
outer membrane transport energization protein ExbB
PEP-CTERM system associated sugar transferase
PEP-CTERM system associated, cell wall protein
Phage integrase, N-terminal SAM-like domain
phenylacetate-CoA ligase
Polysaccharide deacetylase
Predicted thiol-disulfide oxidoreductase YuxK, DCC family
Cystathionine beta-synthase, core domain
Protein-S-isoprenylcysteine O-methyltransferase Ste14
RNA polymerase sigma-70 factor, ECF subfamily
RNA-binding protein, contains PUA-like domain
rRNA pseudouridine516 synthase
Small-conductance mechanosensitive ion channel
sodium/proton antiporter
sulfoxide reductase catalytic subunit YedY
sulfoxide reductase heme-binding subunit YedZ
thiamine binding protein
thioredoxin/CopG transcriptional regulator
transcriptional regulator, AraC family
transposase
tRNA (guanosine-2'-O-)-methyltransferase
tRNA(Met)-cytidine N(4)-acetyltransferase
tRNA/rRNA methyltransferase
tryptophan synthase beta chain
UDP-2,3-diacylglucosamine pyrophosphatase LpxH
UDP-N-acetylglucosamine pyrophosphorylase

Low exclusive of High

16S rRNA pseudouridine516 synthase
DNA-binding response regulator, OmpR family
exopolyphosphatase
FAD-dependent dehydrogenases
Fe-S oxidoreductase
HNH endonuclease
hypotheical [6]
NADH:flavin oxidoreductases, Old Yellow Enzyme family
protease
RIO kinase 1
RND family efflux transporter, MFP subunit
signal transduction histidine kinase
tol-pal system beta propeller repeat protein TolB

Maximum Salinity Tolerance

High exclusive of Low

4-mercaptohistidine N1-methyltranferase
Acyltransferase [2]
Cytochrome C
Diguanylate cyclase

Low exclusive of High

CobN
flagellar assembly protein FliH
formate dehydrogenase accessory protein FdhD
hypothetical [2]

Ferritin
hypothetical [11]
Integrase
Metallophosphoesterase
Methyl-accepting chemotaxis protein [3]
NAD/FAD-dependent oxidoreductase
NADPH-dependent FMN reductase
Permease [3]
putative 4-mercaptohistidine N1-methyltranferase
Response regulator
Rhodanese-like sulfurtransferase
RNA polymerase nonessential primary-like sigma factor
Sensors of blue-light using FAD
Signal transduction histidine kinase
Spermidine synthase
Tat pathway signal protein
Transporter
Transposase
TrkA-C domain-containing protein
Isochorismatase
methylmalonate-semialdehyde dehydrogenase
Predicted lipid carrier protein YhbT
ribonucleoside-diphosphate reductase alpha chain
Transcriptional regulators of sugar metabolism

Optimum Salinity

Low exclusive of High

RNA polymerase sigma-70 factor, ECF subfamily
tRNA(Met)-cytidine N(4)-acetyltransferase
hydrolase

hypothetical

Biome of Isolation

Absent exclusively in Terrestrial

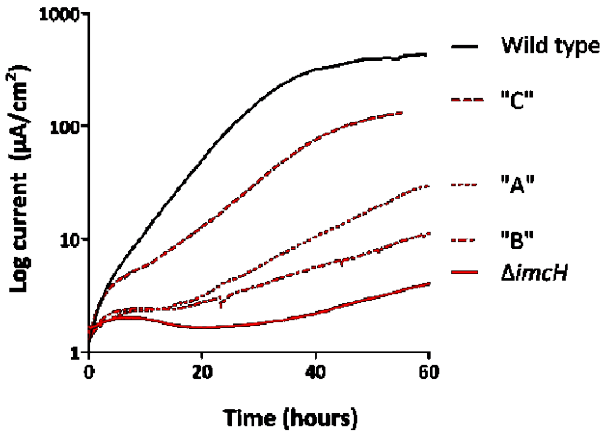
UreA
UreF urease accessory protein
UreG urease accessory protein

*Bracketed numbers indicate a tally of genes with identical annotation

diagnostic to the grouping. The lack of clear delineation by gene content between groupings may be primarily due to differential activity of common genes and regulation, though it is more likely due to multivariate phenotypes which are acquired progressively. Analysis accounting for the contribution of individual genes to cumulative phenotype would likely yield better results. It is worth noting, though not to the exclusion of marine strains, terrestrial isolates exclusively use urea carboxylase, and none have been found to encode a urease.

Activity Status as of *January 1, 2019:*

Progress towards understanding how bacteria utilize electrodes of different voltages: as we found earlier in the project, fluctuations in electrode conditions appear to harm organisms and also cause them to shift their use of different mechanisms. Over the past year, we began searching for ways to 'open the window' of conditions permissible to bacteria on electrodes and in metal solutions. To test this, we began with strains unable to grow at specific potentials, and allowed evolution to select for favorable changes. Surprisingly, many of these changes occurred in genes under study for this project that control electron transfer pathways.



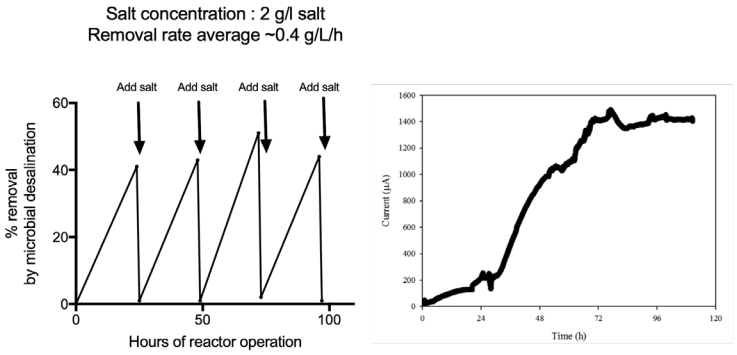
Example of using evolution to find new strains able to grow under new conditions. Solid red line shows original strain (Δ*imcH)*, which cannot produce electricity because it lacks a key electron transfer protein. We hypothesize that other proteins exist which can be adapted to new conditions, and the dotted red traces (A, B, C) show new strains able to perform this task. Sequencing of these organisms revealed changes to a new electron transfer pathway

To test if this approach indeed expanded the abilities, we constructed a strain containing only a single pathway for electron transfer. As expected, this strain could not grow under standard conditions, such as in presence of abundance Fe(III). However, when we made single changes to a gene implicated in voltage-dependent growth, this strain now could grow in the presence of highly oxidized metals. This discovery is important evidence that we are studying the core pathways that control growth with these metals, and provide a method for constructing strains with new and useful abilities.

Progress towards operating microbial desalination cells: As reported in our Jan 1 2017 report, pilot reactors revealed negative microbial effects of 'pulsing' the voltage of electrodes during microbial-powered desalination. This drove research seeking microorganisms tolerant of lower voltages.

