“Designing a Novel Heavy Metal Bioremediation System Utilizing Immobilized Algae Partnered with Heavy Metal Resistant Microbial Isolates Collected From Contaminated Superfund Mine Sites and Identified with a 16S Ribosomal Subunit Analysis”

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Abstract:

Heavy metal contamination in the environment, specifically in aquatic systems, has been a top concern for nearly 100 years. This heavy metal contamination is found at nearly every one of the estimated 500,000 abandoned mine sites in the United States. Of these mine sites, 0.003% are actively funded for cleanup by the EPA, and these cleanup methods cost $300 million annually and are not low-impact. This project seeks to solve this problem through five phases. First, two EPA Superfund mine sites are studied to determine the concentration of heavy metals at different points along contaminated streams, in addition to collecting water samples to be used in Phase 2, which consists of identifying unique morphologies of bacteria found in the water samples and isolating them to be used in a bioremediation system. The isolates are then screened for heavy metal resistance and successful formation of biofilms in high concentrations of heavy metals, two standards a final group of 24 isolates must meet to be identified in Phase 4 with the 16S Ribosomal Subunit Analysis. The identified bacteria are then grouped by genera and partnered with algae in a sodium alginate bead to serve as the heavy metal remediation system.

Acknowledgements:

This examination was designed and conducted by the presenter. Gratitude is extended towards Sally Fenska of Cascia Hall Preparatory School for guidance and mentorship, as well as Dr. Mohammad K. Fakhr, of the University of Tulsa for the allowance of experimentation in the Microbiology lab and the use of its technology, as well as supervision during all protocol conducted in the lab. In addition, gratitude is extended towards Dr. Gerwald Koehler of OSU Tulsa for use of the Plate Reader.

Background:

Acid Mine Drainage (AMD) is one of the results of mining for coal and metal ore, and affects environments around both active and abandoned mines. AMD is created when pyrite and iron containing compounds are exposed to the oxygen in the air and freshwater springs found within mines, creating sulfuric acid and iron oxide, easily identified by its red to orange color. As the AMD flows out and around mine sites, heavy metals such as Lead, Cadmium, Zinc, Cobalt, Chromium, and Manganese are dissolved into the water system. In addition, several heavy metals are introduced into the environment from the large amounts of chat and waste left behind from the mines. These heavy metals, known for their extreme toxicity, have been shown time and time again to pose extremely dangerous effects on both environmental and human health. A detailed explanation of the health concerns associated with heavy metals is outlined below in Figure I.

Figure I: Human Health Concerns Associated with Selected Heavy Metals

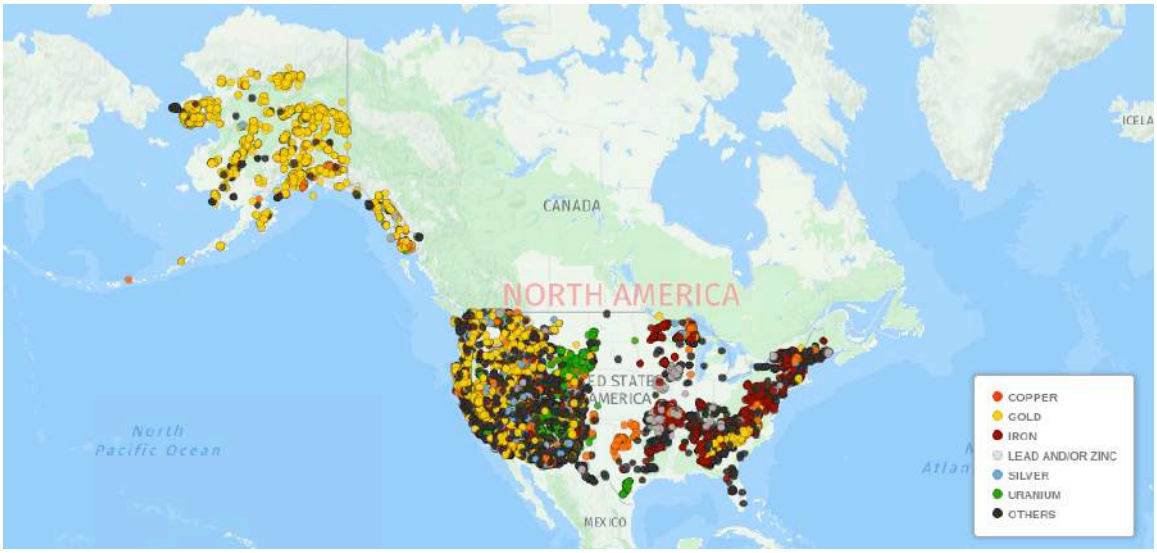
Heavy Metal	Effects on Human Health
Lead	Lead encephalopathy, behavioral disturbances, concentration difficulties, confusion, prolonged reaction times, and memory loss
Cadmium	Kidney damage including tubular dysfunction, skeletal damage including osteomalacia and osteoporosis, cardiovascular disease, and lung, prostate, and kidney cancer
Zinc	Gastrointestinal effects, abdominal pain, vomiting, sideroblastic anemia, and leukopenia
Cobalt	Asthma, pulmonary fibrosis, cardiomyopathy, deafness, blood thickening, thyroid damage
Chromium	Skin irritation and ulcers, liver damage, kidney damage, nerve tissue damage, and respiratory cancer
Manganese	Central and peripheral neuropathies

As described in Figure 1, heavy metals pose an immense threat to human health. In addition, heavy metals have an equally deleterious effect on the ecosystem, with water systems contaminated with AMD experiencing a loss of biodiversity and aquatic life altogether. Heavy metals introduced into the environment as a result of AMD also have an indirect effect on organisms through biological magnification that does not require direct contact with the metals or drinking water containing the metals. For example, heavy metals dissolved in the water of a stream from the AMD of a mine flow down a mountain and into a freshwater river, which then flows into a lake. The heavy metals sink to the bottom, where an aquatic plant species grows. The plant uses the soil, which now contains heavy metals, as nutrients to grow, and is therefore exposed to the heavy metals. A small micro-invertebrate relies on this plant as a food source, and naturally feeds on many of the plants, thus exposing the micro-invertebrate to the heavy metals. Because this organism feeds on multiple plants and ends up being exposed to a greater amount of heavy metals than one of the plants. Next, a small fish feeds multiple micro-invertebrates, so the fish will contain a greater amount of heavy metals than its prey. This cycle builds with each species in the food chain, with each predator species experiencing a greater amount of heavy metals than its prey, essentially magnifying the heavy metal

concentration in the ecosystem. This event could even end with a human consuming fish caught from this lake, being exposed to the greatest concentration of heavy metals in the chain, therefore also being exposed to the greatest risk of health concerns related to the heavy metal toxicity previously discussed.

