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Title of report: Microbial Community Structure: A Biological Assessment Tool for Metal-Impacted Watersheds in the Central Colorado Assessment Project

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Project summary:

The aim of this project was to assess the relationship between sediment bacterial community structure and metal toxicity. Sites identified by USGS collaborators were examined for this work, each site representing varying metal impact. Traditional bacterial culturing methods were combined with advanced molecular genetic methods to ascertain community differences among the sites. The outcome of this work was a preliminary relationship between the composition of the sediment bacterial communities and readily exchangeable metal concentrations. That is, as the concentration of readily exchangeable metals increases, the bacterial community shifts from a predominantly eubacterial community to an archaeabacterial community. Additional sites need to be analyzed to determine the stability of this relationship; however, this early study implies bacteria can be used as indicators of environmental quality.

Project background and objectives:

As a result of their small size (1-2 µm ave.) and intimate contact with their environment, bacteria represent the first biological system type to be affected when metal toxicity is present. Additionally, bacteria form complex communities composed of populations that differentially respond to environmental stressors. Because of their high surface area-to-volume ratio, genetic and metabolic flexibilities and rapid adaptability, bacteria provide a link between chemical biological availability and, therefore, toxicity in environmental systems. One of the specific objectives of the Central Colorado Assessment Project is to identify the effects of toxic metals on water quality and biota associated with impacted watersheds. The proposed research studied the impact of metals on sediment bacterial communities, as a baseline indicator of watershed impacts.

Snake River site.

Even with the use of metal-resistance mechanisms (Roane et al., 2009), metals have been shown to alter microbial community structure (Ellis et al., 2003; Feris et al., 2003, 2004a,b;

Kassab and Roane, 2006; Roane et al., 2009; Stout and Nüsslein, 2005). However, not all bacterial populations are equally metal-resistant allowing for a dynamic compositional response to metal toxicity. Yet, the responses of microorganisms, in general, to metal toxicity are poorly understood. Consequently, this research not only contributed to the objectives of the Central Colorado Assessment Project but has also contributed to the elucidation of microbiological stress responses.

We hypothesized that microorganisms directly respond to metal exposure in terms of absolute numbers and diversity, and, therefore, can be used as direct measures of environmental metal toxicity. We proposed to test this hypothesis by examining the bacterial community structures in metal-contaminated environments through microbial growth experiments in addition to the molecular identification of individual microbial community populations. We expected to find marked differences in the microbial populations present in heavily metal-impacted and lesser metal-impacted sites. Multiple metals were included in this study, e.g., Zn, Cu, Cd, Pb, As, Fe, and Mn. Each of the metals examined has an associated biological toxicity of increasing societal and scientific interest. Based on our hypothesis, the specific objectives of the proposed research were to (1) use bacterial numbers to assess the toxicity of environmental metals and (2) use molecular analysis of bacterial communities to characterize the bacterial population changes that occur upon metal exposure.

Experimental approaches and outcomes:

Field site characterization

Traditional culturing techniques in conjunction with advanced molecular methodologies were used to address the objectives. Field sites chosen represented variously metal-contaminated sediments having direct impact on Colorado watersheds. Sites represented (a) mineralized or altered but not mined; (b) mineralized and mined; and (c) not mineralized or altered. According to USGS and our analyses, each of the sites in this study had some degree of metal impact.

Peru Creek site.

Sample collection: Seven USGS identified sites were sampled by collecting approximately 200 g x 2 in deep grab samples during the summer and fall months of 2006 and 2007. The 2006 samples provided preliminary data. Follow-up at these sites occurred in 2007, the results of which are reported here. Samples were aseptically collected and placed in sterile sample bags and stored at 4^{o}C until analysis. Replicate samples from each site were collected. Field pH, DO (dissolved oxygen), temperature, and conductivity were recorded (Table 1). Once in the laboratory, samples were aseptically divided into sub-samples for the following analyses: bioavailable metal analysis; culturable bacterial counts; and molecular DNA extraction. Particle size analysis (all sediments were sandy) and total metal analysis were performed by Dr. Rich Wanty, USGS Research Chemist with the Mineral Resources Program (Figure 1).

