Letting the Microbes Do the Cleanup: Arsenic Bioremediation of Butte Area Soils

A Proposal to the Butte Natural Resource Damage Restoration Council and the Montana NRDP

October 16, 2015

Dr. Tony Hartshorn
Assistant Professor, Dept. Land Resources and Environmental Sciences
Montana State University
811 Leon Johnson Hall, Bozeman, MT 59717
anthony.hartshorn@montana.edu
406-994-6323

A. Project Summary

Title: Letting the microbes do the cleanup: Arsenic bioremediation of Butte area soils
Sponsor: Montana NRDP
Location: Within Butte Priority Soils Operable Unit boundary: we are currently working with Mr. Eric Hassler to identify the most appropriate site(s) for this project (Figure 1)
Total Dollar Amount: $99,820 NRDP; $14,250 Match
Project Leader: Dr. Anthony Hartshorn, Montana State University
Project Start Date: December 1st, 2015 (or when funding is in place)

This proposal contains a two-phase project that explores an innovative and cost-effective *in situ* soil bioremediation strategy that promotes native plant establishment and growth through the reduction of soil arsenic toxicity within the Butte Priority Soils Operable Unit boundary. The PI, one graduate student, and undergraduate students from MSU will utilize microorganisms capable of transforming arsenic into less toxic and less mobile forms (bioremediation) to treat contaminated soil in place. In a combination of greenhouse and field experiments, we will build upon Montana NRDP-funded work on Butte area metal-tolerant plants (Montana Tech Native Plant Project) to determine if the use of microbes will increase plant growth and plant uptake of arsenic, or in contrast, increase plant growth and avoidance of arsenic. If our findings suggest arsenic can be successfully concentrated into plant shoots through plant uptake, these shoots could be harvested, incinerated, and delivered to the local waste repository at a fraction of the cost of excavating contaminated soil. Alternatively, if our work suggests plant avoidance through arsenic immobilization in the rooting zone, even this outcome should confer greater protection to Silver Bow Creek and downstream water resources. We will compare soil arsenic characteristics and plant growth responses and arsenic uptake following this bioremediation treatment to traditional, more costly soil remediation amendments (i.e., lime and organic matter). This project has the potential for widespread application to improve soil resources and reduce human health risks in the Butte area.