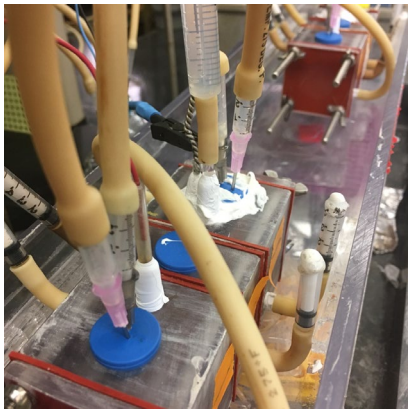
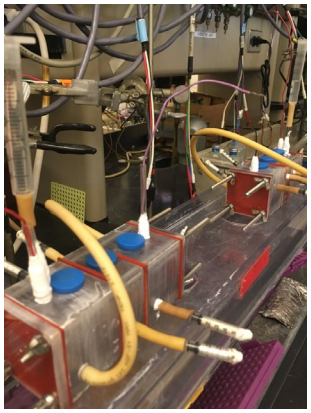
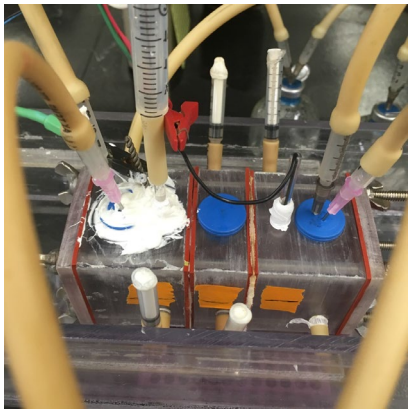
To fulfill Activity 3 (operating functional microbial desalination reactors), we have operated pilot-scale reactors to determine the proper configuration of electrodes and membranes, and achieved 40% salt removal. Studies with Soudan isolates are underway and additional data from these studies will be include in the final Summary.

Pilot studies testing the ability of microbial-powered desalination cells to remove salts from a central membrane chamber over prolonged periods



Pilot reactors were operated with *Geobacter*-powered electrodes, and the conductivity of a central chamber was monitored for changes in conductivity. Within 24 h, reactors typically removed over 40% of added salts, and power increased to a stable value within 3 days.

Final Activity Summary: *Reactor demonstrations.* For our final work periods, we primarily returned to working with strains isolated directly from the Soudan Mine (e.g., *D. soudanensis*). The purpose of these experiments was to show that they could provide the power to drive salts out of model brine solutions during longer incubations in higher salt concentrations. Until these experiments were conducted, *D. soudanensis* had not been used for this purpose. We constructed multiple desalination reactors filled with mine-strength saltwater medium in each chamber, and monitored power production as well as salt removal. We also continued our project-long work of isolating even more salt-tolerant species, which while they are slow-growing represent some of the most unique organisms obtained from this site.

Examples of desalination reactor trials comparing Soudan Isolates with more commonly used organisms. Each of these reactors contains at least seawater-strength brine in all three chambers, and growth of bacteria on electrodes in one of the larger chambers drives electricity production towards a counter chamber. This drives salt ions out of the center chamber to compensate for charge movement, producing a lower-salt product.

While more common laboratory organisms have shown an ability to quickly establish in similar reactors, *D. soudanensis* is adapted to more strictly anaerobic conditions, and initial reactors did not perform as predicted. In subsequent pilot reactors, we included organisms capable of stripping oxygen from the environment, such as *Shewanella* or *Marinobacter* isolated from nearby locations. This co-inoculation procedure produced more reliable operation, allowing establishment of the *D. soudanensis* culture and current production. After these initial trials, we increased the amount of electrode surface area in the reactors, as the harsh conditions slow *D. soudanensis* growth, and tested the effects of sealing reactors from oxygen.

As described in our final summary, these reactors do show an ability to remove salts from a water source, as predicted by inventors of the technology. However, we find they also suffer from many of the limitations of membrane-based reactors, such as mineral build-up and membrane failure after a period of time. Evan at lab scale, this became an issue. If a water source were also contaminated with organic pollutants, this approach could be more desirable, as the bacteria will also remove contaminants from the water as a power source.

ACTIVITY 2:

Description: Fungi-mediated metal removal. Metals, from iron to copper, present special toxicity and solubility issues, and can clog devices designed for water purification. A number of different technologies currently exist for removing metals from water, but many of these technologies are expensive to operate (particularly for waters requiring long-term treatment) or are only partially effective and require other upstream treatments. Microbial metal-removal bioreactors are actively being developed to reduce treatment costs and provide alternative mechanisms for remediation of complex waters. We will develop the strategies for fungi-mediated metal removal of a variety of metals of interest.

Microorganisms can contribute to metal removal by promoting two different processes. The first process is through adsorption of metal to the cell biomass. Essentially, microorganisms can act as natural sponges, sorbing (i.e., removing) metals from the water. Microbial biomass produces surfaces on which these metal sorption processes occur. If the organisms continue to grow in the environment, they represent new surfaces for the metals to sorb. This continual regeneration of sorptive materials will likely drive down maintenance costs of any metal-removal technology.

In addition to metal sorption, some microorganisms can further accelerate removal of metals from water by promoting the formation of metal-containing minerals that are easily removed from water and are less hazardous to human and environmental health. This is a process known as biomineralization, in which an organism transforms a dissolved chemical compound into a solid mineral phase. Some microbially produced “biominerals” also possess sponge-like qualities and can sorb metals from water, thus representing a dual strategy for metal removal.

In our previous work at the Soudan Underground mine, we found microorganisms such as bacteria and fungi thriving in areas heavily contaminated with a variety of metals including copper, cobalt, nickel, iron and manganese. Furthermore, we have identified a diversity of fungal species, including many novel species, that are highly effective in adsorbing certain metals in complex waters (e.g., high salinity and high metals). Because of these observations, we have targeted the fungal microbial community for our metal removal strategy in this project. We will examine how these two naturally occurring processes (metal sorption and mineral formation) can be harnessed and applied to make efficient and cost-effective fungal bioreactors designed to treat metal-rich waters. This passive ‘upstream’ technology could also greatly improve the operation of 'downstream' membrane-based microbial salt removal technologies described in Activity 1.

This Activity is divided into three subprojects:

1. Our earlier work identified a number of organisms that effectively sorb certain metals, thus the first objective of the current project is to identify organisms that promote biomineral formation of manganese (Mn) as a strategy for increasing metal removal from contaminated waters. We will screen the nearly 100 fungal cultures we have from previous work to see which species are capable of Mn oxide biomineralization. We will also return to the Soudan Mine to try to identify and culture additional fungal species missed by the previous culture enrichment techniques. The field work and fungal screening will be started in the summer of 2016 and will be completed by the end of year 1.

2. The second objective is to determine if a single process (sorption or biomineralization) or both processes together is more effective in fungi-promoted metal removal from complex, metal-rich waters. We will compare the growth and metal removal capacity of several different fungal species for different metal contaminants (e.g., Mn and Ni). The culture(s) and processes deemed highly effective (those that reduce at least 50% of the initial dissolved metal concentrations) will be used for further bioreactor development. We will be conducting these experiments throughout the first and second years of the project, and will have all culture experiments completed by the end of year 2.

3. The last objective of Activity 2 is to build and develop prototype laboratory bioreactors to optimize metal removal from contaminated waters. We will build laboratory-scale reactors to simulate the physical and chemical conditions in an industrial metal bioremediation system. We will ensure that conditions remain constant and aerobic throughout the bioreactors. We will seed these bioreactors with fungal cultures and an inert, solid growth substrate in order to retain these cultures within the reactors. These bioreactors can be run independently and in conjunction with downstream salt removal technologies from Activity 1. We will start construction on the reactors in year 2 and optimization will be completed by the end of year 3.