Pennsylvania Mine (Penn Mine) was an additional site included in this study. Penn Mine is an abandoned mining operation. Fed by underground streams, there is a metal-rich effluent stream releasing into Peru Creek, one of the USGS sites. Open to public access, we sampled the effluent for comparison purposes to the other sites.

Table 1. Field characteristics of sampling sites.

	Keystone Gulch	**North Fork Snake River**	**Peru Creek**	**Deer Creek**	**North Fork South Platte**	**Snake River (lower)**	**Snake River (upper)**	**Penn. Mine***
pH	8.1	8.2	5.0	8.1	6.2	5.0	4.5	3.8
Temp.(^{o}C)	4.7	6.7	6.7	3.8	6.0	6.3	6.3	3.4
Conductivity (µS/cm)	96.5	114	270	106	120	200	3,800	1,740
D.O. (ppm)	6.6	6.7	5.8	5.8	5.8	5.8	5.8	4.0

D.O.= Dissolved oxygen *Penn Mine=Pennsylvania Mine.

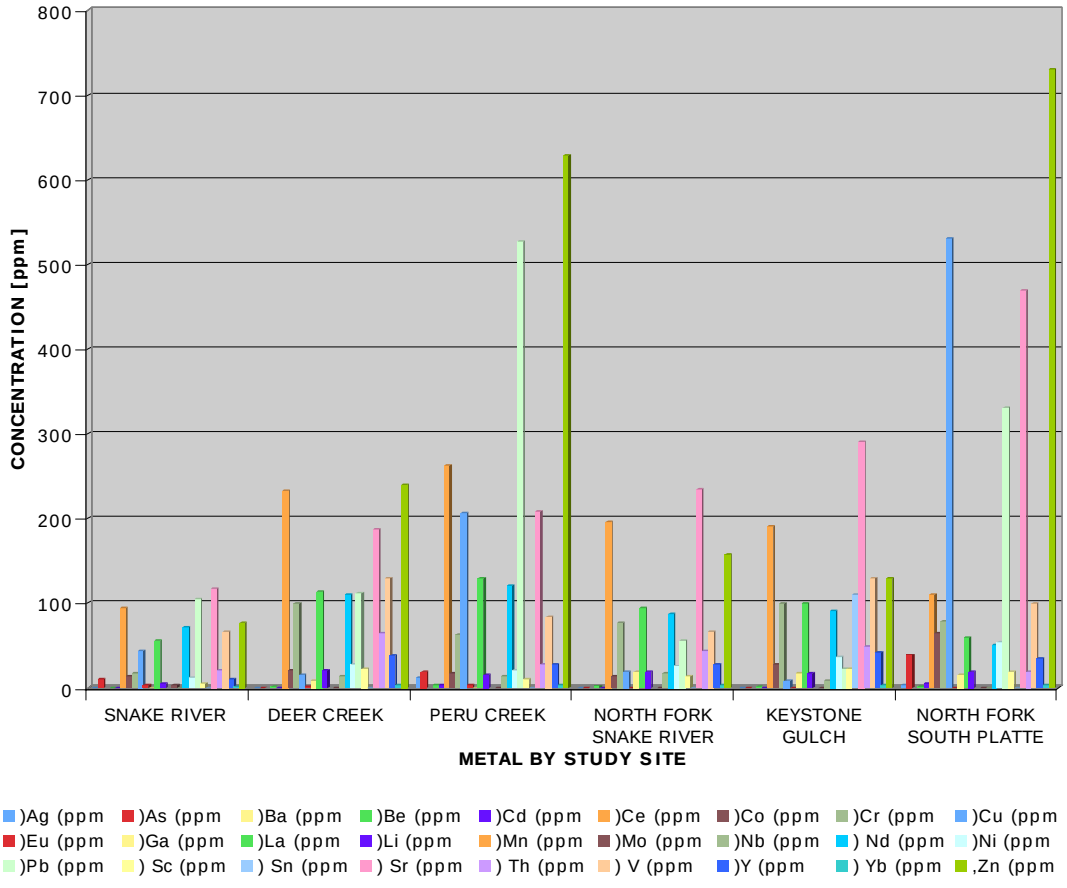

Figure 1. USGS performed total metal analysis for each of the field sites.