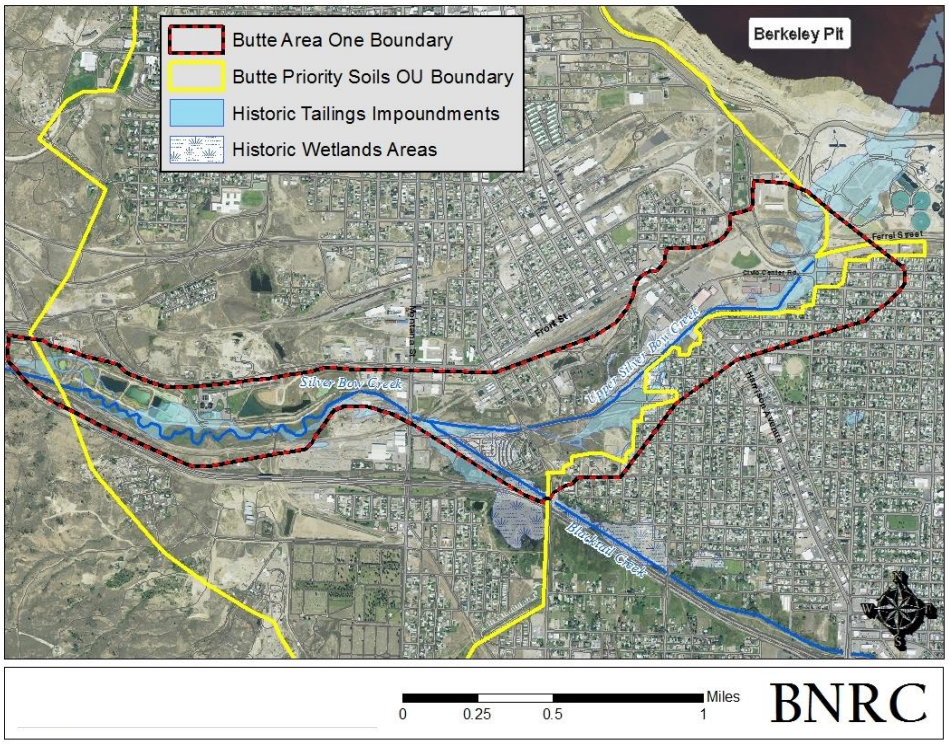

Figure 1. The proposed project demonstration area(s) will be inside of the Butte Priority Soils Operable Unit boundary. The final location(s) will be determined in conjunction with personnel (Mr. Eric Hassler) from the Butte-Silver Bow Residential Metals Abatement Program. Modified from Butte Natural Resources Council, Butte Area One map.

A.2. Background

Here, we leverage decades (and over a billion dollars) of Montana-based mining-related remediation as well as microbial ecology insights by researchers at Montana State University (MSU; e.g., Macur et al. 2004, Qin et al. 2009, Smith et al. 2015, Sánchez-Espinoza et al., in prep.) to rethink how to cost-effectively bioremediate arsenic-contaminated soil. Because these less expensive microbial remediation approaches have the potential to be effective in a wide range of environments, they show promise for large-scale application where funding does not exist for more comprehensive—but also expensive—remediation tactics such as excavation, isolation and storage. In Butte, the arsenic cleanup levels for zoned industrial, recreational and residential areas are 1000 parts per million (ppm), 500 ppm, and 250 ppm, respectively. Due to the nature of arsenic in soil and its ability to blow or seep across landscapes, however, these levels may not be sufficiently low to enable vigorous plant establishment and growth, which could minimize human health risks. This proposal provides a unique opportunity to test a cost-effective bioremediation approach in areas that will not receive any treatment under Butte's current arsenic soil cleanup standards, but still have observable detrimental impacts to plant community health and development.

Soil microorganisms can transform toxic forms of arsenic (As) that are taken up by plants into less toxic and less mobile forms (Sánchez-Espinoza et al. 2015). Pure cultures of two strains of a bacterium, *Agrobacterium tumefaciens*, have been isolated by the McDermott Lab at Montana State University (Wang et al. 2015); the wildtype strain can oxidize trivalent arsenic (AsIII) to pentavalent arsenic (AsV; "oxidizer"), a form less toxic to plants, whereas the mutant strain cannot convert AsIII to AsV ("reducer"). Basin wildrye (*Leymus cinereus*) grown in metal- and metalloid-contaminated Deer Lodge Valley soils amended with a combination of 5% organic matter (OM) and the oxidizing strain of *A. tumefaciens* showed sharply better performance than those grown with 5% OM and 5% lime (the traditional soil remediation amendment combination), or the combination of 5% OM and the reducing strain of *A. tumefaciens* (Figure 2). In the same way that wastewater treatment plants rely on microorganisms to bioremediate wastewater, use of arsenic-oxidizing bacteria under field conditions could lead to substantial cost-savings in terms of amendments (purchase and application), or more comprehensive approaches like capping or soil removal. In theory, any organic matter amendment could be pre-inoculated with the bacterium (e.g. wildtype *A. tumefaciens*) prior to seeding, just as legume seeds are inoculated with *Rhizobia* to facilitate nitrogen fixation.