Unfortunately, heavy metals released into the environment as a result of AMD are not a rare occurrence. It is predicted that there are over 500,000 abandoned mines in the United States, a map of which can be seen below (Figure 2). Each of these mines produces AMD, which then introduces heavy metals into the water system. Through rain, snowmelt, and other water flow, the contaminated water can easily spread to new rivers, lakes, and streams, putting these new ecosystems at risk. Heavy metal contaminated water, therefore, is a major environmental and health concern affecting a vast majority of the population of the United States.

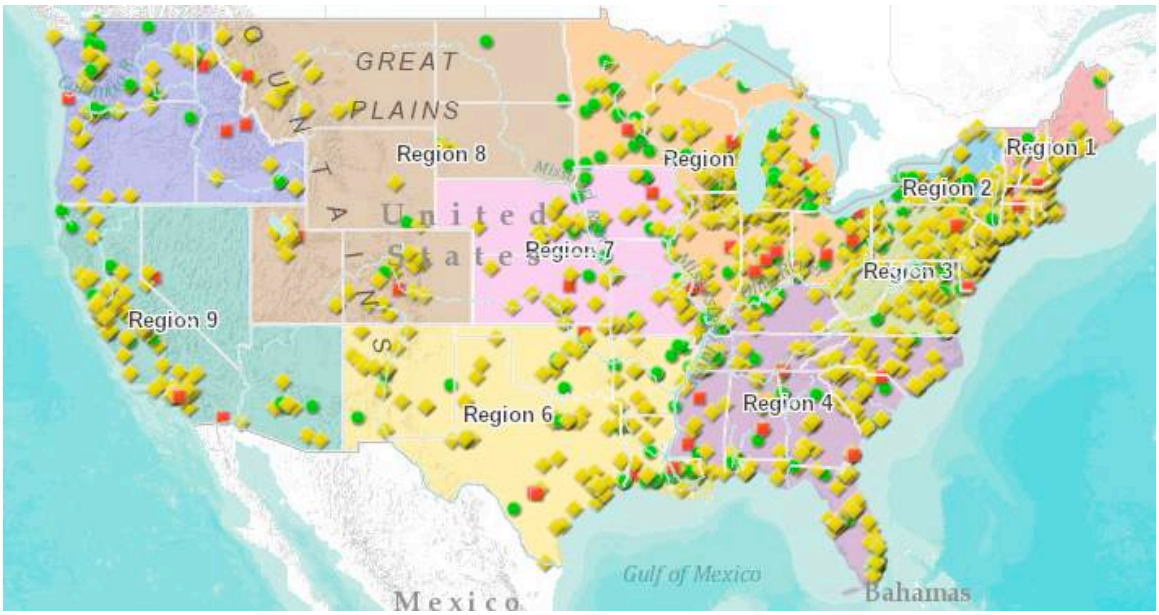
Figure II: Abandoned Mine Sites in the United States



www.skytruth.org/2015/09/inactive-metal-mines/

In 1980, the Comprehensive Environmental Response, Compensation and Liability Act (CERLA) was passed, enabling the Environmental Protection Agency (EPA) to enact a program known as Superfund, which targets the remediation of mines and surrounded areas contaminated by heavy metals as a result of AMD. Figure 3 below shows the mine sites that are currently under the control of the Superfund program. However, the first major problem in the heavy metal remediation effort can easily be observed when figures II and III are compared. There are a multitude of abandoned mine sites that do not have any funded cleanup efforts, as they are left out of the Superfund program.

Figure III: Abandoned Mine Sites Maintained by the EPA under the Superfund Program



www.epa.maps.arcgis.com/apps/webappviewer/index.html?id=33cebcdfdd1b4c3a8b51d416956c41f1

The exclusion of so many abandoned mine sites simply comes down to funding. Each site requires millions of dollars in funding to cover varied methods of remediation, which brings about the second major problem: there is not a cost-effective, low-impact method of heavy metal remediation that can be easily implemented into contaminated water systems. Many of the current approaches to remediating the contaminated areas are very expensive. Private institutions, such as the University of Oklahoma, for example, have used millions of dollars in funding to conduct research on the Tar Creek Superfund site, even building a heavy metal water treatment facility. Even this expensive and cutting edge, large-scale operations do not prove to be significantly efficient in remediating the heavy metal concentrations in the water. Secondly, many Superfund sites take to diverting water flow as it comes out of the mine by digging channels in planned locations, however these diversions simply move the toxic water to a new location, further damaging the environment, and making no significant progress in heavy metal remediation.

Purposes and Experimental Design:

Based upon the fact that thousands of mines are neglected in the Superfund remediation program and there is not a cost-effective, low-impact method of bioremediation, the purpose of this project is to develop a low cost, low impact method for the bioremediation of heavy metals, which can be easily implemented into water sources at and around all of the abandoned mine sites. Each phase of this examination has a unique purpose established to move towards the creation of a bioremediation system utilizing algae and environmentally isolated bacterial species found at and around Superfund mine sites in an immobilized form.

The first phase consists of performing two field studies at two different EPA Superfund mine sites. The first being Pennsylvania Mine in Dillon, Colorado, and the

second being Tar Creek, in Miami, Oklahoma. At each site, water samples are collected to be used for the growth of bacteria species native to each environment and measure the concentration of selected heavy metals at each test site. The purpose of Phase I consists of examining the EPA Superfund mine sites and their water systems better understand the concentrations of heavy metals found at different points along contaminated streams in addition to collecting the water samples that will provide bacterial isolates to be screened and analyzed as candidates for the bioremediation system.

Each of three water samples from each test site are plated on nutrient agar independently. Later, the independent colonies on each plate for all test sites are analyzed based on their morphology, with unique colonies being isolated into nutrient broth as a candidate for the remediation system. The purpose of Phase II, therefore, consists of determining which bacteria of all the bacteria grown should be isolated for further analysis and screening for the bioremediation system.