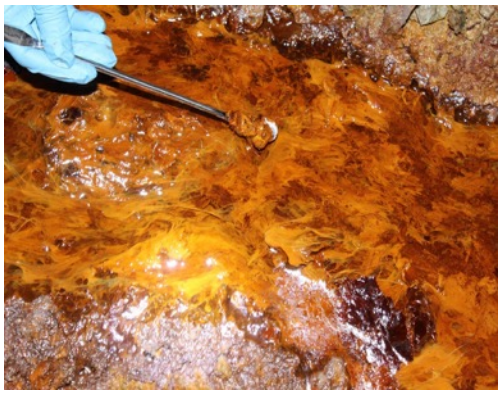
Summary Budget Information for Activity 2:

ENRTF Budget: $ 219,642
Amount Spent: $ 216,610
Balance: $ 3,032

Outcome	Completion Date
1. Identify fungal species from the Soudan Underground Mine that can promote formation of solid Mn oxide biominerals from metal-rich waters	July 2017
2. Identify the key fungal species and most effective metal removal pathways (sorption and/or biomineral formation) in batch laboratory experiments	July 2018
3. Build and operate laboratory scale reactors to optimize metal removal from complex, metal-rich waters	July 2019

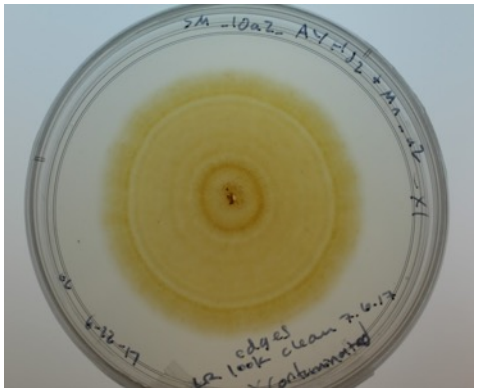
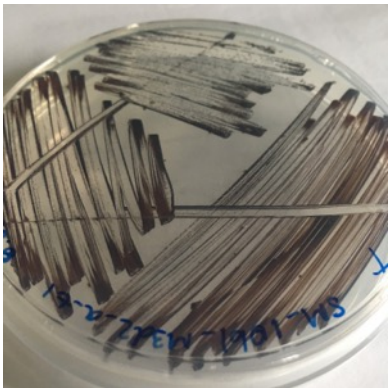
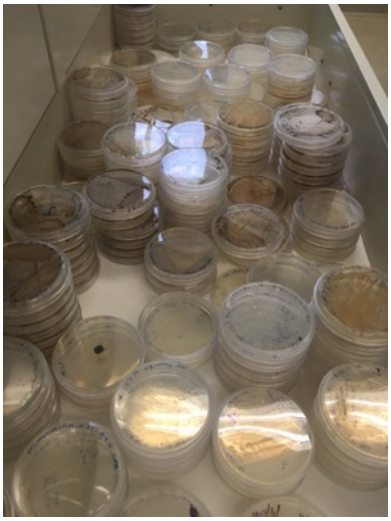
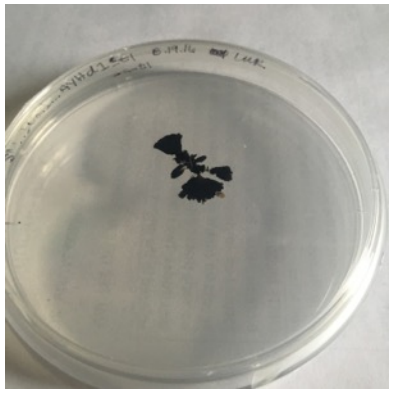
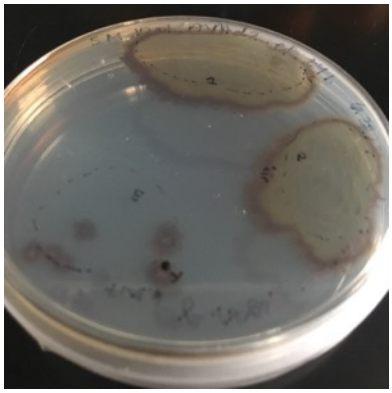
Activity Status as of *January 1, 2017***:** We completed exploratory trips and sampling trips to the Soudan Mine to identify new sites that could host novel fungi for metal recovery. In particular, we sampled from copper-rich sites and other regions of high metal concentration. During this period of initial sampling, enrichment, and isolation, we also began our first Objective of developing screening methods to measure metal binding and precipitation by our collection of other fungi isolated from metal-impacted sites.





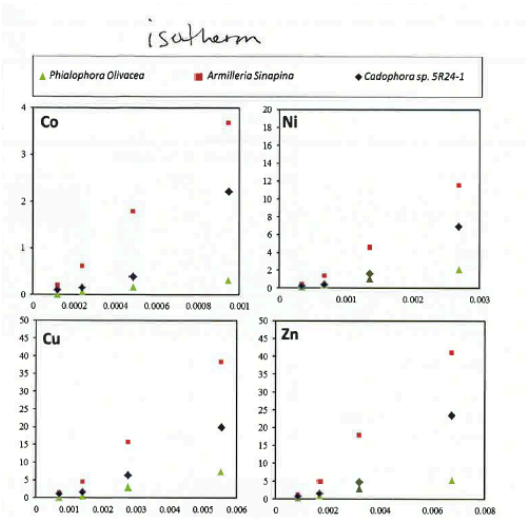
Above, images from Soudan mine sampling expeditions to obtain fungal enrichments, searching for new metal-binding microbes. Samples were taken directly from copper- and iron-rich biofilms, and inoculated into liquid medium. Samples were taken from wood or other non-metal substrates for comparisons. In the laboratory, positive enrichments are streaked onto solid medium to obtain pure cultures and incubated with metals for evidence of binding or oxidation activity.

Activity Status as of *July 1, 2017***:** Hundreds of potential isolates were obtained to purity, and incubated on solid medium to screen for metal precipitation activity. Examples of the wide variety obtained from this first period are shown below. In each case, black and brown coloring is indicative of metal (typically manganese) precipitation and binding by isolated fungal strains. Obtaining such precipitated metals is a goal of this project, as these metal oxides typically are able to act as sorbents for other toxic compounds in water and waste streams.

With the identification of many novel metal binding and oxidizing strains, the second objective of this project was to quantify the ability of promising strains. This involves growth of cultures in the presence of toxic metals such as cobalt, nickel, copper and zinc (Co, Ni, Cu and Zn), followed by digestion and analysis of the fungal biomass. In parallel, we are also analyzing the different forms of manganese oxides produced by cultures to determine the exact types of minerals being produced, so we may identify the highest surface-area or most stable forms that would be best suited for remediation.

Examples of liquid cultures (top two images) producing manganese oxides when exposed to metals in solution. Each image shows a fungal culture precipitating manganese, producing the dark mineral, compared to the lighter control flask lacking metals. Both of these strains are highly active in metal recovery.



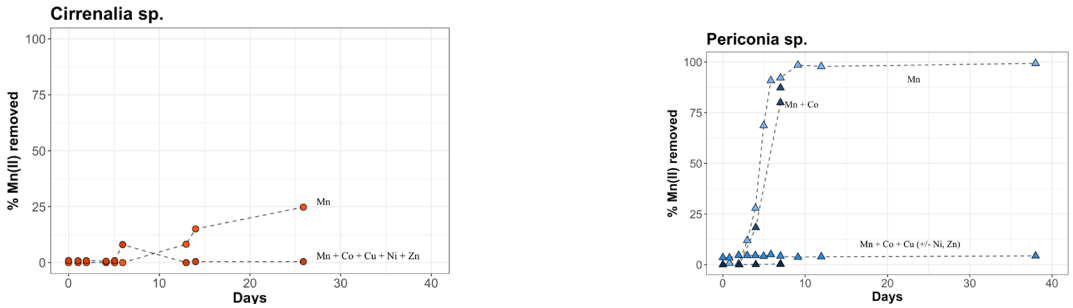
Sorption isotherm data for Armilleria S., Phialophora, and Cadophora. X axes are the initial metal concentration in mmol, and Y axes are the final metal concentration sorbed in mmol metal / kg of dry biomass.

Left, examples of minerals prepared for characterization, from Soudan Mine isolates, via high-energy analyses methods able to discriminate between different mineral structures. Right panel shows an example of metal binding assays used to test removal of different heavy metals by each strain. For each strain and condition, the x-axis indicates metal concentration in the medium, and the y-axis the amount of metal bound to the fungi. The Soudan *Armilleria* strain binds nearly ten times as much metal as the *Philaphora* strain.