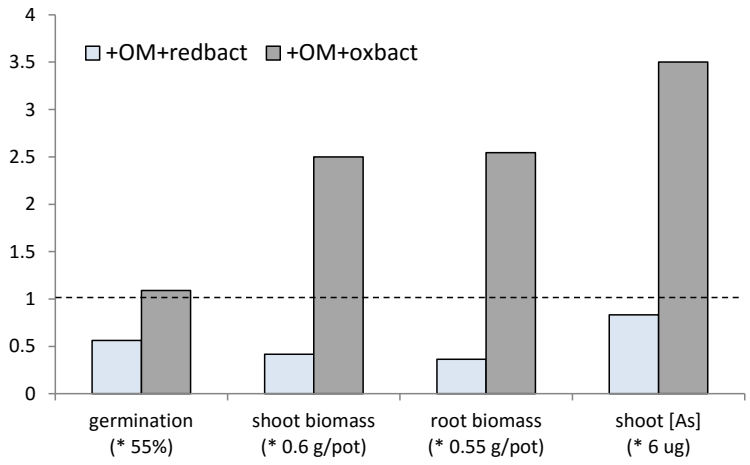

Figure 1. Summary of Sánchez-Espinoza et al. greenhouse results. The dashed line represents results (*) for arsenic-contaminated soils amended with 5% lime and 5% organic matter (OM). All other results are normalized to this treatment. Note basin wildrye grown in soils amended with 5% OM and a reducing strain of A. tumefaciens showed poor performance compared with plants grown in soils amended with 5% OM and an oxidizing strain.

As promising as these greenhouse results are, they leave several questions unanswered:

(i) Will plant species other than basin wildrye – remediation-specified seed mixes currently being developed by Montana Tech (*Koeleria macrantha* [prairie junegrass] , *Pseudoroegneria spicata* [bluebunch wheatgrass], and *Stipa spp.* [needlegrass]) – yield comparable results in the greenhouse, and in field contexts where drought, herbivory, and competition by other plant species are likely to reduce plant growth? and

(ii) Will combinations of the above-listed plants and arsenic-oxidizing bacteria isolated from Butte soils (other than *A. tumefaciens*) yield comparable results?

B. Project Goals and Objectives

The primary goal of this project is to identify the most successful and cost-effective bioremediation strategy for arsenic-contaminated soils in the Butte area.

The objectives of this project are:

(i) catalogue promising species of bacteria capable of oxidizing AsIII to AsV, qualitatively assessing microbial AsIII oxidizing ability with a technique refined by the McDermott lab;

(ii) identify the most promising combinations of plant species and AsIII-oxidizing bacteria for use in the field; and

(iii) initiate and complete a Butte–based field study that builds on data from (i) and (ii),as well as decades of prior MSU work and recent Montana Tech Native Plant Project findings.

All work will address a dual focus: quantifying return-on-investment relative to traditional soil remediation treatments (i.e. liming and/or the addition of OM), and promoting greater public awareness of Butte-specific arsenic bioremediation approaches and their potential benefits. We believe the novel arsenic oxidation abilities of the strains isolated by the McDermott lab at MSU, coupled with NRDP-sponsored research on metal tolerant plants at Montana Tech, constitute a synergistic breakthrough worthy of the greenhouse and field tests proposed here.

C. Project Benefits

This project will result in a number of benefits to Butte citizens and their natural resources outlined in the following paragraphs.

Arsenic in soils is most commonly found as either AsIII or AsV – any remedial approach must consider that we cannot simply make arsenic disappear. When the contaminated soil itself cannot be removed or capped, transforming the arsenic to its less toxic form (oxidized; AsV) is the most viable solution. This project will develop better tools for managing revegetation in arsenic-contaminated soils by determining which mechanism plants use to either avoid or take up the microbially-transformed arsenic. The proposed bioremediation will concentrate arsenic in either plant roots or plant shoots, thereby minimizing potential transfers (e.g., leaching) of soil arsenic into Butte-Silver Bow groundwater or surface water resources. If our findings suggest arsenic can be successfully concentrated into plant shoots, these shoots could be harvested, incinerated, and delivered to the local waste repository—at a fraction of the cost of excavating contaminated soil. Alternatively, if our work suggests plant avoidance

through arsenic immobilization in the rooting zone, even this outcome should confer greater protection to Silver Bow Creek and downstream water resources.
As previously mentioned, this bioremediation approach promises to be more cost-effective than the traditional *in situ* soil amendments, and much cheaper than capping or removing the contaminated soil. Another way in which this project maximizes already allocated funds is through a collaboration with the Montana Tech Native Plant Project, whose PI, Dr. Robert Pal, is providing us with seeds from his Montana NRDP-funded work on metal-tolerant plant species from the Butte area.

