Bacterial Soil Rejuvenation, A Process to Initiate Viridescence, Restoration and Repair of Natural Resources Damaged by Mining that Impact Butte Area One

A Proposal to the Montana BNRC/NRDP

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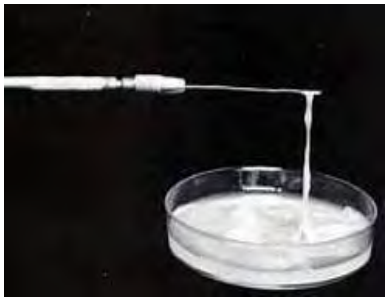

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"Here is the means to end the great extinction spasm. The next century will, I believe, be the era of restoration in ecology."- E.O. Wilson

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Restoration and Repair of Natural Resources Damaged by Mining that Impact Butte Area One

A. Contact Information

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B.1. Project Summary and Map

Microbial Biorestoration is the practice of renewing and restoring degraded, damaged, or destroyed soil ecosystems by active human intervention. In the case of Butte, Montana that soil ecosystem was destroyed by mining activities and/or the subsequent deposition of tailings which stripped the soil of organic carbon and indigenous bacteria. As a result, the soil ecosystem died; it has lost its ability to recycle essential nutrients through normal biogeochemical processes, thus preventing most plant life and other organisms from growing. Sheoran (2010) found that when soil layers were disturbed by mining, that microbial activity was slow to resume independently. The reestablishment of free living, nitrogen fixing soil bacteria and other associated microorganisms that were lost, will recover the natural soil ecosystems to restore damaged areas by transforming them into normal, steady state, biodiverse ecosystems which will become gradually viridescent again. (Zhan 2011). Viridescence is a term used to describe something becoming green or alive again. In this case, it would be the brown mine tailing soils beginning to support life again. Researchers have demonstrated that by reintroducing nitrogen fixing bacteria can reestablish stable soils that have been depleted of bioavailable nitrogen and restore microbial activity. As a result, there are many commercial agricultural inosculates available; however, these organisms will not fix nitrogen in acidic soils of below pH 4. J.H. Becking (1961) discovered that organisms in the genus *Beijerinckia* were exceptions to this nitrogen physiology axiom and that they could fix nitrogen in acidic, metal rich soils. These organisms are ideal for initiating microbial biorestoration of mine waste sites and reestablishing the microbial soil ecosystem that was once present. These extremophiles also have tremendous potential for the initiation of natural revegetation of mine waste areas due to the generation of bioavailable nitrogen. First, these bacteria do not require good quality soil; they produce it by the generation and secretion of copious amounts of extracellular polysaccharides (see cover page), and bioavailable nitrogenous compounds. These two components are well known to improve soil aggregation and progressively affect plant growth.

The rehabilitation of mine dumps or disturbed areas by native and/or nonnative vegetation is usually a very slow or even a nonexistent natural process. The removal of mine waste, capping and replacement of soil, and subsequent replanting of the affected areas is usually necessary. This removal/capping restoration is an expensive project, and when a responsible party cannot be identified to pay for this restoration, the sites remain barren. Consequently, rains and wind continuously transport the contaminants from the overburden into the aquifer. Microbial communities and their associated organisms will slow or prevent this process. They create rich soil by building up organic material from their growth and by trapping silt and soil from the environment

(wind and rain). Huang (2011) found that nitrogen fixing bacteria were essential during the primary succession on copper mine tailings. Finally, microbial communities are self-maintaining without supplemental irrigation (Sinisbaugh 2015). These organisms are not only resistant to metals and metalloids, but they also adsorb these toxic substances and remove them from the surrounding soil. In this way, microbial communities provide a foundation for a natural succession of other plant communities to build a stable ecosystem.

This grant proposes a specific research goal. The practical application is to produce large amounts of lab grown *Beijerinckia* in culture vats (aspirator bottles), and subsequently introduce them back into the disturbed areas to initiate soil restoration in test areas of BAO (see Maps 1-5). A mixture of *Beijerinckia* medium with 2% sugar medium will be applied to these test sites to improve the establishment and growth rates of nitrogen fixing bacteria and subsequently phototrophs. This data will provide an understanding of how researchers can initiate repair and restoration of mine waste environment soil by use of acidophilic, polysaccharide producing, free living, nitrogen fixing bacteria. This proposal requests $40,421 for a PI to study and restore mine waste damaged ecosystems from extreme to mildly contaminated soil areas affecting the BAO (Butte Area One) watershed. Funding of this proposal will be a significant opportunity to invest in the future of restoration and repair of natural resources by investigating the soil repair potential of these extremophile microbes.