In Phase III the environmental isolates are screened for heavy metal resistance. First, each isolate must successfully grow in heavy metal conditions, isolates showing no growth are removed and are no longer candidates for bioremediation. Secondly, each remaining isolate is screened as to its ability to form biofilms in even stronger heavy metal conditions. The key to the bioremediation system is that the immobilized bacteria will grow and form biofilms, and the biofilms break down the heavy metals. The purpose of this phase is to narrow down the number of isolates and use established criteria to determine which of the isolates are the best candidates to later be used in the bioremediation system.

Phase IV consists of performing a 16S Ribosomal Subunit Analysis on 24 isolates selected from the previous phase. This will identify each isolate as a bacteria species and allow for further analysis before the remediation system can be fully designed. The purpose of this phase is to identify the environmental isolates so that they can be appropriately grouped and further analyzed, ultimately so that the design for the bioremediation system is successful in utilizing bacterial biofilms to break down the heavy metals.

Phase V is the long term and final goal of the project. In this phase, the selected algae are grouped together by common genus, as bacteria of the same genus share many common qualities. Each genus will be partnered with mixed green algae, immobilized in Sodium Alginate Beads, and placed in independent solutions of Lead, Iron, Cadmium, Zinc, Manganese, Chromium, and Cobalt. The overall purpose, therefore, is to successfully develop a novel, cost-effective, and low-impact method for the bioremediation of heavy metals.

Hypotheses:

Many phases of the project are simply procedures designed to take the necessary steps of selecting and analyzing the environmental isolates for the bioremediation system. Therefore, Phases I, III, and V are the only aspects of the project where numerical analysis is used.

Phase I:

H_0: There is no significant difference in the concentrations of Lead, Iron, Cadmium, Zinc, Manganese, Chromium, and Cobalt between the test sites at both field studies: Pennsylvania Mine and Tar Creek, indicating that all upstream sites, mine sites, and sites downstream from the mine all measure the same amount of each heavy metal.
H_A: There is a significant difference in the concentrations of Lead, Iron, Cadmium, Zinc, Manganese, Chromium, and Cobalt between the test sites at both field studies: Pennsylvania Mine and Tar Creek. It is therefore predicted that sites upstream from the mines will have significantly less amounts of heavy metals, the mine sites themselves having the highest measure of heavy metals, and the downstream sites, although not as high as the mine sites, will still show a considerable amount of each heavy metal, indicating that the heavy metals introduced several miles upstream in a water source contaminate the environment in a damaging way a great distance downstream from the point of heavy metal introduction.

Phase III:

H_0: There is no significant difference between the absorbance readings on each isolate in the biofilm screening process. Therefore, each heavy metal had no effect on the growth and formation of biofilms, and all wells have a relatively similar absorbance reading.
H_A: There is a significant difference between the absorbance readings on each isolate, indicating that the heavy metals utilized do have an effect on the growth and formation of biofilms of specific isolates. Therefore, some of the environmental isolates show a better ability of heavy metal growth and resistance than others, as shown by higher absorbance readings in all of the metal wells than other isolates.

Phase V:

H_0: There is no significant difference in the heavy metal concentrations after the algae-bacteria remediation system has been placed in the solutions for two weeks. This indicates that there was not successful remediation of heavy metals, as the concentration did not increase.
H_A: There is a significant difference in the heavy metal concentrations in the solutions tested with the algae-bacteria remediation system. Specifically, a significant decrease suggests that the system was effective, as the algae and bacteria partnership successfully broke down heavy metals dissolved within the solution. This system therefore shows extreme potential for further development and implementation into heavy metal contaminated water systems.

Materials:

Phase I:

Vernier™ Lab Quest II
Vernier™ Optical Dissolved Oxygen Probe
Vernier™ Digital pH Probe
Vernier™ Temperature Probe
Vernier™ Conductivity Probe
LaMotte™ Cadmium Test Kit
Vernier™ Turbidity Colorimeter
Vernier™ Stream Flow Rate Sensor
LaMotte™ Smart Colorimeter III
LaMotte™ Lead Test Kit
LaMotte™ Iron Test Kit
LaMotte™ Zinc Test Kit

LaMotte™ Manganese Test Kit
LaMotte™ Chromium Test Kit
LaMotte™ Cobalt Test Kit

Phase II:

Sterilized petri dishes
Nutrient agar
Inoculation loops
Nutrient broth
Micro-tubes

Phase III:

Sterilized petri dishes
Lead Nitrate
Iron Nitrate
Cadmium Nitrate
Nutrient agar
Nutrient broth
Steam autoclave
96 Micro-well plates
Crystal Violet Stain
30% Acetic Acid
BioTek Synergy 2 Plate Reader
Gen 5 Software (Plate Reader)

Phase IV:

Sterilized petri dishes
Tryptic Soy Agar
PCR tubes
Sterile toothpicks
Proteinase K
Thermo cycler
TE buffer
Micro centrifuge
PCR Master Mix
PA and PH Primers
TBE Buffer
Agarose
Electrophoresis Chamber
Ethidium Bromide
Gel Box Imaging System
Gene Sequencer
NCBI BLAST Website
Finch TV Software
MEGA Software
Distilled Water

Phase V:

Lead Nitrate
Iron Nitrate
Cadmium Nitrate
Zinc Nitrate
Manganous Nitrate
Chromium Nitrate
Cobaltious Nitrate
Calcium Chloride
Mixed Green Algae (Carolina Biological)
Grow Lamps
LaMotte™ Lead Test Kit
LaMotte™ Iron Test Kit
LaMotte™ Cadmium Test Kit
LaMotte™ Zinc Test Kit
LaMotte™ Manganese Test Kit
LaMotte™ Chromium Test Kit
LaMotte™ Cobalt Test Kit
Sodium Alginate

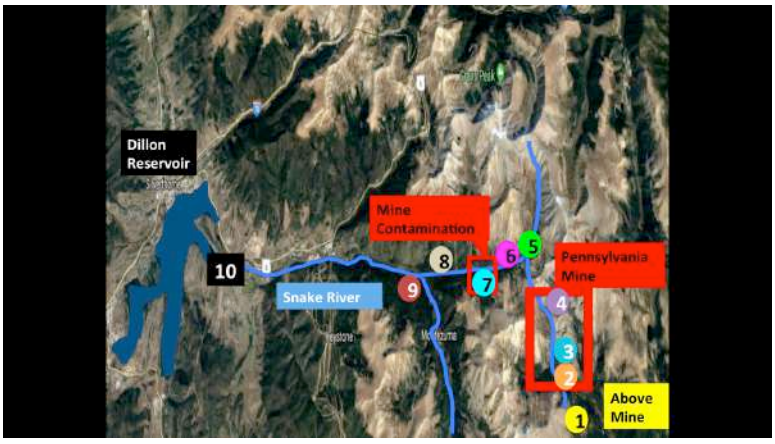
Phase I:

Phase I is designed to accomplish two main goals through two separate field evaluations. The first is to provide exploratory information as to how AMD is effecting the health of environmental water systems as well as determining the extremity of heavy metals contamination in the water systems, both in distance from the source of the mine and concentration of heavy metals in the water. Secondly, Phase I provides the water samples needed to extract unique bacteria and continue with screening processes for heavy metal resistance in later phases, all working towards the end goal of having a select group of bacteria isolated from the environment itself that will be used for a low-impact yet extremely effective method for heavy metal bioremediation.

As previously mentioned, Phase I includes two field examinations. Both sites are EPA Superfund Mine Sites. Each study contains at least one site upstream from all mine contamination to serve as a control, allowing for the comparison of heavy metal concentrations and bacterial isolates between a “clean” water system and the same water system once it has been contaminated with heavy metals. Secondly, each study has multiple sites known as “mine sites”. These mine sites are points along the streams at which the heavy metal contamination is the strongest. The mine sites are the actual locations along the streams at which the stream is directly exposed to a mine or mine drainage. Finally, each study includes many downstream sites. These sites are at multiple points along each stream to analyze how heavy metal concentrations in the water system change as distance from the point of contamination increases. Downstream sites also offer a comparison of bacteria between the upstream and mine sites. The field studies are discussed n detail below.

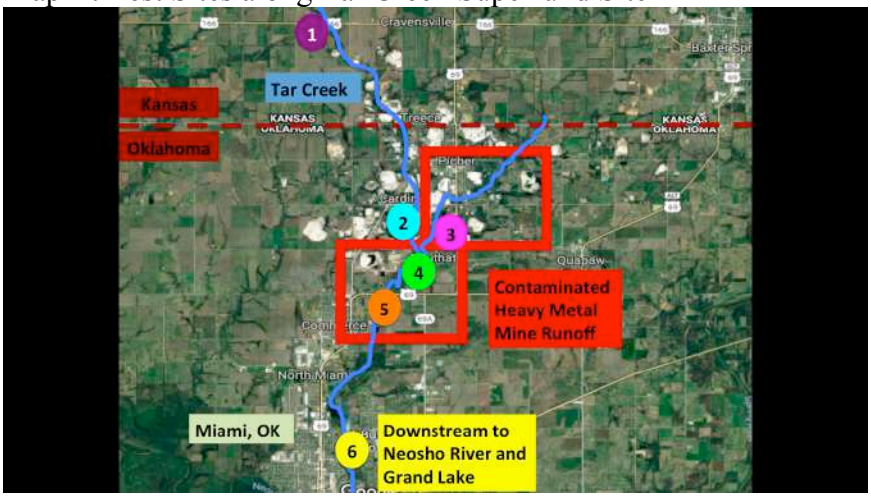
The first field study was conducted along Peru Creek to Snake River and into Dillon Reservoir to track how contamination from Pennsylvania Mine, just outside of the Keystone Ski Resort in Colorado, is affecting the stream. A map of the study is provided below. The source of the stream is spring snowmelt, so, to match the original quality of the source of the stream as accurately as possible, snow that had fallen the previous day was used as the upstream site (Site 1). Sites 2, 3, and 4 are all at different points along the actual mine. Water was flowing out of the mine into engineered pools and channels; so three different pools were analyzed for the mine sites before the water made contact with Peru Creek. Sites 5 and 6 are downstream sites after the mine drainage has entered the stream. Site 7 is another mine contamination site. This case is unique, as it was not expected. While surveying the stream, it was found that at the point that was tested as site 7, AMD was leaking out of the side of the mountain through an underground spring and into Peru Creek, so this site served as an additional source of mine data and bacteria. Sites 8 and 9 are downstream sites, with site 9 being the intersection between Peru Creek and Snake River. Site 10 is the final site, which is the Dillon Reservoir, about 16 miles away from the mine. This lake is owned by the City of Denver as a drinking water source and also serves as a major recreation and fishing area. If the reservoir contains high levels of heavy metals, anyone who drinks the water, including animals, or eats the fish, could be effected by the negative health effects associated with excessive exposure to the metals, as discussed in the background information of this study.

Map I: Test Sites Along Peru Creek to Dillon Reservoir



The second field examination was conducted at the Tar Creek Superfund Site near Miami, Oklahoma. This site is infamous for its enormous chat piles that leach heavy metals into Tar Creek, including high amounts of lead, that lead to the desertion of the towns of Pitcher and Cardin, Oklahoma. Harvard University has even come to do studies and found that elementary school children from Miami, Oklahoma had a high concentration of lead in their bloodstreams. Site 1 of the study is upstream from all mine contamination and is in fact across the Oklahoma border into Kansas, to ensure there is no contamination. The second upstream site, site 2, is just upstream from the intersection between the contamination and the healthy stream, and could show higher concentrations of heavy metals from spring floods. Sites 3 and 4 are the mine sites where the heavy metal contamination is the strongest, with site 3 being just the contaminated water and site 4 being Tar Creek a few feet after the introduction of the contamination. Sites 5 and 6 are downstream sites. Site 6 is near downtown Miami, and Tar Creek will continue from that point to the Neosho River and Grand Lake, another major recreation and fishing area, again increasing the potential that humans and wildlife could be exposed to the health effects associated with heavy metals previously discussed, should either source contain heavy metals. A map can be found below.

Map II: Test Sites along Tar Creek Superfund Site



At each site, readings of Dissolved Oxygen, Temperature, pH, Conductivity, Turbidity, and Flow Velocity were taken using Vernier™ equipment. These readings were taken for general analysis to provide more information about the streams, but are not essential to the main purpose of this study. In addition, three bottles of water samples were taken at each site and transported relative to the stream's temperature to ensure that all bacteria in the samples were in their optimal conditions to continue growth. In Colorado, the samples were transported on ice, as some of the water was collected through broken ice, and the Tar Creek samples were kept at room temperature. The bacterial analysis and isolation is done in Phase II. The final component of Phase I includes using the LaMotte™ Smart Colorimeter III, its test kits, and their protocol to measure the amounts of heavy metals at each site along the streams studied. A summary of this data compared to the EPA Drinking Water Standards can be found below.