Bioavailable metal analysis: As soluble or readily exchangeable metal concentrations are thought to be more reflective of biological toxicity than total metal concentrations, readily exchangeable metal concentrations were determined using graphite atomic absorption (AA) analyses (Table 2). Exchangeable metal concentrations were determined from 1:10 soil: glycerophosphate (0.1% w/v) extracts. Glycerophosphate is a charge neutralizer and was used to release loosely bound metals from organic materials and from soil particles.

Table 2. Field Site Readily Exchangeable Metal Concentrations (ppm).

	Peru Creek	**Snake River (upper)**	**Snake River (lower)**	**Deer Creek**	**North Fork South Platte**	**Keystone Gulch**	**North Fork Snake River**	**Penn. Mine***
Metal								
As	5.2	11.4	6.5	4.2	3.7	1.2	1.3	<1
Se	0.5	0.2	1.8	1.5	0.8	2.4	0.5	1.0
Ag	<1	<1	0.3	<1	<1	<1	<1	<1
Cd	1.7	1.2	1.4	2.4	1.8	0.4	0.8	21.3
Mn	787.3	504.0	190.2	12.8	440.7	24.1	72.0	3,944.6
Co	0.8	1.3	0.7	0.9	1.0	1.4	0.7	8.9
Cr	<1	11.9	0.8	<1	<1	<1	<1	0.9
Cu	84.7	64.2	31.2	12.6	75.5	77.5	20.2	796.9
Fe	976.3	43,199.0	6,010.8	553.5	680.3	ND	ND	299.9
Ni	2.5	15.8	8.3	2.1	6.0	2.9	5.6	30.4
Ba	6.4	6.0	6.7	268.2	36.8	32.5	46.7	45.4
Pb	23.2	14.1	6.5	238.3	8.4	1.0	2.3	5.4
Sb	0.7	0.8	1.0	0.3	0.3	0.6	0.5	0.4
Tl	<1	0.9	<1	<1	2.2	13.7	1.1	1.4
V	<1	<1	<1	32.7	0.4	<1	<1	<1

ND = not determined. *Penn Mine=Pennsylvania Mine

Each of the sites in Table 2 and Figure 1 has unique metal fingerprints containing different levels of the metals represented. Biologically essential metals, such as Fe and Ni, for example, are less of a toxicity concern than metals as As, Cd and Pb, which represent metals of no known biological function. However, it should be noted that in excess biologically essential metals can be toxic. In aquatic systems, typical background levels of metals such as As, Cd, Cu, and Pb are trace, 0.06, 0.63, and 0.06 ppb, respectively. Typical background soil levels of the same metals are 0.49, 0.6, 296, and 99 ppb, respectively. Accordingly, all sites had elevated levels of metals of the greatest toxicity concern, which according to the Environmental Protection Agency (www.epa.gov; 2009) include As, Cd, and Pb. As expected, all sites had lower concentrations of soluble metals as compared to total metal concentrations.

Objective 1. Use of bacterial numbers to asses the toxicity of environmental health

Upon return to the laboratory, sediment samples were immediately processed for culturable bacterial numbers (Figure 2). Bacteria were extracted from the soils using glycerophosphate as a charge neutralizer. Extracts were then diluted and plated onto R2A, a

heterotrophic minimal bacterial growth medium. Following incubation at 28°C, bacterial growth was enumerated with a visual inspection of colonial growth.