Activity Status as of *January 1, 2018***:** While our first phase work obtained many isolates able in the laboratory to precipitate metals, making them candidates for treatment solutions, these experiments were performed

under ideal conditions. The second phase of our work aims to screen these cultures for those able to both grow and bind metals even in high salt and high metal conditions, to eventually learn the basis for this useful trait. For these experiments, we created a medium based on Soudan water analysis to contain the same salt and nutrient levels as would be experienced in treatment conditions, then added specific metals to fungal cultures to determine possible binding or removal. Of particular interest is whether additional metals alter the fate of the final product, as the minerals themselves become a surface for binding other organic contaminants.

Shown below are example experiments from this phase simulating real-world incubation, under salt and nutrient conditions similar to a reservoir or holding tank containing high salt, metal-rich waters. The *Periconia* species shows some of the highest growth and binding abilities, removing both Mn(II) and Co(II) within 10 days.

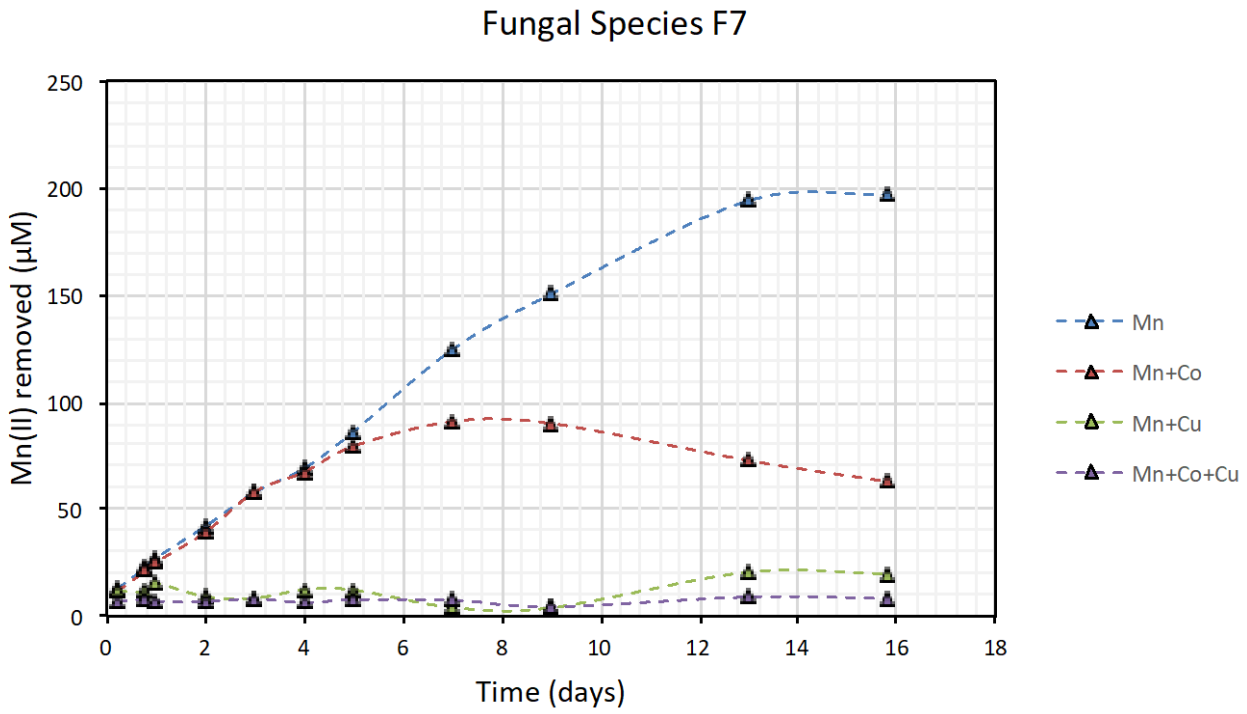


Example data showing the first direct comparisons of fungal isolates from the Soudan mine, incubated in medium with the same high salt and metal concentrations as found in mine effluents. The *Cirrenalia* isolate is able to grow and remove Mn(II), but when other metals are added, growth is inhibited. In contrast, the *Periconia* isolate can grow in the presence of both Manganese and Cobalt, and remove these minerals via sorption. Other metals remain inhibitory.

Activity Status as of *July 1, 2018:* To date, from our sampling and screening of strain from the Soudan Mine, we have narrowed our research down to 4 different species of fungi that oxidize Mn and make Mn oxide biominerals under metal-rich conditions. After testing in the lab during this period, we found that 2 of the four fungal species grow quickly enough to be used for a bioremediation strategy. We are experimenting with how well these fungi simultaneously remove Mn with Co, Cu, Ni, and Zn from salty mine waters under scaled-up conditions and verifying that minerals are attached to the fungi for easy removal or later use .

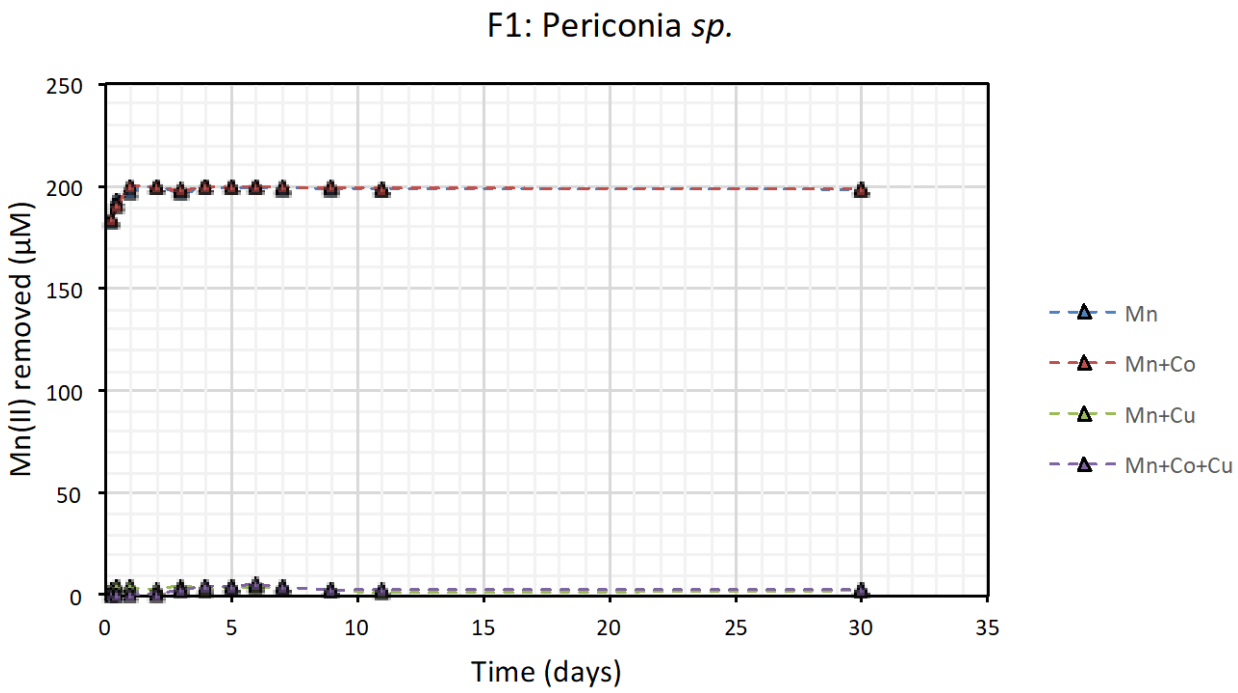
Examples of fungi able to precipitate metals under high salt concentrations at the surface of brine waters (left) and as metals precipitated on the fungi (right). The fungal mass becomes heavy with minerals and easy to remove from reactors.