Finally, we are committed to sharing the results of this project through multiple outlets. All plant and soil data, along with the interpretation of the results, will be presented to the NRDP in the form of an annual technical report. In addition to the technical report, the graduate student will use this study to complete his dissertation and submit our findings to a peer-reviewed journal for publication. We also look forward to providing educational talks and study site visits for Butte area citizens and other interested parties to promote these types of innovative Butte-specific remediation strategies.

D. Project Implementation

The proposed project is composed of two phases – a greenhouse study and a field study. Results from the greenhouse study and preliminary analyses of soil from the study site will guide decisions on plant species and treatments for use in the field study. In addition, the greenhouse phase of the proposal is a cost-effective way to confirm the potential for promising field results before moving forward with the field-based phase.

D.1. Phase One: Soil Collection and Greenhouse Study

Soil samples will be collected from the proposed Butte field site at 10-cm interval depths (up to 30 cm) and prepped for physical, chemical, and microbiological characterization. The results of the microbiological characterization of the native soil will be used to isolate a bacterium to test in addition to *A. tumefaciens*.

Additionally, we will collect soils from our proposed Butte field study site to conduct a 12-week greenhouse study to assess performance of three Butte area remediation-preferred plant species (prairie junegrass, bluebunch wheatgrass, and a needlegrass) on As-contaminated soils. Dr. Robert Pal of Montana Tech has agreed to provide seeds from the Native Plant Project for the greenhouse study. The three plant species will be seeded and grown in pots of Butte soil in a Montana State University greenhouse facility. Each plant species will be subjected to every combination of two treatment types - an amendment treatment and a bacteria treatment – with five replicates for each combination. The amendment type consists of 4 treatments: a control (no amendment), a 5% organic matter (OM) amendment, a 5% lime amendment, and a 5% lime + 5% OM amendment. The bacteria type will consist of three treatments: a control (no bacteria), addition of *A. tumefaciens* bacteria, and addition of an As-oxidizing bacteria isolated from our field study site. There will be 180 total pots in the study: 3 plant species x 4 amendment treatments x 3 bacteria treatments x 5 replicates. The treatment combinations will be applied to the soil directly prior to seeding. Twelve weeks after seeding, data on plant growth (root and shoot biomass), foliar As concentrations, and soil As levels will be collected.

D.2. Phase Two: Field Study

The two top performing plant species in the greenhouse study will be used in the field experiment (Figure 3). Physical and chemical soil properties will be used to determine *in situ* amendment application rates (i.e., lime and organic matter) for the field portion of this study, and will help quantify the soil variability across our field sites.

The field study will be implemented during Spring 2016 across 72 1.5-square meter subplots according to the treatment design outlined in Figure 3. Treatments will be applied to the subplots prior to seeding – each of the two species will be used to seed half of the subplots. The field treatment combinations will be the same as those used in the greenhouse study; both plant species will be subjected to every combination of the two treatment types, with 3 replicates for every treatment combination by species (2 plant species x 4 amendment treatments x 3 bacteria treatments x 3 replicates = 72 subplots).

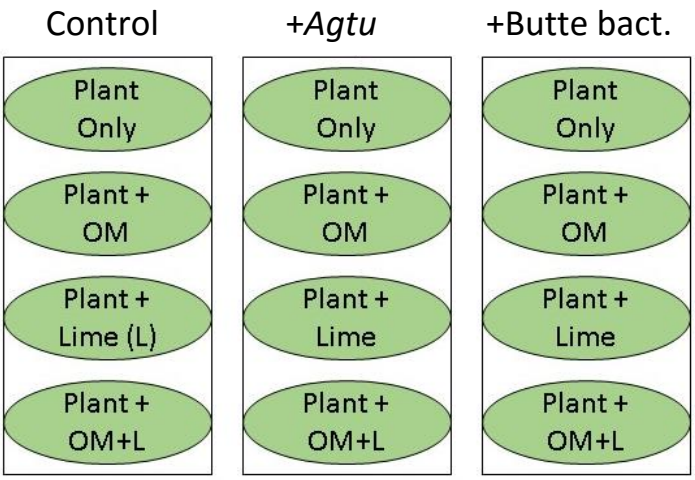

Figure 3 The above treatment design will be applied twice in the field-based study – once for each of the two top-performing metal tolerant plant species determined from the greenhouse trials. Each treatment combination will be replicated 3 times. OM=Organic matter; Agtu=Agrobacterium tumefaciens; bact.=bacterium.. Total number of subplots: 72.