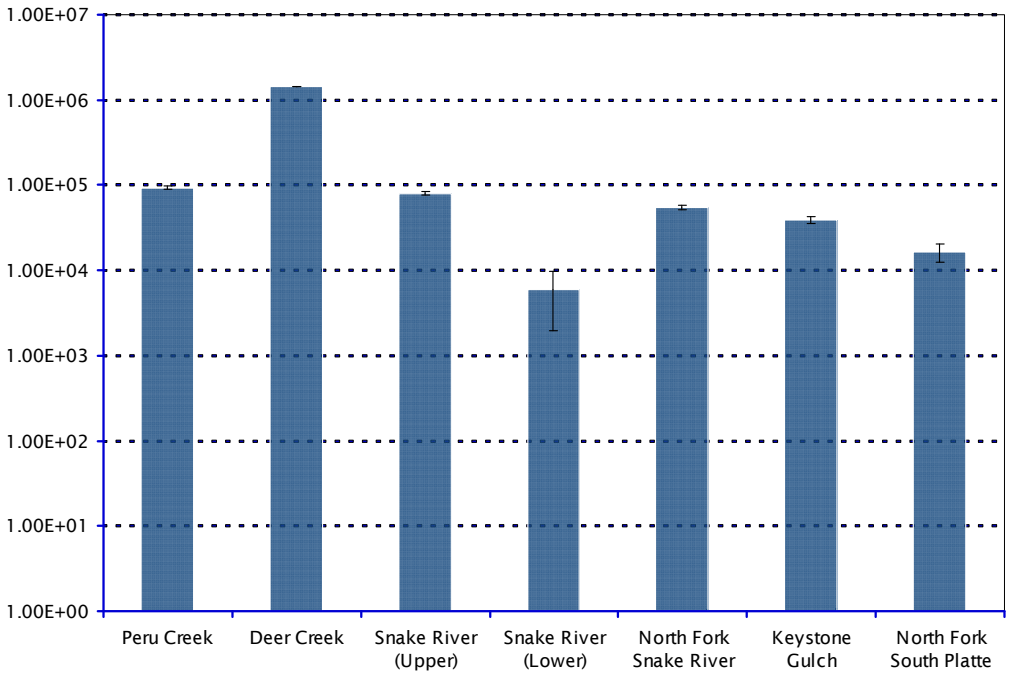

Figure 2. Culturable bacterial numbers grown on R2A medium. The numbers reflect maximum growth upon incubation at 28°C.

No correlation was observed between the degree of metal-contamination and bacterial growth. One of the reasons for this may be the limitations of culturing bacteria in the laboratory which include the lack of a universal medium to grow all bacteria in a sample due to their different nutritional requirements.

Objective 2. Use of molecular analyses of bacterial communities to characterize the bacterial population changes that occur upon metal exposure.

Total DNA from sediment samples were collected using the Powermax Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). The total DNA was then subjected to 16S rDNA PCR using DNA primers designed to isolate and amplify bacterial genetic material. The resulting genetic material was then sent to a colleague, Dr. Gary Anderson, Center for Environmental Biology, Molecular Microbial Ecology, at the Lawrence Berkeley National Laboratory, for microarray analysis. We originally proposed to use a molecular technique called DGGE (denaturing gradient gel electrophoresis) to genetically analyze the bacterial communities; however, preliminary analyses of the communities showed too much complexity in the community composition to allow for accurate analysis (Figure 4). At this time, we were using the PhyloChip microarray (Affymetrix, Inc., Santa Clara, CA) technology for bacterial community analysis in other projects and so decided to use it for the proposed work (Figures 5 and 6). Note that PhyloChip data analysis for the remaining sites (North Fork Snake River, Keystone Gulch, Snake River, Peru Creek) is still underway.

The PhyloChip microarray provides the ability to detect the presence of nearly 32,000 eubacterial and archaeal taxa via unique 16S rDNA gene sequences attached to the microarray chip. The PhyloChip microarray is increasingly being used for bacterial community characterization (www.lbl.gov/Tech-Transfer/techs/lbnl2229.html). An advantage of the PhyloChip microarray is the ability to classify bacterial taxa into eubacterial and archaeabacterial. Bacteria are divided into the two categories based on structural and biochemical differences: the eubacteria representing "typical" bacteria, e.g., *Bacillus*, *Pseudomonas* spp.; and the archaeabacteria thought to represent more "extreme" bacteria capable of withstanding harsher conditions than the eubacteria, e.g., *Crenarchaeota* and *Eukaryarchaeota*. The relationships between these two groups are not understood and are only now being investigated.