Activity Status as of *January 1, 2019:* Laboratory experiments have confirmed the effectiveness of one promising new fungal species grown in various metal-enriched condition and its ability to form Mn oxides minerals. This species responded well to growing in waters containing manganese (Mn) and cobalt (Co), but not Cu-containing waters. Even when grown in waters contaminated with both cobalt and manganese, the fungi was able to grow and remove nearly half (~100 µM) of the manganese from solution. Analyses are currently underway to verify removal of other metals, but in other experiments these become incorporated into the matrix as well.



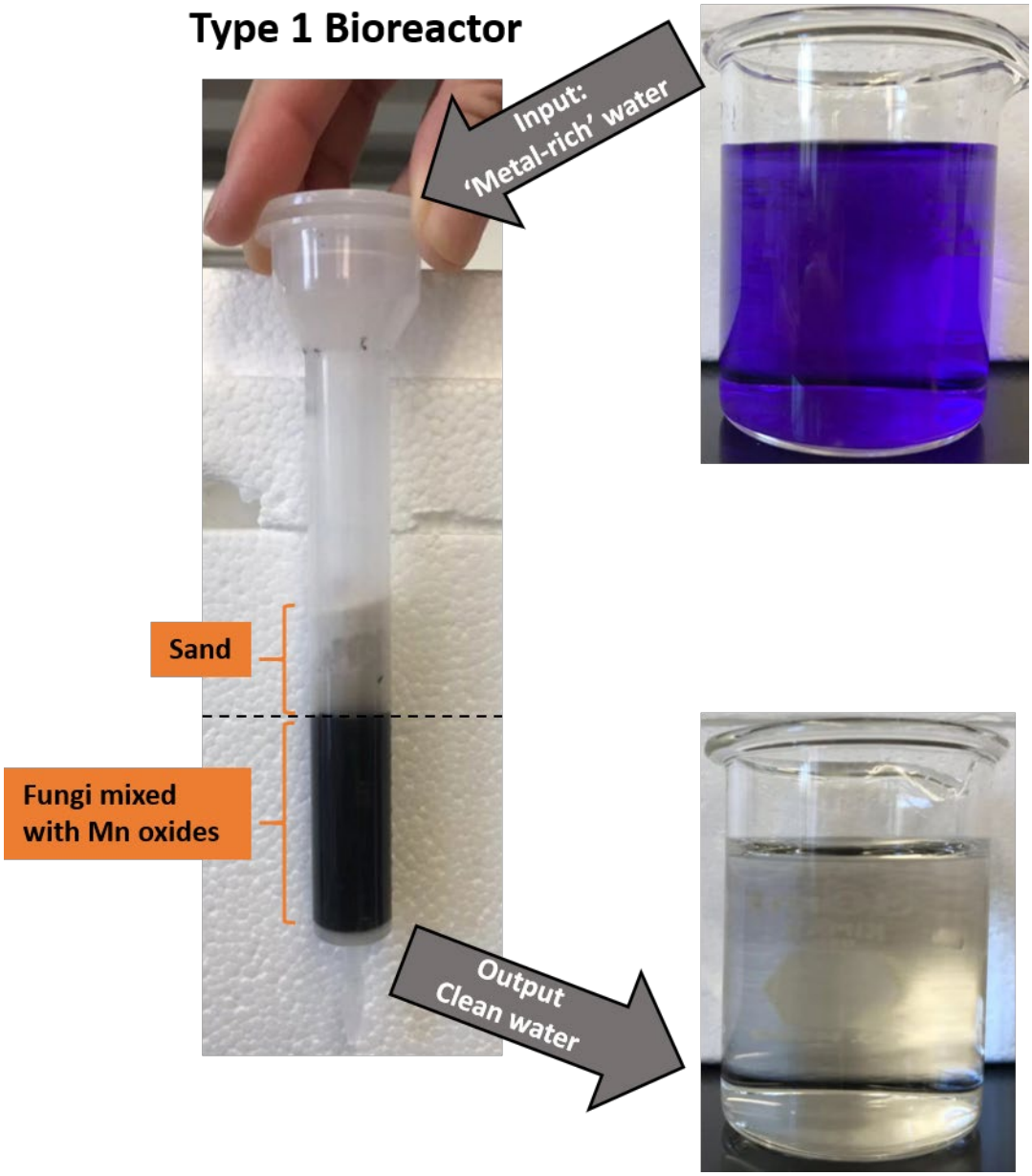
The amount of Mn(II) removed from the media as a function of time for fungi F7 grown in brine with 200 µM $MnCl_2$ (blue marker), 200 µM $MnCl_2$ + 104 µM $CoCl_2$(orange marker), 200 µM $MnCl_2$ + 109 µM $CuCl_2$ (grey marker), and 200 µM $MnCl_2$ + 104 µM $CoCl_2$ + 109 µM $CuCl_2$(yellow marker)

A second fungal species tested (F1) also responded very well to growing in waters in the laboratory containing manganese (Mn) and cobalt (Co), but not Cu-containing waters. When grown in Co-rich waters, the fungi was able to grow and remove nearly all (~200 µM) of the manganese from solution **within just 3 days of growth**. Presumably, this fungus also removed the cobalt present in solution, but these analyses are currently underway.



The amount of Mn(II) removed from the media as a function of time for F1 (*Periconia sp.*) grown in brine with 200 µM $MnCl_2$ (blue marker), 200 µM $MnCl_2$ + 104 µM $CoCl_2$(orange marker), 200 µM $MnCl_2$ + 109 µM $CuCl_2$ (grey marker), and 200 µM $MnCl_2$ + 104 µM $CoCl_2$ + 109 µM $CuCl_2$(yellow marker)

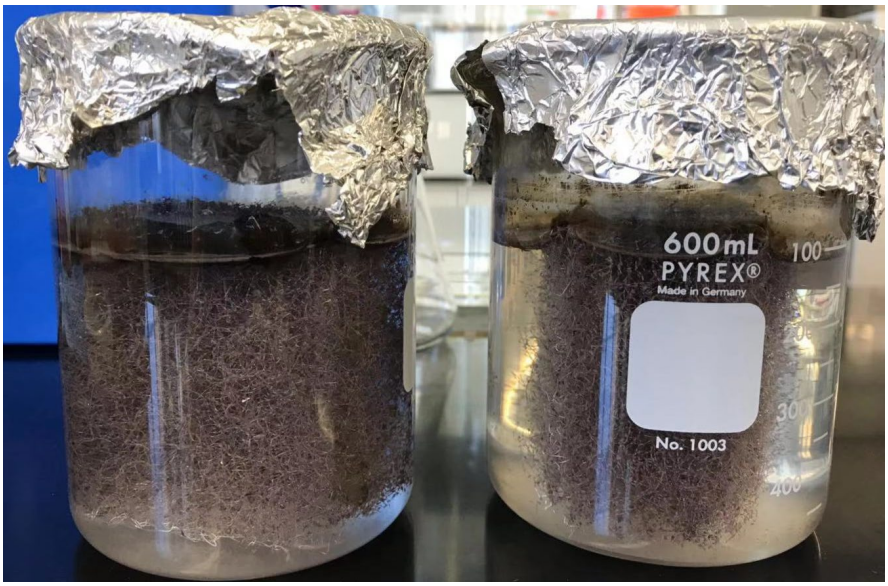
Final Activity Summary: Two types of laboratory scale bioreactors have been built to optimize or demonstrate metal removal by fungi from contaminated waters. The Type 1 bioreactor is specifically developed for outreach purpose. This bioreactor consists a thick layer of biomass + Mn oxides at the bottom and a layer of sand on the top. The input crystal violet dyed solution, representing contaminated waters, turns clear after passing through the reactor. Type 1 bioreactor is a simple and safe demonstration of a bioremediation strategy showing that fungi have potential to remove pollutes from contaminated waters. We will do demonstration experiments use type 1 bioreactor to general audiences on August 22nd, 2019 at the Minnesota State Fair with Market Science. Several of these bioreactors are being sent to the Soudan Underground Mine to be used on the park tours to demonstrate the research being done, and the potential for fungi from the mine to be used for sustaining a healthy environment.