Table I: The Average Measure of Heavy Metals at Colorado Field Study Sites Compared to EPA Drinking Water Standards

Metal	Above Mine	Mine Sites	Dillon Reservoir	EPA Drinking Water Standards
Lead	0.00 ppm	21.567 ppm	9.51 ppm	0.00 ppm
Iron	0.00 ppm	93.79 ppm	15.55 ppm	0.3 ppm
Cadmium	0.00 ppm	2.85 ppm	0.25 ppm	0.005 ppm
Zinc	0.00 ppm	14.793 ppm	1.851 ppm	5.00 ppm
Manganese	0.00 ppm	9.967 ppm	0.95 ppm	0.05 ppm
Chromium	0.00 ppm	0.64 ppm	0.22 ppm	0.1 ppm
Cobalt	0.00 ppm	0.973 ppm	0.517 ppm	None Given

Table II: The Average Measure of Heavy Metals at Tar Creek Field Study Sites Compared to EPA Drinking Water Standards

Metal	Above Mine	Mine Sites	Downstream Site	EPA Drinking Water Standards
Lead	1.817 ppm	12.783 ppm	1.027 ppm	0.00 ppm
Iron	7.183 ppm	80.91 ppm	10.113 ppm	0.3 ppm
Cadmium	0.517 ppm	3.2 ppm	1.483 ppm	0.005 ppm
Zinc	0.6 ppm	16.08 ppm	1.377 ppm	5.00 ppm
Manganese	0.2 ppm	35.233 ppm	3.4 ppm	0.05 ppm
Chromium	0.037 ppm	2.567 ppm	0.12 ppm	0.1 ppm
Cobalt	0.043 ppm	2.947 ppm	0.077	None Given

The null hypothesis is rejected. There is clear evidence to suggest that the concentration of each tested heavy metal differs drastically based on the location of the test site in relation to the heavy metal contamination.

It can be seen in the summary tables above that all of the mine sites are unsurprisingly above the EPA Drinking Water Standards. However, on every heavy metal test with the exception of Zinc conducted on the downstream sites of both studies, the concentration was far above the EPA Drinking Water Standards. This indicates that the problem of AMD and heavy metal contamination reaches far beyond the few miles within the mine. Thousands of people could be exposed to these heavy metals without even being aware of their presence. Worse, there is no guarantee that the water filtration process will remove all of the heavy metals from the water; so the millions of people in Denver are also exposed to a major health risk, thus further demonstrating the need for a new method of heavy metal remediation.

Phase II:

Phase II is a short yet crucial phase of the project, as it consists of isolating the environmental bacteria for further screening and analysis to determine which of the isolates are candidates to be used in the heavy metal bioremediation system with the algae in an immobilized alginate bead.

Nutrient agar plates and nutrient broth are prepared according to given protocol. Each of the three water samples from each test site of both field studies are plated on an individual agar plate by adding 200μL of each sample after each bottle has been thoroughly inverted. To ensure that bacteria from each study are in their optimal growing conditions, the Colorado plates were placed in the refrigerator for the 24 hour growing period, while the Tar Creek plates were left at room temperature. After the growing period, individual colonies appeared on each plate. Each colony was analyzed based on its morphology, or shape, color, and other visible characteristics. Unique bacteria were identified and one colony of each unique bacteria was removed with a sterilized inoculation loop and placed in nutrient broth, becoming one of the isolates. Each of the three plates for each test site was analyzed, and after the two field studies had been fully analyzed and isolated, there were 250 environmental isolates that were to be used in Phase III.

Phase III:

The goal of Phase III is to evaluate which of the 250 environmental isolates are the best fitted to serve in the bioremediation system in high concentrations of heavy metals. This was done by designing two different screening processes that eliminate isolates that do not show the capabilities necessary for the remediation system, such as a strong heavy metal resistance and the capability to form biofilms in strong heavy metal concentrations.

The first screening process was designed to determine whether or not the isolates were capable of growing in heavy metal conditions. This is important because not all of the bacterial isolates were from heavy metal contaminated sites. Each of the 250 isolates were streaked from the original culture onto two individual plates: one with nutrient agar containing 20 ppm lead, and the other with nutrient agar containing 100 ppm iron. These concentrations were based off of the concentrations of lead and iron found at the mine sites in the field studies from Phase I. This is a logical choice because the bacteria need to be able to grow in both lead and iron conditions, which are present at nearly every mine, and specifically at those concentrations, as the remediation system would be implemented into similar environments with similar metal concentrations. After a 24 hour growth period, the isolates were analyzed. To advance to the second screening process, an isolate must show successful growth on both the lead and iron plates, therefore no growth at all or no growth on one of the plates results in the removal of the isolate from the study. 189 of the 250 original isolates advanced to the second round of screening.

Screening process II was designed to focus less on whether or not the bacteria grow, and focus more on how the bacteria function in high heavy metal concentrations, specifically, aquatic environments. When bacteria grow, they grow into both planktonic cells and biofilms. The planktonic cells are the free floating particles in a nutrient broth, for example, whereas biofilms are the result of each bacteria cell releasing a sticky film substance and binding together as one mass. These biofilms are hypothesized by many to have the potential for remediating heavy metals. Therefore, for the bioremediation system, a bacterial isolate must be able to form biofilms successfully in very high concentrations of heavy metals in order to remediate them. In the second screening process, each bacterium is placed in a single column of 4 micro wells on a 96 micro well

plate. A control negative is composed of nutrient broth, a control positive is used containing nutrient broth and the isolate, and three individual wells containing nutrient broth with 100 ppm of lead, 100 ppm of iron, and 100 ppm of cadmium with the isolate added to all three. Finally, recent studies in early 2018 show that biofilms grow more successfully along the outer wells of a micro well plate, so to remove any chance of error, the outer wells were filled with distilled water and only the inner wells were used.