For the initial molecular analyses, three sites were chosen: Penn Mine (considered highly contaminated); Deer Creek (considered moderately contaminated); and North Fork South Platte (considered least contaminated), with respect to Cd, Co, Cu, and Mn (metals with known toxicities) (Figures 3a and 3b).

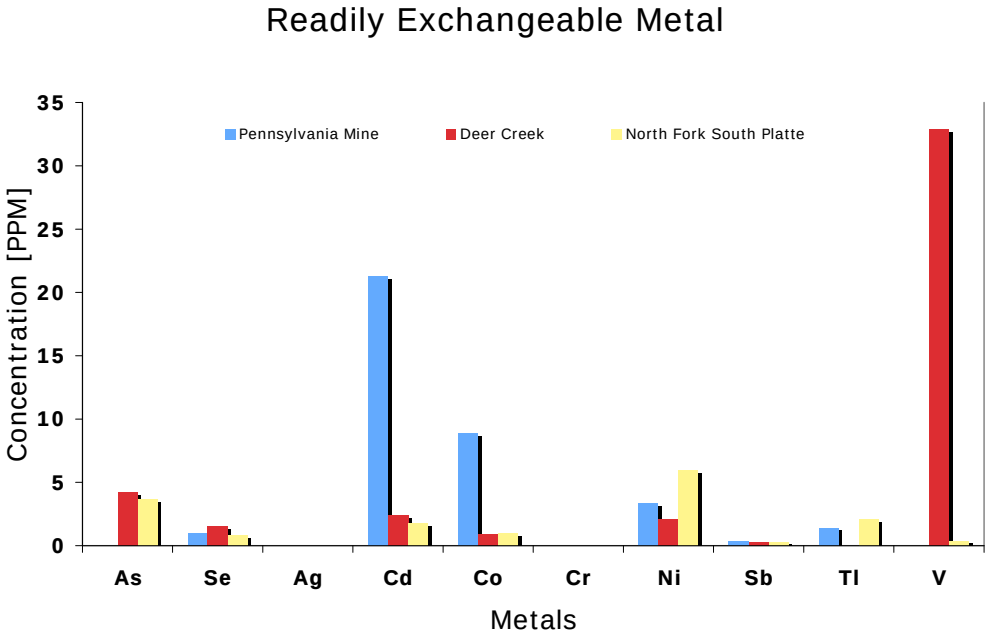

Figure 3a. Readily exchangeable metals in the Pennsylvania Mine, Deer Creek and North Fork South Platte sites.