B.2. Background

Beijerinckia is an organism in the Family: Beijerinckiaceae, Phylum: Proteobacteria and class: Alphaproteobacteria. The Genus name proposed by Derx in 1950 honors the Dutch Microbiologist whom is quoted as saying “Everything is everywhere, but, the environment selects” ‘Geobiologie of inleiding tot de milieukunde’ (Geobiology or introduction to the science of the environment) by Professor Lourens Gerhard Marinus Baas Becking 1934. Historical records document that *Beijerinckia* has been isolated from over 392 soils ranging from temperate to tropical soils throughout the world including North America (Snake River Plain, Idaho) (J-H Becking 2006), and they probably occur in fertile Montana soils as well. These nonpathogenic bacteria are known for their ability to grow on acidic soils, rich in metals and promote soil fertility by nitrogen fixation and polysaccharide production among their other metabolic activities (Becking 1961). They have the ability to survive in a spectrum of environmental conditions: from nutrient-rich to nutrient-poor media, from acidic soils of pH 3 to more basic soils of pH 10, under varying levels of oxygen availability, temperature fluctuations, varying degrees of moisture saturation, and within certain forms of contamination, of which is remediated (Dong-Hee 2010). They have even been known to metabolize toxic petroleum distillates including xylene. They are also well known for their copious polysaccharide slime production which helps to revitalize the soil (front cover).

Preliminary experiments (McLain Biology undergraduate thesis 2012) demonstrated that the Badger State Mine Soil treated with *Beijerinckia indica* in the lab began to revitalize the soil as demonstrated by increased bacterial numbers and increased soil viridescence by growth of organisms including, fungi, mosses, algae (Figure 1-3). In McLain’s experiments either 2% glucose or moss

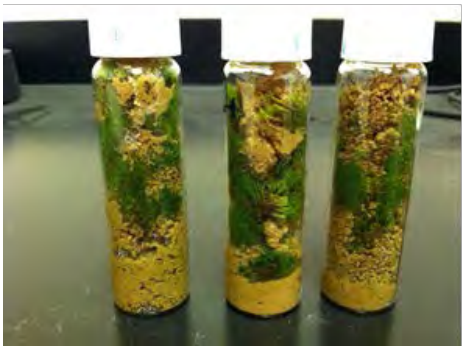
was added as a carbohydrate source or carbohydrate producer respectively. In both cases, after 6 weeks, bacterial cell numbers increased an order or magnitude (e.g. ~$1.7x10^7$ to $2.2x10^8$). In treatments where no moss or phototrophs were added there was no significant increase in bacterial numbers or viridescence. The 2% glucose addition seemed to be a good "starter" to activate the bacteria until carbohydrate producing phototrophs could develop to make the soil a self-sustaining ecosystem. Once photosynthetic organisms develop in the soil they will produce a constant supply of secreted organic carbon to maintain bacterial populations and restore the soil ecosystem and restore the normal biogeochemical cycles. The reintroduction of these bacteria to the soil can help reestablish microbial mats which many scientists consider to be the best restoration technology available for soils and also substrates for the initiation of stable, plant community succession.

Butte, Montana is an area rich in mine waste sites that need revegetation, restoration and repair of its natural resources. It is easy to find hundreds of sites characterized by years of mining, milling, and smelting waste to test the bacterial restoration processes. Thus, the research potential for this restoration approach is tremendous. Nevertheless, the only solutions to restoration are often chemical in nature or involve physical removal of soils, and researchers' often overlook biological aspects. When they restore a site, implementation of the species composition of these sites is critical, because not all organisms will grow at any particular site. Thus, it is critically important that organisms used to restore the specific site are suited to the specific substrates in question. This is why a microbial mat is the ideal solution for revegetation of mine waste; it can be applied to any site and revitalize the soil and encourage pioneer organisms to grow. Introducing nitrogen fixing bacteria to the soil is not a new idea. Environmental restoration companies are currently implementing soil amendments in many parts of the world to initiate revegetation. However, this technology has not worked previously for mine waste areas because of the pH 4 limitation for Nitrogen Fixation. To my knowledge, although *Beijerinckia* has been isolated from wastelands of copper mine tailings during the process of natural ecological restoration (Zhan 2011, Huang 2011), it has never been purposefully introduced to accelerate the process. This research proposes to implement and speed up this natural successional process.