A common biofilm crystal violet staining assay was then performed to determine how successful each isolate was in forming biofilms in each of the three heavy metals. After a 24 hour growth period, the plates were forcefully dumped out to remove the broths, water, and any planktonic cell growth. Then, 1% crystal violet stain was added to each of the wells and left for thirty minutes, and then fully rinsed out. This stain stained all of the biofilm growth on the sides of the wells. Finally, 30% acetic acid was added to solubilize the crystal violet stain so that the micro well plates could be read for absorbance readings with a plate reader. A higher absorbance reading indications the darker the well, indicating that there was more biofilm growth. A BioTek Synergy II Plate Reader was used at the OSU Tulsa microbiology lab by the presenter to determine these absorbance readings.

Phase IV of the examination is a 16S Ribosomal Subunit Analysis, which is very detailed and laborious process, so a set number of 24 isolates was chosen to limit the number of isolates moving on for additional screening as candidates for remediation. Therefore, to ensure that the final selection of environmental isolates was as diverse as possible and did not include multiple isolates from the same site or same species, criteria were developed to decide which of the 189 isolates advanced to become one of the 24. First, the bacteria must have a high absorbance reading in all of the heavy metals tested, suggesting the ability to form biofilms in high concentrations of heavy metals is present. Secondly, the test site of the bacteria is analyzed to ensure that bacteria in the final round represent many different levels of heavy metal exposure along both of the streams tested. Finally, the morphology of the isolates is further analyzed to determine whether or not a bacterium is unique, as 24 isolates of the same genus or species does not represent the environment as a whole. Table III below shows a summary of the bacterial isolates selected for Phase IV of experimentation.

Table III: Bacterial Isolate Screening Summary

Environmental Isolate Screening Process Summary	
Total Isolates: 250	
Total Heavy Metal Resistant Isolates (Screening 1): 189	
Total Isolates Selected for 16s Ribosomal Subunit Analysis: 24	
Number from Colorado Study: 12	Number from Tar Creek Study: 12
Number of Colorado Sites (out of 10) represented by final isolates: 7	Number of Tar Creek Sites (out of 6) represented by final isolates: 6
Number of Final Isolates from Contaminated Mine Sites : 11	
Number of Final Isolates from Upstream/Downstream Sites:13	

Although the extensive biofilm absorbance data is not shown, 24 isolates could be selected based on the above criteria. Therefore, the null hypothesis is rejected, as indicated by the fact that the absorbance readings did differ for each metal between the isolates, suggesting the heavy metals did have an effect on the growth and formation of biofilms on some isolates.

Phase IV:

Phase IV focuses on analyzing the 24 environmental isolates shown to be the top contenders for heavy metal remediation based on the screening process. Phase IV consists of performing a 16S Ribosomal Subunit Analysis on each isolate to identify each genus and species. This data is important to know as each bacterial isolate will then be known to show heavy metal resistance and further research about each genus and species can be pursued. In addition, this identification helps with the design process of the remediation system.

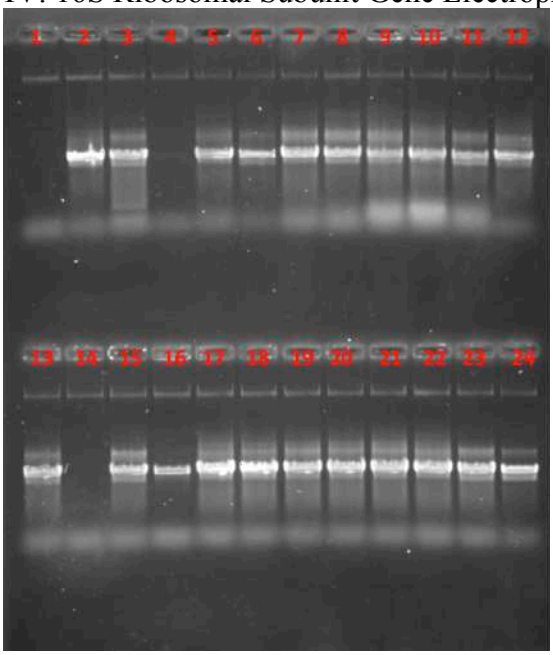
The first step is the extraction of a pure colony from the isolates. Each isolate culture is streaked on TSA plates with long, continuous back and forth motions to try and isolate colonies. After 24 hours, a single colony is picked with a sterile toothpick into PCR tubes containing single cell lysing buffer and restriction enzymes that will "clean" the DNA, "cutting out" the 16S Ribosomal Subunit Gene. The tubes are placed in the thermo cycler overnight.

Next, the DNA is extracted and stored for later use. A Master Mix as well as PA and PH forward and reverse primers are added to clean PCR tubes. This ensures that as the DNA is separated via heat in the PCR machine, each strand of the DNA gets replicated from the 5 Prime to 3 Prime ends. Therefore, two copies of each piece of extracted DNA exist. Some of the extracted 16S Ribosomal Subunit Analysis Gene DNA is then added to the tubes. The tubes are placed into the PCR Machine overnight for DNA amplification, or the constant replication of this piece of the 16S Ribosomal Subunit Gene to ensure that enough DNA is present for accurate results in further analysis.

The final step of the process is gene sequencing, and a contaminated sample will skew the results, as the gene will not be correctly sequenced. To ensure that each isolate contains a pure sample of the correct gene, the 16S Ribosomal Subunit Gene, Electrophoresis is used to determine the purity of each isolate. A standard agarose gel is prepared and samples of the amplified DNA of each isolate are added into each well. The gel and electrophoresis chamber is then filled with TBE Buffer. The electrophoresis chamber is connected to the electric controls and the gel runs for a duration of 1 hour. The gel is then removed and stained in Ethidium Bromide and rinsed in distilled water. The gel is photographed in the gel box imaging system, and the photograph can be seen below.