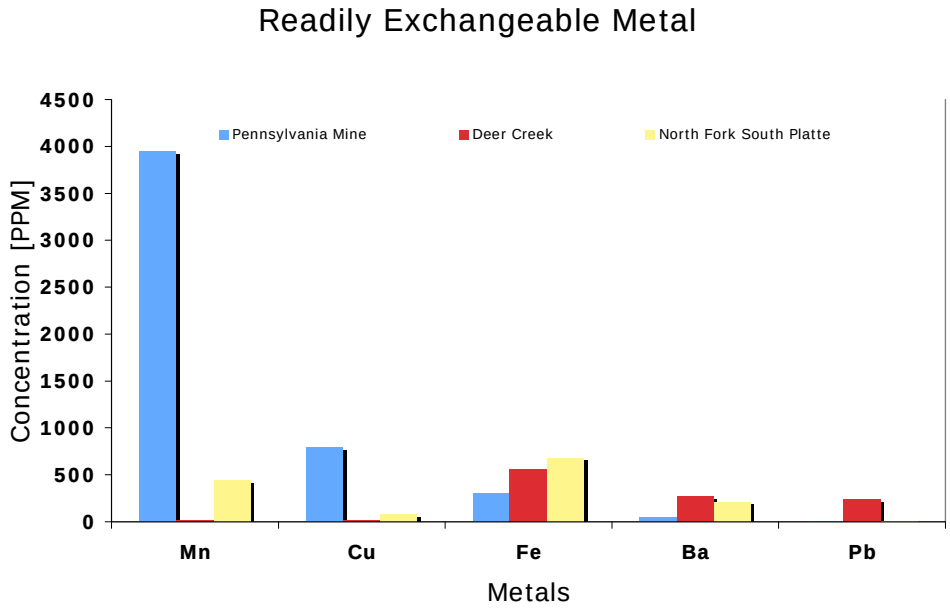

Figure 3b. Continuation of the readily exchangeable metals in the Pennsylvania Mine, Deer Creek and North Fork South Platte sites.

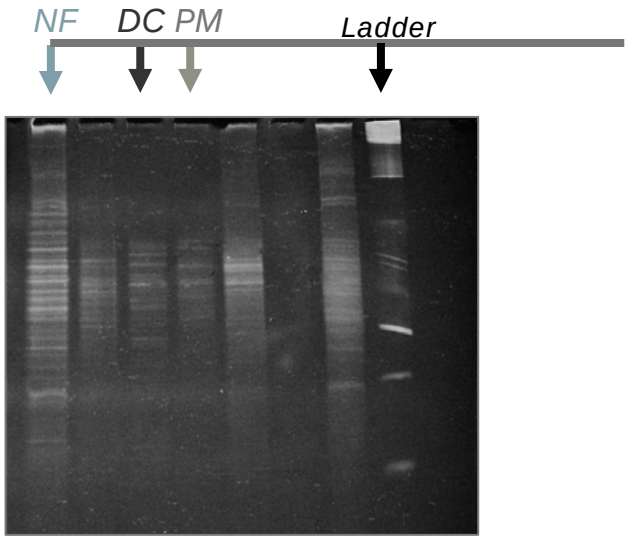

Figure 4. Example DGGE gel (40-80% denaturing gradient) showing too many bacterial populations to accurately analyze. Note each study site (NF=North Fork South Platte; DC=Deer Creek; PM=PennMine*) showed differences suggesting compositional differences in the bacterial communities. Because of the complex banding patterns on the gels, bacterial community analysis was switched to the PhyloChip microarray for bacterial identification. The ladder refers to a reference DNA size ladder.