Figure 1: Treated and innoculated Winogradsky Tubes on incubation day 1. (McLain 2012).

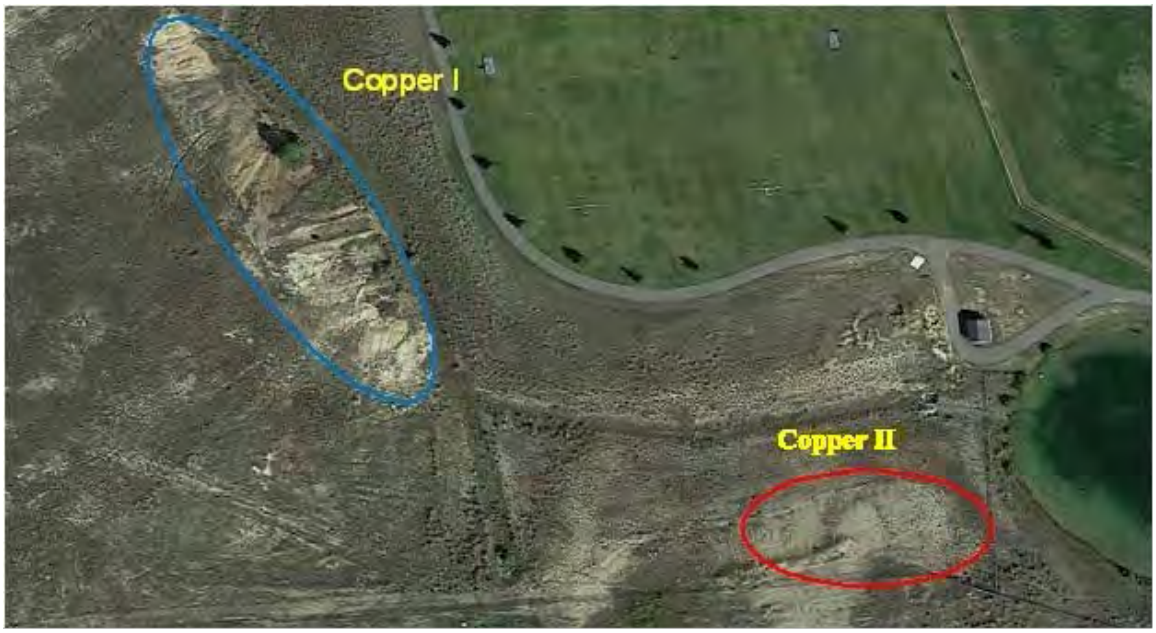
Figure 2: New moss growth. bacteria + moss in Winogradsky Tube after 6 weeks incubation (McLain 2012).

Figure 3: Bacteria + moss treated Winogradsky Tubes at week 6 of incubation. From L to R (pH): 5.4, 6.4, 7.4. (McLain 2012).



Map 1. Copper Mountain Recreation Complex-Arial View**.**

Map 2. Copper Mountain Recreation Complex-close up.



Map 3. Ryan Mine and Walkerville Drive

Map 4. Ryan Mine Close-up.

Map 5. Walkerville Drive -Close-up

C. Project Goals and Objectives

The Primary goal of this project is to develop an easily replicated method to restore nitrogen fixation to mine impacted soils not designated for natural resource repair, sites that are cost prohibitive to address, locations for which there is not a responsible party, or overburden that is found on private land. This technology would address natural resource recovery for the many locations affecting BAO for which the expensive removal and replacement or capping and replanting of the affected areas does not occur, for the previously listed reasons. Without soil restoration of some kind, the persistent barren mine waste sites ensure the continued erosion and transport of the contaminated overburden into the BAO aquifer. Secondary goals of this project will involve the development of bacterial application mixture for mine waste areas.