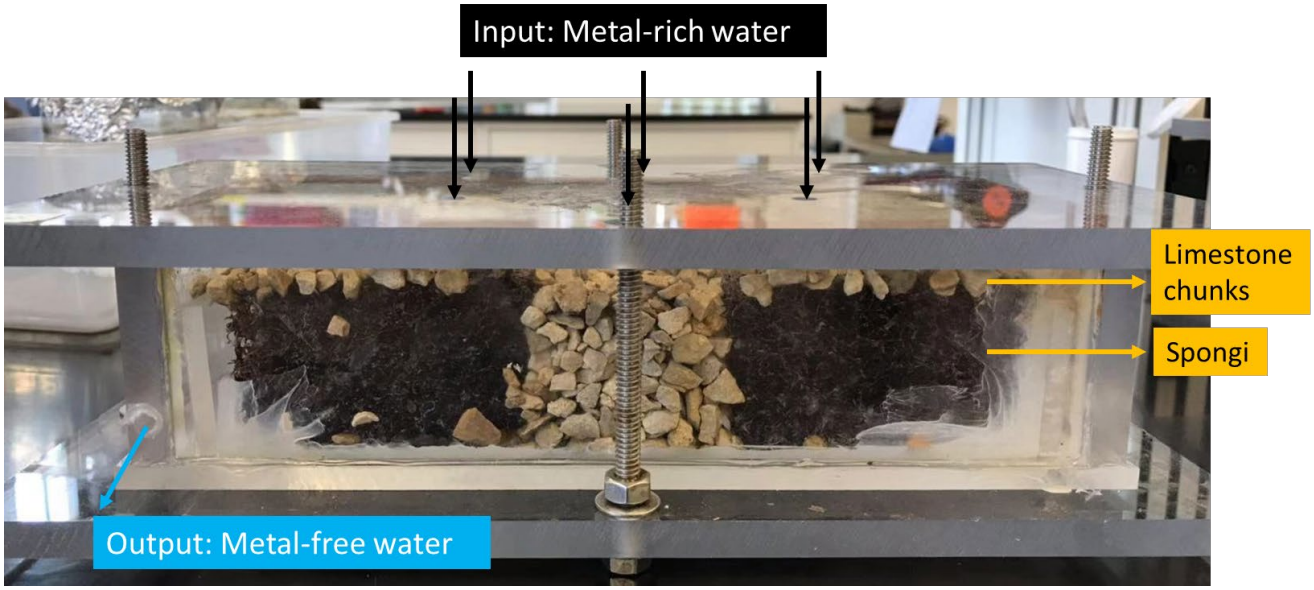
The above figure is type 1 bioreactor, consisted of biomass (F1) mixed with Mn oxides and covered by sand. The input purple solution is water dyed by crystal violet, representing metal contaminated water. When passing type 1 bioreactor, the purple solution becomes clean, resulting in the output clean water.

Type 2 bioreactor (flow through reactor) has been built to optimize metal removal by fungi from salty, metal-contaminated waters. This is similar to the design for development of a field-scale bioreactor at the Soudan Mine or other environments where multiple metals co-occur in waters (e.g., industrial waste water; Mn-rich municipal waste water). This bioreactor is loaded with "spongi" and covered by limestone chunks. Spongi is prepared by growing fungi F1 together with sterile sponges in brine (salty) + manganese media. After two to three weeks of inoculation, spongi will accumulate enough biomass and can to be loaded into the reactor. The input solution is complex, metal-rich waters with their flow rates controlled by a programmable continuous

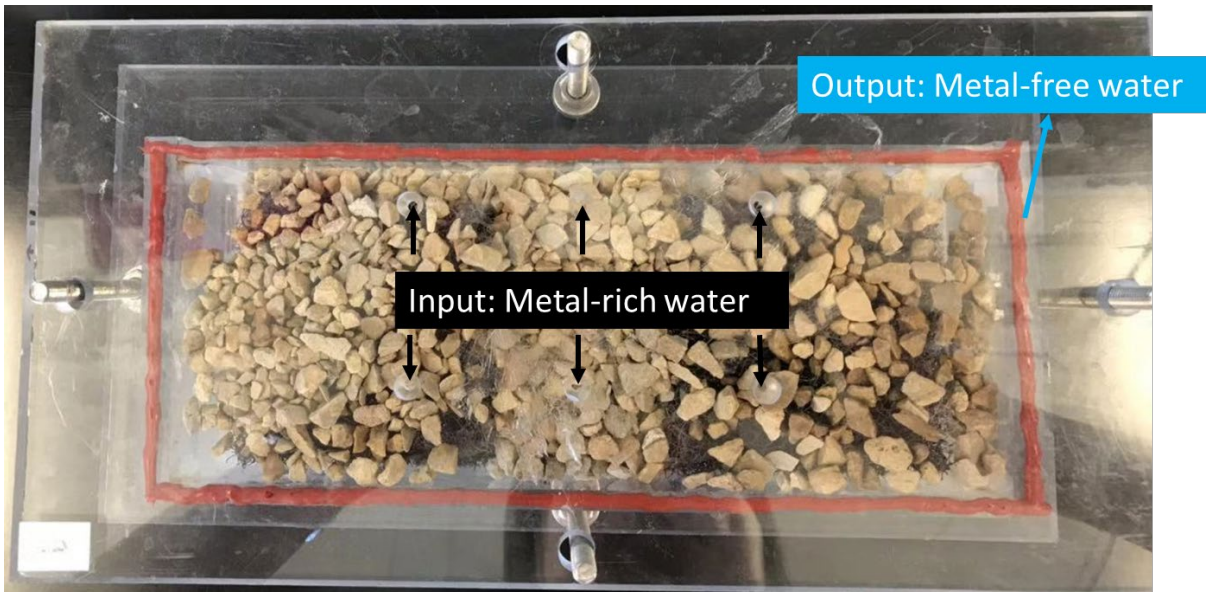
infusion syringe pump. Our test experiments use brine + Mn media with 104 µM $CoCl_2$ as the input solution at a flow rate of 40 mL/h result in the outlet solution. The preliminary results suggest that this type 2 bioreactor can effectively remove Co from lab prepared brine solution with concentrated Co, though analyses are still ongoing. This indicates that that these reactors may also be effective to remove Cu or other metal of interests from real metal-contaminated waters, but these analyses are currently underway. More experiments targeting different metal contaminates, using lab prepared or real mining waters and optimizing related parameters (e.g., sponge size, spongi inoculation time, and flow rate) are needed to build the final products.



Examples of spongi (fungi F1 grown in sponges in brine + manages media) growing in briny media for two weeks.



The above figure is the side view of type 2 bioreactor. The input metal-rich water will pass through the reactor at constant flow rate controlled by a programmable continuous infusion syringe pump.



The above figure is the top view of type 2 bioreactor.