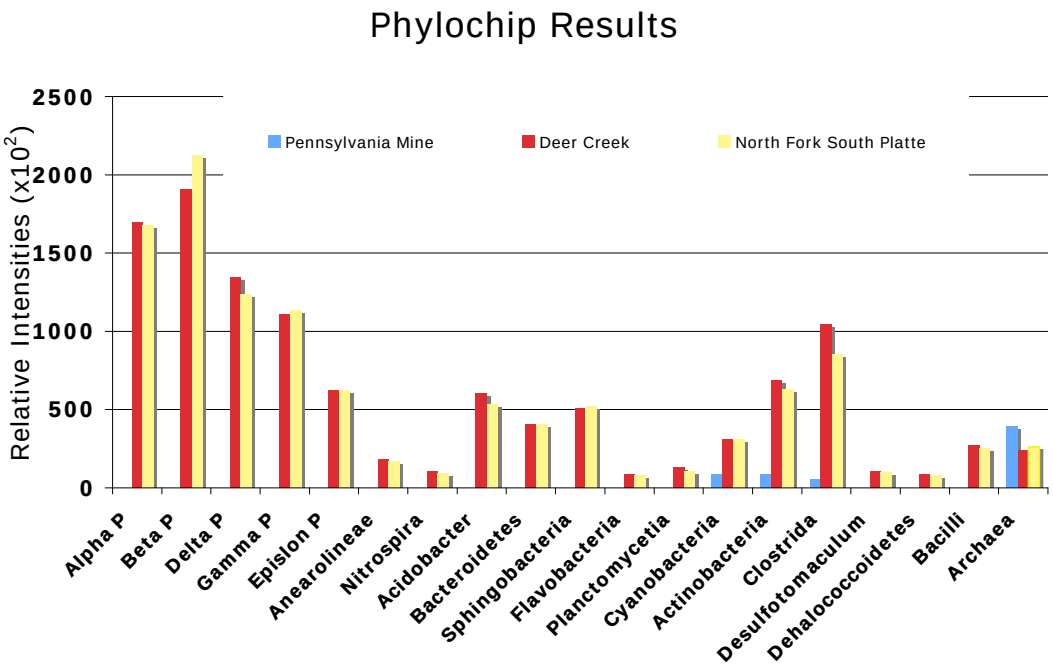

Figure 5. Analysis of the bacterial taxa composition of the sediment communities of Penn Mine, Deer Creek and North Fork South Platte sites. When compared to the Deer Creek and North Fork South Platte sites, the absence of significant eubacterial populations in the more metal-contaminated Penn Mine site was noted and seemed to correspond with an increased presence of archaeabacteria.

The intense banding patterns observed in the DGGE gels (Figure 4) revealed a high degree of bacterial community complexity in each of the sites. The apparent diversity observed could not be efficiently deciphered and so the PhyloChip microarray was used to elucidate the community compositions to allow for comparison among the sites. PhyloChip analyses implied two things: (1) the bacterial communities in more heavily contaminated sites are dominated by archaeabacterial populations as opposed to eubacterial populations; and (2) the archaeabacterial populations in more heavily contaminated sites seem to be more diverse than those observed in lesser contaminated sites (Figures 5 and 6).