The Project Goals and Objectives pursued under this project are described under Project Benefits below:

D. Project Benefits

To summarize, the project benefits/objectives of this research includes:

1. Restoration of nitrogen fixation and repair of mine waste affected areas.
2. Restoration of a native soil microbial ecosystem that existed before mining in Butte.
3. Provide a suitable substrate for the initiation of stable soil ecosystem.
4. Development of an easy and cost-effective protocol to restore soil ecology.
5. Provide a soil water retention ecosystem that will reduce transport of contaminated water runoff and windblown soil into the BAO aquifer.
6. This Project represents a prototype of a low cost, easily reproducible method to revitalize barren mine waste and other areas.
7. Information obtained from this project will be used to construct a poster for display purposes for various venues.
8. Publish a step by step “how to” pamphlet, so that individuals and other agencies may continue to use this application technology.

It is important that this technology be easily transferable to anyone who wishes to implement it, and not be buried in a scientific publication.

E. Project Implementation

E.1 Task 1. Bacteria Collection

Beijerinckia indica indica (B-4324) bacteria will be the primary strain used in this experiment. It will be obtained as a freeze dried culture from the United States Department of Agriculture Culture Collection National Center for Agricultural Utilization Research Unit (http://nrrl.ncaur.usda.gov). The USDA maintains cultures for this type of research and provides them free of charge to researchers. Other species of B*eijerinckia* will also be obtained for testing to determine the best strain including: *B. derxii congensis, B. derxii derxii, B. derxii*

venezuela, B. indica lacticogenes, B. fluminensis, B. mobilis, and Derxia gummosa. Isolation of naturally occurring nitrogen fixing bacteria from Butte mine waste soils will also be attempted to determine if natural populations exist or may be stimulated to grow by the addition of *Beijerinckia* medium.

E.2. Task 2. Bacteria Propagation

Bacteria will be propagated large culture vats (aspirator bottles) using sterile technique with standard *Beijerinckia* medium: (Becking, J. H. 2006)

Distilled water	1 liter
Glucose	20.0g
K_2HPO_4	0.8g
KH_2PO^4	0.2 g
MgSO4 $_2$O7H	0.5g
$FeCl_3 \cdot 6H_2O$	0.05g
$Na_2MoO_4 \cdot 2H_2O$	0.005g
$CaCl_2$	0.05g

During this growth task, techniques for rapid bacteria propagation will be developed so that large scale application and implementation methods will be practicable in the future. This process will be continuous since bacteria will be needed for the duration of the soil study.

E.3. Task 3. Bacteria Strain Testing

Different Bacterial strains will be tested on mine waste soil in the lab to determine their optimal growth rates and best species to use. This will be determined by analysis of bacterial numbers/gram of soil and amount of bioavailable nitrogen present. Also, a variety of carbon sources at 2% solution will be tested including sucrose (table sugar), glucose and Soil Moist Starch$^{TM.}$

E.4. Task 4. Soil Analysis

Soils from each site will be characterized for metals and nitrogen by the University of Georgia Laboratory for Environmental Analysis (LEA) so that for future work specific bacteria may be associated with metal concentration for continued revegetation projects. pH and other physical soil parameters will also be collected. Soils from the four sites will also be characterized for total number bacteria/gram and total nitrogen.

E.5. Task 5. Bacteria Application.

Application will begin as soon as bacteria can be grown in sufficient quantity and applied to each of the four (4) sites (see maps B.3., Maps 1-5. Only the fastest growing and best nitrogen fixing species from lab trials will be used. Optimal carbon source from lab trials will also be used. Bacteria/*Beijerinckia* Medium mixture will be applied by a commercial backpack sprayer at an application level determined by laboratory experimentation. In addition to application, two controls will also be used: 1) *Beijerinckia* medium without bacteria, and 2) no treatment as delineated in Table 1.

Table 1. Treatment Matrix.

	Beijerinckia	**No *Beijerinckia***	**No treatment**
Beijerinckia	*Beijerinckia*		
No *Beijerinckia*		No *Beijerinckia*	
No treatment			No Treatment

E.6. Task 6. Dissemination of Results

Data, results and their interpretation will be reported to BNRC/NRDP in the form of technical reports. A report and presentation will be available to BNRC each year with a final report submitted spring 2018. In addition, a step by step "how to" pamphlet will be produced on how to make use of the technology of bacteria for soil restoration and repair of natural resources. Finally, a poster will also be produced that summarizes our findings and how to implement Bacterial soil restoration. This will be presented to BNRC and made available to any other interested parties on request.