Final Activity Summary:

We have identified several fungal species from the Soudan Underground Mine that can promote the formation of solid Mn oxide biominerals in metal-rich, salty waters. Isolate F1 (*Periconia sp.)* has been identified as the best fungal species for metal removal from metal-rich waters. Fast rates of metal removal were achieved with this species when exposed to multiple metals. We completed batch laboratory experiments to identify metal removal pathways by F1. Our results show that nearly all Mn is removed through formation of solid Mn oxide minerals within two days; Co and Ni is removed by biogenic Mn oxides through adsorption and/or structural incorporation, usually within 5 days. Based on the quick removal of Mn, Co, and Ni, we have built different types of laboratory scale reactors using F1-mediated substrates. Our flow through bioreactor (type 2) shows great potential to remove metals from metal-rich briny waters. This could ultimately lead into the design and testing of a field-scale bioreactor using the fungi isolated from the Soudan Mine.

V. DISSEMINATION:

The primary mode of dissemination will be via peer-reviewed articles and status reports. Late in the project, we will also convene at least one joint meeting with our entire LCCMR project group and potential collaborators/partners from UMN-Duluth, NRRI, DNR and other interested parties who may be able to adopt our technology as we begin construction of pilot-scale reactors.

Description:

Status as of *January 1, 2017***:** None to report
Status as of *July 1, 2017***:** None to report
Status as of *July 1, 2018***:** None to report
Status as of *January 1, 2018***:** None to report

Final Report Summary of Dissemination:

Our primary scientific dissemination activities are manuscripts crediting this project, two of which are under revision or submission and not available online at the time of this report. We presented our results at the 2nd Geobiology Society Conference in Banff, Canada, in June 2019 in the poster section titled as "Remediation of Metals by Mn-Oxidizing Fungi in Minnesota Soudan Iron Mine". Other examples of local exposure include also the Mycological Society of America 2019 Annual Meeting in Minneapolis, MN, in August 2019. In October, the research results will be presented at the Society for Mining Engineers conference in Minneapolis, MN.

As part of this project we conducted outreach activities to show the potential offered by bacteria powering salt-removal devices. Some examples of outreach during this project include: three 'Market Science' events, bringing demonstration devices to farmer's markets in the Twin Cities area, three events as part of the Bell Museum's 3rd and 4th-grade science camps where students constructed microbial powered devices and meet scientists in our laboratory, assisting two local Lego League teams who were incorporating microbial power into their demonstration projects and providing materials for their devices (one group progressed to the State competition), hosting a short workshop training graduate students in construction of microbial electrochemical devices, participation in the MN clean water summit and the American Society for Microbiology science outreach series. Our other stated goal was to facilitate group meetings with other collaborators and interested parties so this work could expand or continue. Due to these collaborations, work initiated in this project in terms of searching for new organisms from metal-impacted environments will be able to continue in a 5-year NSF-funded project to be based in the Soudan Mine, fulfilling a key goal described in our Long Term Strategy. We have also applied for new support from other state-based programs (such as MNDrive) to support the scale-up of new technologies for bioremediation.

We will continue to share these results, including demonstration experiments about bioremediation strategy use the type 1 bioreactor to general audiences on August 22nd, 2019 at the Minnesota State Fair with Market Science. Further, these type 1 bioreactors will now be regularly prepared for Sound Underground Mine State Park science tours of the mine. Mine tour guides will demonstrate these at the mine, and the cultures have been shared for observation under a microscope in collaboration with State Park staff.

Examples of outreach activities with Bell Museum campers, Hamline Elementary students, and workshops for students educating students about microbial-powered devices. These images involve minors and should not be shared via social media or other platforms.

Outreach activities with Market Science and other Twin Cities locations. The white-topped devices are powered by bacteria, and provide a blinking light to demonstrate how even mud can be a power source with the right microorganisms. These images involve minors and should not be shared via social media or other platforms.

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget Overview:

Budget Category	$ Amount	Overview Explanation
Personnel:	$ 430,900	1 postdoc ($157,109) for 3 years, and 1 graduate student (~$113,849) for 3 years, to lead enrichment, isolation, and analysis of bacteria essential to microbial desalination, along with construction and operation of desalination reactors. 1 graduate student for 2 years ($84,356) , plus summer salary support for two faculty for 3 years ($75,856) leading isolation and characterization of new fungi able to bind and precipitate metals from water.
Professional/Technical/Service Contracts:	$ 0	
Equipment/Tools/Supplies:	$ 154,600	Parts for construction of new reactors (~$11,100), operation of larger reactors ($28,000) reagents for molecular biology (~$30,200), DNA sequencing and analysis, geochemical analysis such as water and XRD measurements (~$18,200), electrochemistry supplies such as membranes and electrodes ($19,500), laboratory consumables (~$27,200).
Travel Expenses in MN:	$ 6,000	Routine sampling of Soudan and other areas, obtaining water for desalination and metal recovery
Other:	$ 4,500	Peer-reviewed journal costs.
TOTAL ENRTF BUDGET:	**$** 596000	

Explanation of Use of Classified Staff: N/A

Explanation of Capital Expenditures Greater Than $5,000: N/A

Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation: N/A

Number of Full-time Equivalents (FTE) Estimated to Be Funded through Contracts with this ENRTF Appropriation: N/A

B. Other Funds: The University of Minnesota will provide administration, laboratory space, and maintenance at no cost, estimated value below.

Source of Funds	$ Amount Proposed	$ Amount Spent	Use of Other Funds
Non-state			
	$	$	
State			
No indirect cost recovery is being claimed on this proposal. Based on UMN policy of 52% indirect costs on all non-graduate student fringe and non-equipment funds, approximately $273,000 in administrative and overhead support is being provided by the University of Minnesota	$ 273,000	**$ 273,000**	Salaries for Bond and Gralnick, University administration and maintenance, etc.
TOTAL OTHER FUNDS:	**$ 273,000**	**$ 273,000**	

VII. PROJECT STRATEGY:

A. Project Partners:

Team Leader: Dr. Daniel Bond (UMN) is an Associate Professor of Microbiology and the BioTechnology Institute. He performed many of the original experiments discovering microbial electricity production by metal-reducing bacteria and will direct construction of microbial desalination reactors.

Dr. Jeff Gralnick (UMN) Associate Professor in the Department of Microbiology and the BioTechnology Institute is an expert in electron transfer by bacteria, and led the LCCMR project that discovered the bacteria tolerant of extreme conditions used in this proposal, and pioneered the genetic analysis of these organisms using high-throughput methods.

Dr. Brandy Toner (UMN) Associate Professor in the Soil, Water and Climate Department is an expert in geomicrobiology and toxic metals, and responsible for all mineralogical and metal analyses. Dr. Toner conducted the original experiments to document metal binding by Soudan Mine fungi.

Dr. Robert Blanchette (UMN) is an expert in fungal biology, and discovered the fungi able to adsorb metals in our previous LCCMR project.

Dr. Cara Santelli (UMN) is an expert in fungi active in mining and metal-impacted sites, and discovered fungal strains able to directly precipitate metals on their surfaces. She will be responsible for new fungal discovery and reactors operated upstream of microbial desalination cells.

Jim Essig (DNR Park Manager of Soudan Mine State Park) is an additional partner (not funded by ENRTF) include who will help coordinate research activities.