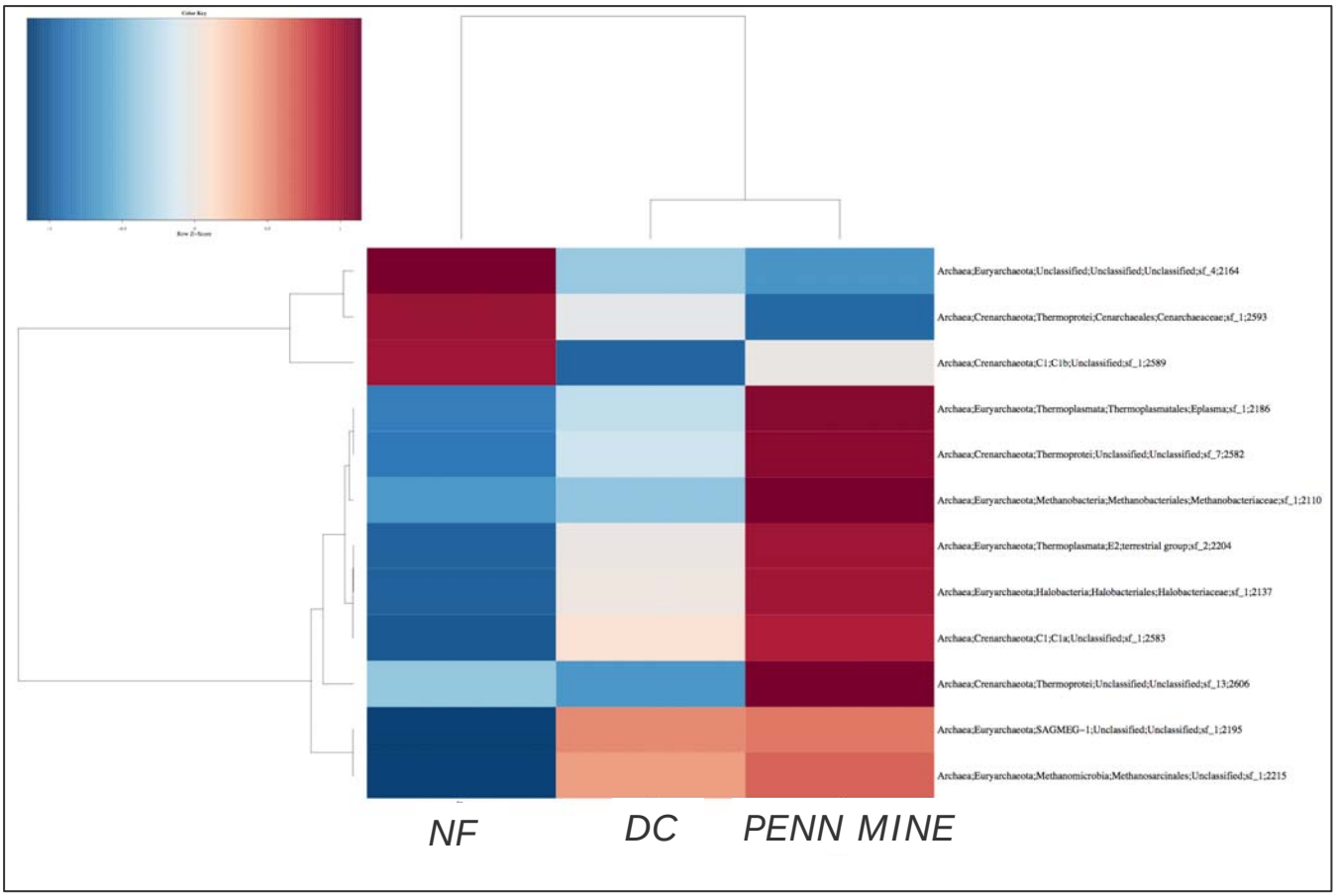

Figure 6. Heat plot of the diversity of archaeabacteria in the Penn Mine, Deer Creek and North Fork South Platte sites. Red boxes indicate a higher number of the corresponding bacterial group as compared to the less present blue boxes. The Penn Mine site, representing the most highly contaminated of the three sites, also had the greatest archaeal diversity. Note that the type of archaea present in each site varies.

Project discussion:

Based on our hypothesis, we expected to find community composition differences among the differently contaminated sediments. Not only did we find composition differences but the differences seem to be indicative of metal toxicity in that the bacterial communities in "lesser" contaminated sites were dominated by eubacteria and had less archaeabacterial diversity. The contaminated sites progressively shifted from eubacterial dominated communities to archaeabacterial dominated communities. The culturing work revealed little useful information as only limited colony diversities were observed as compared to the genetic diversity revealed through the molecular work. Future studies will focus on molecular approaches for community diversity assessments.

While the molecular analyses of the bacterial communities indicated a diversity pattern associated with metal concentration (e.g., loss of eubacteria and the increasing presence of archaeabacteria), this work will have to be extended to many more sites to conclusively establish the pattern. PhyloChip data analyses of other sites sampled, including Keystone Gulch, North Fork Snake River, Peru Creek, Snake River (lower) and Snake River (upper), are currently underway.

This study was unique in its attempt to use bacterial communities as biological indicators of metal toxicity. The outcome of this study was a unique bacterial community fingerprint indicative preliminarily of readily exchangeable metal concentration and, thus, toxicity. We hope to obtain additional funding to continue analysis of other metal-contaminated sites. If the observed pattern holds, bacterial communities will provide an additional biological indicator of environmental health.

Citations for project publications (*student presenter):

2009 Kester*, M. and T.M. Roane. Use of Bacterial Community Structure in Assessing Metal Toxicity in Sediments, May 2009. American Society for Microbiology, Poster Q-217.

2009 Kester*, M. and T.M. Roane. Molecular Profiling of Bacterial Communities Associated with Mining Sediments. University of Colorado Denver, Research and Creative Activities Day.

2008 Kester*, M. and T.M. Roane. Analysis of Sediment Bacteria as Indicators of Metal Toxicity, May 2008. American Society for Microbiology, Poster Q-570.

2008 Kester*, M., M. Albuthi, and T.M. Roane. Investigation of bacteria associated with metal-impacted sediments, April 2008. Rocky Mountain American Society for Microbiology.

2008 Kester*, M. and T.M. Roane. The Study of Soil Bacteria as Signals of Metal Toxicity. University of Colorado Denver, Research and Creative Activities Day.

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