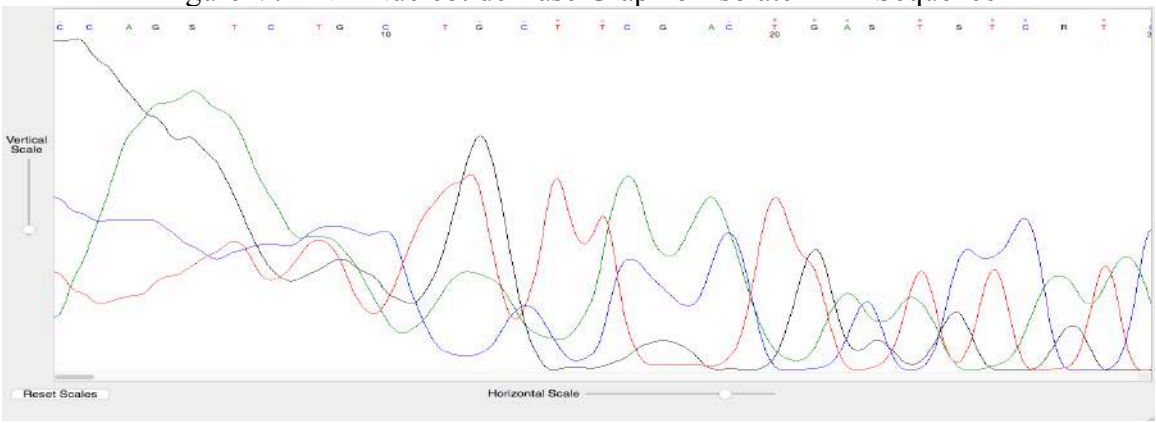
It can be seen on the gel that there is no detection of DNA for isolates 1, 4, and 14. This indicates that there is an error, and these isolates are excluded from further analysis. All of the isolates show one, clean, similarly sized bar, indicating the 16 S Ribosomal Subunit Gene has been correctly extracted and amplified, and the isolates are ready for sequencing. Isolate 24 was not sequenced, as there is some formation of an additional bar, indicating possible contamination.

Figure IV: 16S Ribosomal Subunit Gene Electrophoresis



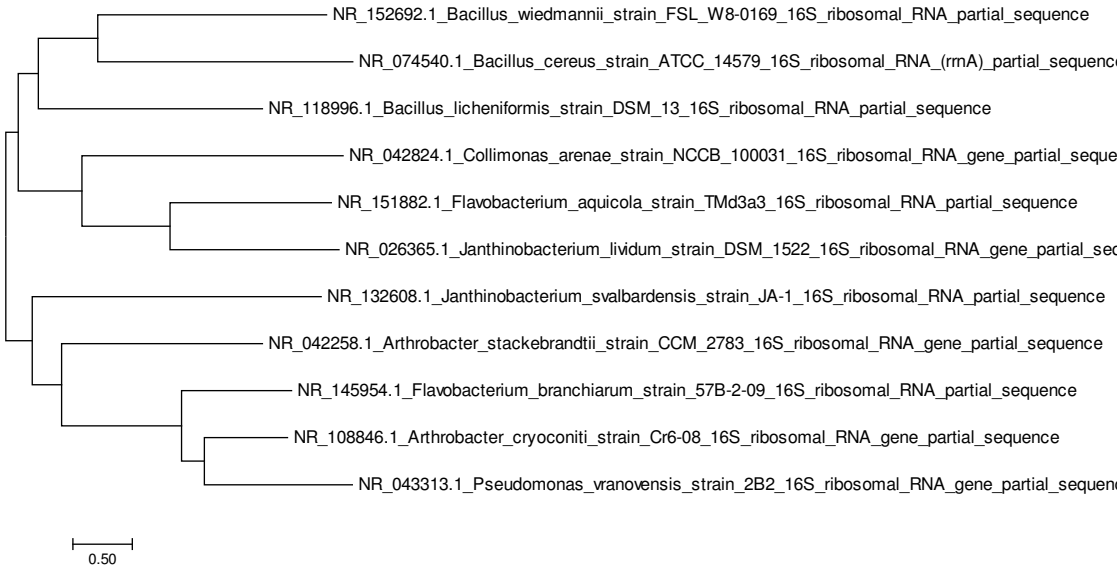
Finally, the isolates are prepared for gene sequencing. The remainder of the amplified DNA is added to a plate along with the master mix and either the PA or PH restriction enzyme. So, because 20 isolates are sequenced, and they are each sequenced twice, once with the PA primer and once with the PH primer, there are 40 sequences given, with the PA of one sample being the reverse sequence of the PH sequence, and vice versa. The plate is inserted into the gene-sequencing machine at the University of Tulsa and run for the pre-set amount of time. The resulting data is analyzed with the Finch TV program and each DNA sequence is added to a word document for analysis, the PA and PH of each isolate kept together. All of the sequences contain some errors, where no base is detected, so the DNA nucleotide base graph associated with each isolate is consulted to ensure that each sequence is accurate and complete. A small picture of this graph can be seen below.

Figure V: DNA Nucleotide Base Graph of Isolate 2 PH Sequence



Once each sequence is complete, the first twenty and last twenty bases are deleted from the sequence, as error can sometimes occur when sequencing the ends of a gene. The middle portion of each sequence is inserted into the NCBI BLAST nucleotide website and matched with bacteria showing extremely high similarity. The bacteria genus and species is recorded and accepted as the most likely identification of the isolate. This is done for both the PA and PH sequences of each isolate to ensure that the results are the same for both. Finally, this data is utilized to construct a cladogram depicting the relationship of each bacteria species in relationship to one another in the MEGA software program. There were a few duplicate bacteria identified, hence the fact that there are 11 species instead of 20.

Figure VI: Cladogram of the Relationship Between Sequenced 16S Ribosomal Subunit Genes from Environmental Isolates



Phase V:

The sequenced environmental isolates are completely analyzed and are believed to be the best candidates for the bioremediation of heavy metals as indicated by the previous four phases of the project. Now, the overarching goal of the project, the remediation system, can be designed.

The system combines mixed green algae and the environmentally isolated bacteria in an immobilized sodium alginate bead. This symbiotic relationship between the algae and bacteria is the key to the success of the system. The algae photosynthesizes, providing the bacteria with a food source and oxygen source so that the bacteria can grow and form biofilms. The biofilms of the bacteria then remediate the heavy metals in the stream via complex mechanisms for each specific metal. As the metal concentration decreases, the algae can continue to grow and thrive, while also raising the dissolved oxygen in the stream as a result of photosynthesis. Every reaction and impact of this unique partnership benefits the other organism and the stream. In addition, native species of bacteria are used that are already shown to be heavy metal resistant, and neither the

bacteria nor the algae are introduced directly into the stream, as they are completely contained in the alginate beads.