B. Project Impact and Long-term Strategy:

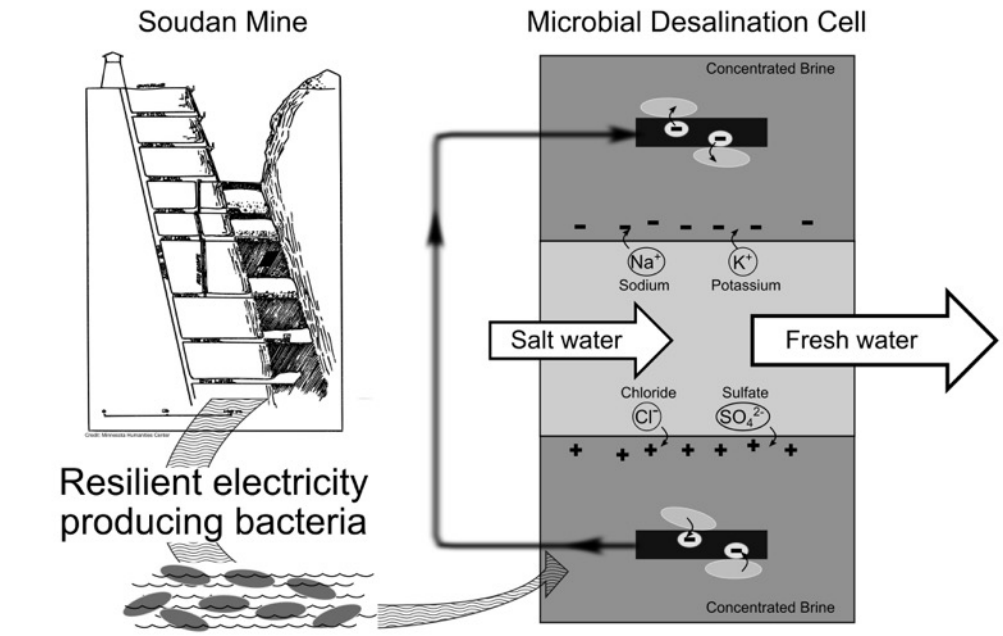
The proposed work is based on a discovery from our current LCCMR program, which first discovered a range of exotic life in the abandoned mine, then explored the unique microbiology of the Soudan Mine in search of new drugs, bacteria, and remediation strategies (LCCMR 2010-2013 and 2013-2016). We are now prepared to demonstrate an application of these newly discovered bacteria as some of these microbes can be used in industrial and environmental settings. This funding is essential to the scale-up and demonstrations that protect the Intellectual Property of the microbial component, while we will obtain federal funding (National Science Foundation, Department of Energy, United States Department of Agriculture) to support future work understanding the biology enabling this technology. We will share our reactors and organisms with collaborators at NRRI and the University of Minnesota-Duluth for implementation studies related to bioremediation.

C. Funding History:

Funding Source and Use of Funds	Funding Timeframe	$ Amount
ENRTF, ML-2010-"Science and Innovation from Soudan Underground Mine State Park", The first LCCMR project to support visits to the Soudan Mine with bacterial sampling, led to discovery of bacteria on different levels of Soudan Iron Mine.	2010-2013	$545,000
ENRTF ML 2013-03f "Harnessing Soudan Mine Microbes: Bioremediation, Bioenergy, and Biocontrol", This project focused on isolation of bacteria producing antibiotics and agents for control of White Nose syndrome, as well as bacteria that could possibly produce electricity at electrodes. Two strains discovered in 2013-03f will be utilized in the project proposed here.	2013-2016	$838,000

VIII. FEE TITLE ACQUISITION/CONSERVATION EASEMENT/RESTORATION REQUIREMENTS: N/A

IX. VISUAL COMPONENT or MAP(S):



X. RESEARCH ADDENDUM: Submitted in a separate document.

XI. REPORTING REQUIREMENTS:

Work plan status update reports will be submitted after January 1, 2017, July 1, 2017, January 1, 2018, and July 1, 2018. A final report and associated products will be submitted by August 15, 2019.

Environment and Natural Resources Trust Fund
M.L. 2016 Project Budget

Project Title:Reducing salt and metal removal costs with microbes
Legal Citation M.L. 2016, Chp. 186, Sec. 2, Subd. 04o
Project Manager: *Daniel R. Bond*
Organization: *University of Minnesota*
M.L. 2016 ENRTF Appropriation: $596,000
Project Length and Completion Date: 3 ***Years, June 30, 2019***
Date of Report: 8/2019 (Final)

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Activity 1 Budget	Amount Spent	Activity 1 Balance	Activity 2 Budget	Amount Spent	Activity 2 Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	***Microbe-powered desalination***			***Fungi-mediated metal removal***				
Personnel (Wages and Benefits), Overall Costs:	$270,958	$270,958	$0	$159,942	$159,942	$0	$430,900	$0
Postdoc *#1 : Design, build, operate microbial desalanation cells. (83% salary, 17% benefits), 1 FTE/y for 3 years, estimated at $157,109*								
Graduate Student *#1 : Cultivate, test, and provide bacteria capable of electricity production and metal precipitation (81% salary, 19% benefits) 1 FTE/y for 3 years, estimated at $113,849*								
Graduate Student *#2 : Cultivate, test, and provide fungi capable of metal recovery (81% salary, 19% benefits) 1 FTE/y for 2 years, estimated at $84,356*								
Assistant Professor Cara Santell *: mentor undergraduate research and graduate student #2, assist with field work and all activity 2 objectives (1 month summer salary, fringe benefits, 3 years). One month of summer salary is $8,716 in the first academic year with 3% salary increase for the following project years. The fringe benefit for Nine - Month B - term faculty is 33.7% based on the University regulation, estimated at $36,022*								
Associate Professor Brady Toner *: mentor undergraduate research and #2, assist with field work, mentor and assist with geochemistry analyses (1 month summer salary, fringe benefits, 3 years). One month of summer salary is $12,800 (in the first academic year with 3% salary increase for the following project years. The fringe benefit for Nine - Month B - term faculty is 33.7% based on the University regulation, estimated $39,564*								
Equipment/Tools/Supplies: Overall Costs:	$98,400	$98,400	$0	$56,200	$56,200	$0	$154,600	$0
Machine shop charges for desalination and metal removal reactors at lab scale, including membranes, ports and gaskets ~$1500/each, estimated total $11,100								
Bioreactors: power supplies, peristalitic pumps, gas controllers, temperature, salinity and pH monitoring sensors to document water quality, estimated total $28,000								
Laboratory consumables; gloves, sterile pipet tips, tubes, syringes, stoppers, glassware for cultivating bacteria in the absence of oxygen, ~$3200/y per active researcher, estimated total $27,200 based on historical average								
Molecular biology reagents: polymerase chain reaction enzymes, restriction enzymes, plasmid and PCR miniprep kits, cloning reagents, sequence verification, DNA synthesis, ~$3800/y per researcher, estimated costs $30,200								
Electrochemical consumables: reference electrodes, wire and electrodes, anaerobic grade gasses and catalysts to remove oxygen, membranes. ~$3500/y per researcher (2 researchers), estimarted costs $19,500								
DNA sequencing (Mayo clinic or UMN sequencing center, $1500/genome or metagenome sample) Imaging of electrodes ($40/h), software licenses ($300/y), estimated costs $20,400								
Geochemical analyses for measuring concentrations of metals in batch experiments and laboratory bioreactors ($32/sample; 500 samples = $16,000); X-ray diffraction mineral analyses $32/hour; 20 hours = $640; columns and detectors for HPLC/GC/IC analysis ($500/y), estimated costs $18,200								
Travel expenses in Minnesota								
Routine sampling and research trips to Soudan Mine (~4x/y), includes one night lodging and vehicle costs according to University of Minnesota reimbursement rates	$4,000	$1,154	$2,846	$2,000	$391	$1,609	$6,000	$4,455
Other							$0	
Publications and dissemination of results in Open Access (non-restricted) journals	$3,000	$1,915	$1,085	$1,500	$77	$1,423	$4,500	$2,508
COLUMN TOTAL	**$376,358**	**$372,427**	**$3,931**	**$219,642**	**$216,610**	**$3,032**	**$596,000**	**$6,963**