Sampling of the field-based study will occur post-implementation during peak plant biomass in late Summer 2016. We will collect data on plant establishment and growth, and harvest a representative subsample from each treatment for foliar As concentrations and biomass. Soil sampling will include soil As characterization (AsIII or AsV), soil As concentrations, and quantifying the persistence of bacterial inoculum treatments in the soil.

The results of this field-based study will provide a ready-to-implement bioremediation tool for arsenic-contaminated soils in the Butte area – a tool that connects already-existing work on metal-tolerant plants to an innovative and cost-effective way to transform soil arsenic through microbial processes.

D.3. Dissemination of Results

All plant and soil data, along with the interpretation of the results, will be presented to the NRDP in the form of an annual technical report. In addition to the technical report, the graduate student will use this study to complete his dissertation and submit our findings to a peer-reviewed journal for publication.

This project will also promote the abundant educational opportunities surrounding the legacy of mining in western Montana in general, and Butte in particular. After all, our way of life depends on the metals that were extracted from this watershed. We will work with the NRDP, and other interested parties, to provide informational talks and study site visits that celebrate the opportunities for these types of innovative Butte-specific remediation strategies.

E. Project Schedule

- Soil samples will be collected for extraction of promising bacterial isolates and rRNA sequencing, in conjunction with determining soil physical (e.g., texture, OM, bulk density, etc.) and biogeochemical (e.g., pH, CEC, available nitrogen, arsenic species) properties in late Fall 2015.

- Fieldplots will be established in late Fall 2015/early Spring 2016, with amendment application and seeding in early Spring 2016.

- Sampling of the field-based study will occur post-implementation during peak plant biomass in late Summer 2016. Soil samples will be used to quantify the persistence of the original bacteria inoculum over time as a function of treatments, target plant species and soil As concentrations. Plant biomass and foliar metal(loid) concentrations will be measured from representative subsamples of treatments.

F. Monitoring Activities

While we have only budgeted this proposal for a single year, there is potential to make this a multi-year effort in order to assess the long-term efficacy of our bioremediation project. Therefore, we will be pursuing additional funding opportunities in order to extend monitoring efforts beyond a single year.

G. Budget

Study	Costs	NRDP	Match	Total Project
Greenhouse	1. Salary			
	Hartshorn	0	0	0
	McDermott	0	0	0
	Grad Student	4,800	3,800	8,600
	Undergraduate	8,500	0	8,500
	2. Benefits			
	Hartshorn	0	0	0
	McDermott	0	0	0
	Grad Student	0	0	0
	Undergraduate	170	0	170
	3. Supplies	4,000	0	4,000
	4. Analytical	7,500	0	7,500
	5. Travel	1,000	0	1,000
	6. Total Direct	26,170	3,800	29,970
	7. IDC's	6,524.50	0	6,525
	8. Total project	**32,695**	**3,800**	**36,495**

1. 14 weeks greenhouse salary is requested for one Montana State graduate student and four Montana State undergraduate students (2 student teams of 2 at $15/hr for 10 hrs/wk). This cost includes setup and monitoring of the greenhouse study, formative and summative analyses and workflow

development, which is most of the work described in the greenhouse portion of the proposal. In-kind contributions include the graduate student's $3,800 Nielsen Graduate Research Assistantship.

2. Estimated undergraduate student benefits for Spring/Fall are <1%.

3. Greenhouse costs include general setup supplies (e.g., pots, etc.), amendments (OM and lime) and inoculum of two bacteria for 180 pots (n =180).