F.1. Project Schedule

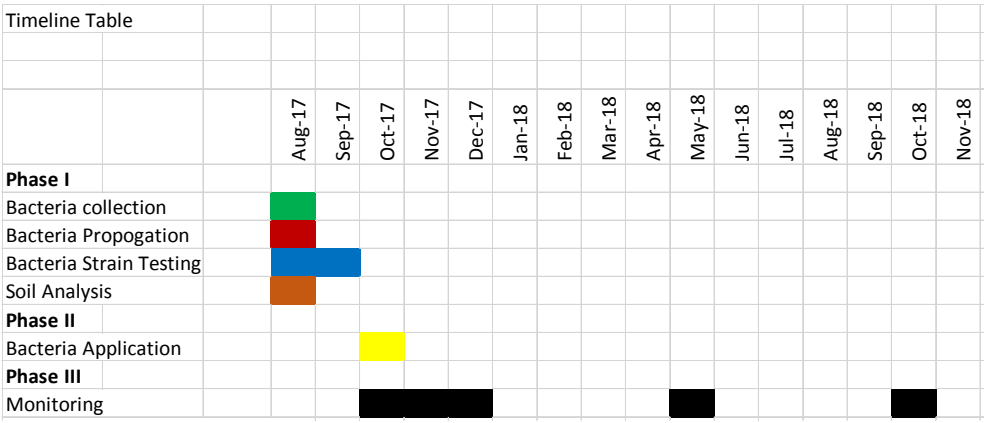
The proposed work will begin as soon as funding becomes available and as soon as the supplies can be delivered. The work plan for this one-year project will consist of three phases:

Phase I will consist of bacteria propagation, strain and substrate testing and, soil analysis.
Phase II is application of bacteria to the damaged sites.
Phase I, II and III will occur all within the first year of the project, monitoring will be the next year.
Phase III will consist of monitoring beginning in year one (2017) and continue until fall 2017. Initial monitoring will be conducted every month the first year and May and October, when there is no snow coverage.

A report and presentation will be available to BNRC/NRDP after the first year with a final report submitted fall 2018.

G. Monitoring Activities

Initial monitoring of quadrats for the four sites studied will be conducted every month the first year and May and October, when there is no snow coverage. After application to the complete area of the four sites, data will be sampled by observing random quadrants along a transect line. In order to determine project effectiveness, the increase in bacterial numbers/gram, total nitrogen present, and appearance of moss and other phototrophs (algae and plants) will be assessed in terms of increases in both species diversity and plant growth percent cover and explained by bio statistical analysis. Below is a tentative schedule that will have to be modified depending on when funding becomes available.



H.1. Project Budget

	Monthly	# of		# of	Cost
A. SALARIES AND WAGES	**Rate**	**Months**	**BNRC**	**Months**	**Share**
Grant Mitman	$9,990	2	$19,980	2	$19,980
Subtotal			**$19,980**		**$19,980****
B. FRINGE BENEFITS					
46% Contract Professionals			$9,191		$9,191
Subtotal			**$9,191**		**$9,191**
C. OTHER DIRECT COSTS					
Supplies			$10,000		
Travel			1,250		
Subtotal			**$11,250**		**$0**
D. TOTAL COSTS			**$40,421**		**$29,171**

** If the project funding begins after 9/1/2017, the cost share salary and fringe amount will be prorated and will not extend past 5/15/2018. Salary cost share is only available during the 2017-2018 academic year.

F.2. JUSTIFICATION OF BUDGET

The research supplies requested for this proposal covers the goals for this research to evaluate *Beijerinckia* for soil restoration.

Salaries: Two months of salary are requested as salary for Grant Mitman (PI) from BNRC/NRDP and will matched by 2 months of time (1:1) as a match by Montana Tech.

Benefits: Benefits are 46% of contract professional salary.

Other Direct Costs

Supplies: Aspirator bottles (bacterial growth bottles), $2500.00, Soil analysis University of Georgia (LEA) $2,500.00, Backpack sprayer $375.00, Soil $375.00, Growing trays $1250.00, other supplies (to include lab consumables and shipping) $3000.00. Total $10,000.00.

Travel: $1,250 to support travel costs to the testing sites.

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