Each of the 20 bacterial isolates is recultured in fresh nutrient broth. To test for the best remediation system, the bacterial isolates are divided up by genus, as each genus of bacteria share many similar traits. So, the six geniuses are Bacillus, Janthinobacterium, Arthrobacter, Pseudomonas, Flavobacterium, and Collimonas. Each of the new bacterial isolate cultures from each genus are combined into a common culture, so there are six different genus cultures in the end.

Mixed green algae is grown and centrifuged multiple times to ensure that a decent amount of algae is present in the beads. A similar process is repeated for each genus of bacteria, with the concentrated bacterial pellet being added to a tube until there was 2.5 mL of bacterial solution present. Then, 2.5 mL of the green algae was added. The two solutions were mixed well. 2.5 mL of sodium alginate is added to the tube, and plastic pipettes were used to drop the solution into calcium chloride, which seals the beads. This process is repeated for each genus of bacteria, so that there are six types of beads in the end, all containing mixed green algae and a different genus.

5 parts per million (ppm) solutions of lead, iron, cadmium, zinc, manganese, chromium, and cobalt are prepared. Ten alginate beads of each bead type are added to tubes containing the 5 ppm solutions, with one tube being left with only the solution as a control. For example, the metal lead contains seven test vials. Each of the first six contains 10 beads of a different bacterial genus, and the seventh is left empty as a control. All vials are left under grow lamps for two weeks. This is the setup used for each of the heavy metals being tested. The setup can be seen below.

Figure VII: Bioremediation Test Set Up

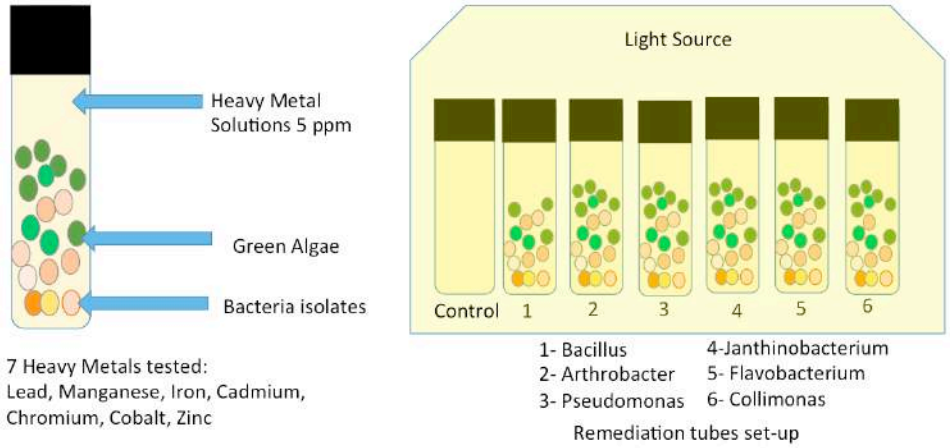


Table IV: Results of Bioremediation System

Colli-monas	Bacillus	Pseudo-monas	Heavy Metals	Control	Flavo bacteri
1.2%	87.27%	44.6%	Lead	0%	38.4%
92.8%	83.6%	69.4%	Iron	0%	87.2%
80.2%	79.4%	81.2%	Cadmium	0%	73.6%
80.4%	86.8%	75.4%	Zinc	0%	88.0%
16.0%	42.0%	10.0%	Manganese	0%	42.0%
89.6%	95.8%	93.8%	Chromium	0%	89.0%
88.6%	91.8%	89.8%	Cobalt	0%	93.0%

50% Change > 80% No Change

The results of the bioremediation system test demonstrate the extremely significant potential this system holds. In the table above, it can be seen that there was no change in the concentration of the 5ppm control solutions of each heavy metal. However, all of the boxes in dark green show an over 80% decrease in heavy metal concentration, an astounding amount considering only 10 beads were present in each solution for a two week period.

Due to lack of funding, the EPA Superfund Program only includes 1,332 mines out of the estimated 500,000 abandoned mines in the United States, which translates to 0.003% of the total number of mines. The remediation system presented consisted of 600 individual alginate beads, with a cost totaling only $10. Recently, the EPA has spent around $300 Million annually on the Superfund Program. The development and implementation of this system into the environment therefore would allow for a massive financial benefit, thus allowing a very large majority of the 500,000 mine sites to be remediated. The fact that the majority of these systems remediated over 80% of the heavy metal concentration in such a short time also signifies the groundbreaking success has in the mission to remove heavy metals from the environment; a problem humanity has been embattled with for nearly 100 years.

Conclusions:

Heavy metal contamination as a result of AMD is among the top problems facing the environment today. Only, the environment is not the sole concern. Field studies in this examination demonstrate that heavy metals are found many files outside of their original source, and these concentrations are well above EPA Drinking Water Standards. Even through water purification systems, no system is 100% effective and humans could still easily be exposed to heavy metals and the negative, life-threatening effects associated with exposure.

In addition, heavy metal contamination has the potential to effect human and animal life in an indirect way as well, as heavy metal exposure in the food chain results in biological magnification, or the increasing of heavy metal concentration within a predator organism, obtained from its prey, which was in one way or another exposed to heavy metals. This means that a practice as common as fishing could be dangerous in areas where heavy metal contamination was unconsidered.

There are few problems as large and widespread affecting the population of the United States in such a way. With over half a million abandoned mines, and only a small fraction being maintained by the EPA, heavy metal exposure is inevitable. Not to mention the tens of millions of dollars spent on remediating abandoned mines, yet no significant results have been accomplished. Secondly, the remediation efforts currently in practice are often not significantly effective or low impact, as entire ponds and remediation plants have been built.

These problems give clear evidence to suggest that there is an immense need for a new method of remediation. The proposed method utilizes clean sources, native isolates from the stream, to accomplish the goal at stake. In addition, the new method is very low impact and protects the stream from the remediation species and the remediation devices can be easily inserted and removed from the stream systems due to the immobilization of the remediation species. Finally, and perhaps most importantly, this new method shows promise as far as funding goes. This method is extremely cost effective, compared to the little progress that the millions of dollars in government funding have accomplished over the years.

This study utilized environmental isolates, determined to be heavy metal resistant through a detailed screening process, and sequenced in an in depth 16S Ribosomal Subunit Analysis to develop a heavy metal bioremediation system to attempt to fill the massive gap left in this problem. There is an extreme problem that requires a novel, out of the box solution, and the proposed method and the pending results are great examples of the required detail and dedication a successful solution requires.

Resources:

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