4. Analytical costs include all microbial analyses, which will be performed by a private lab, and soil analyses, which can run $15/sample, not including specialty As speciation work. We have budgeted here for at least 1 foliar and 1 soil analysis per pot (n=360; ~$5400). Only a subset of foliar and soil samples will be run for As speciation.

5. Travel costs are requested for 10 round-trip visits to Butte to determine field sites, collect greenhouse material, as well as pre- and post-project debriefs and meetings.

7. Montana State indirect costs are 25% of total direct costs.

Note: The proposed project is composed of two phases – a greenhouse study and a field study. Results from the greenhouse study and preliminary analyses of soil from the study site will guide decisions on plant species and treatments for use in the field study. In addition, the greenhouse phase of the proposal is a cost-effective way to confirm the potential for promising field results before moving forward with the field-based phase.

Study	Costs	NRDP	Match	Total Project
Field	1. Salary			
	Hartshorn	0	5,000	5,000
	McDermott	3,000	0	3,000
	Graduate	5,000	4,000	9,000
	Undergraduate	12,000	0	12,000
	Equipment Op.	15,000	0	15,000
	2. Benefits	0	0	0
	Hartshorn	0	1,050	1,050
	McDermott	630	0	630
	Grad Student	500	400	900
	Undergraduate	1,200	0	1,200
	3. Supplies	6,370	0	6,370
	4. Analytical	7,500	0	7,500
	5. Travel	2,500	0	2,500
	6. Total Direct	53,700	10,450	64,150
	7. IDC's	13,425	0	13,425
	8. Total Project	**67,125**	**10,450**	**77,575**

Combined Total Direct	79,870	14,250	94,120
Combined Total IDCs	19,950	0	19,950
Combined Total Project	**99,820**	**14,250**	**114,070**

1. Two months summer salary is requested for one Montana State graduate student ($2500/month) and 4 months for two Montana State undergraduate students (1 student team of 2 at $15/hr for 25 hrs/wk) who will perform much of the work described in the field portion of the proposal. MSU's, Dr. Tim McDermott, will be retained as a project consultant for 1-2 week(s). Matching funds include the graduate student's $4000 summer support and two weeks of my summer salary totaling $9,000. Equipment operator salary is estimated for two weeks at $200.00/hr.

2. Estimated undergraduate benefits for summer is 10%. Matching funds include two weeks of my summer salary benefits. Estimated benefits for Tim McDermott (MSU faculty) is 21%.

3. Field costs include OM, lime and inoculum for 72 – 1.5m x 1.5m plots.

4. Analytical costs include all microbial analyses which will be performed by a private lab, as well as additional biogeochemical characterization of soils, before, during, and following treatments.

5. $2500 is requested for travel and per diem costs during the spring/summer field season.

7. Montana State estimates indirect costs to be 25% of total direct costs.

Works Cited

Macur, R., H. Langner, B. Kocar, W. Inskeep. 2004. Linking geochemical processes with microbial community analysis: successional dynamics in an arsenic-rich, acid-sulphate-chloride geothermal spring.

Qin, J., C.R. Lehr, C. Yuan, X. Chris Le, T.R. McDermott, B.P. Rosen. 2009. Biotransformation of arsenic by a Yellowstone thermoacidophilic eukaryotic alga. *PNAS*. **106**: 5213-5217.

Sanchez-Espinoza, D.N. 2015. Interactive effects between lime, organic matter, and bacteria in the establishment of *Leymus cinereus* in mine tailings. M.S. thesis. Land Resources & Environmental Sciences, MSU.

Smith, M., A. Rocha, C. Smillie, S. Oleson, C. Paradis, L. Wu, J. Campbell, J. Fortney, T. Mehlhorn, K. Lowe, J. Earles et al. 2015. Natural bacterial communities serve as quantitative geochemical biosensors. *mBio*. **6:** doi: 10.1128/mBio.00326-15.

Wang, Q., D. Qin, S. Zhang, L. Wang, J. Li, C. Rensing, T.R. McDermott, and G. Wang. (2015) Fate of arsenate following arsenite oxidation in *Agrobacterium tumefaciens*, GW4. Environmental Microbiology. 17:1926-1940