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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Masterthesis

Mine site remediation enhanced through exploitation of local microbiome

Tori Langill

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :

Prof. dr. Jaak VANGRONSVELD

dr. Sofie THIJS

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Universiteit Hasselt
Campus Hasselt:
Martelarenlaan 42 | 3500 Hasselt
Campus Diepenbeek:
Agoralaan Gebouw D | 3590 Diepenbeek

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"Be grateful for what you already have while you pursue your goals. If you aren't grateful for what you already have, what makes you think you would be happy with more?" – Roy T. Bennett

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List of abbreviations and definitions

Abbreviations

ACC-deaminase. 1-Aminocyclopropane-1-Carboxylate deaminase
ARISA. Automated method of ribosomal intergenic spacer analysis
GRO. Gentle remediation options
IAA. Indole-3-acetic acid
ICP-OES. Inductively coupled plasma optical emission spectrometry
MetaPhlan2. Metagenomic Phylogenetic Analysis 2
MG-RAST. Metagenomic Rapid Annotations using Subsystems Technology
PEAR. Paired-End reAd mergeR
PCR. Polymerase chain reaction
PGP. Plant growth promotion
PGPR. Plant growth promoting rhizobacteria
QIIME2. Quantitative Insights Into Microbial Ecology 2
qPCR. Quantitative polymerase chain reaction
ROS. Reactive oxygen species
SEM. Scanning electron microscopy
VAMPS. Visualization and Analysis of Microbial Population Structures
VAPs. Vertical agar plates

Definitions

Accumulators. Plants that concentrate metals within their shoots
Bioleaching. The extraction of metals from ore through living organisms, usually bacteria
Excluders. Plants that restrict metals to their roots and do not allow root to shoot translocation
Historic Tailings. Tailings of a mine that is no longer active
Hyperaccumulators. Plants that concentrate metals within their shoots at concentrations >1000mg/kg
Shotgun sequencing. A method used for sequencing long strands of DNA
Tailings. Mine waste product, including but not limited to, waste rock, effluents, water, heavy metals

Samenvatting (Nederlands)

Inleiding: Koper is een zeer gewild metaal omwille vanzijn gebruik in groene technologie, unieke antimicrobiële oppervlakte en zeer goedgeleidende eigenschappen. Het ontginnen van koper veroorzaakt echter schade aan de omgeving. Mijnbouw leidttot ontgonnen gebieden die niet enkel een gevaar vormenvoor de menselijke gezondheid door grondwater verontreiniging, maar ook voor de natuur door ernstige bodem verontreiniging. Planten herintroduceren in deze ontgonnen gebieden is een belangrijke stap in hun sanering. Fytoremediatie is het gebruik van interacties tussen (1) planten en bacteriën, (2) planten en bodem en (3) bodem en bacteriën om vervuilende stoffen te verwijderen of te stabiliseren om herstel van het ecosysteem aan te moedigen. Deze thesis onderzoekt het potentieel van het introduceren van planten op verontreinigdesites door het gebruik van natuurlijk voorkomendemicrobiële gemeenschappen van een mijnsite onder herstel (Kamloops, Canada, Hoofdstuk 1) en een actieve mijnsite (Galicië, Spanje, Hoofdstuk 2).

Material & methods: Verschillende technieken werden gebruikt om het volledige plaatje van bacteriën en planten van deze mijnsites te onderzoeken. Plantengroei promotie testen (PGP) werden uitgevoerd op de cultiveerbare bacteriën, en ICP-OES werd uitgevoerd op planten- en bodemstalen om de mate van metaal verontreinigingvast te stellen op alle sites. Ion-Torrent Sequencing werd uitgevoerd op stalen van beide sites, en shotgun sequencing werd uitgevoerd op stalen van de historische mijnsite. Een uitlogingstest werd uitgevoerd op stalen uit Spanje om de capaciteit van de bacteriën om het meer biobeschikbaar maken van metalen te testen. Veranderingen in de bacteriële gemeenschap werden geanalyseerd met behulp van qPCR. Analyse van de gemeenschapstructuur werd uitgevoerd op alle stalen om verder te onderzoeken waarom precies deze gemeenschappen gedijen op de mijnsites. Uiteindelijk werden twee soorteni*In plantae* tests uitgevoerd: één waarbij het effect van individuele bacteriesoorten op wortelgroei onderzocht werd, en een tweede waarbij het effect van een consortium op kieming, planengroei en koper-opname op verontreinigdebodems onderzocht werd.

Resultaten: Er werd vastgesteld dat een consortium bacteriën van een verontreinigdesite plantengroei bevordert en een significant effect heeft op kieming ($p<0.05$; $n=12$). De rhizosfeer van de metaal-uitsluiter *Holcus lanatus* is interessant voor fytoremediatie, door het feit dat hij de hoeveelheid koper in de bodem verlaagt door het meer biobeschikbaar maken van koper door uitlogen. Koper werd vervolgens in grotere mate opgenomen door planten die geen uitsluiters zijn. Onderzoek van de bacteriële gemeenschap toont aan dat de structuur van de gemeenschap verandert volgens de omgeving. Ondanks het feit dat een diverse bacteriële gemeenschap een teken is van een herstellende omgeving, is diversiteit geen noodzakelijk drijvende kracht achter bodemsanering.

Discussie & conclusie: Uit de resultaten van deze thesis blijkt dat bacteriën van mijnsites de noodzakelijke kenmerken bezitten om te helpen bij het (her)introduceren van planten. Bacteriën van metaal accumulators en uitsluiters zijn meer geschikt dan bacteriën van historische mijnsites. Dit kan te wijten zijn aan het feit dat deze bacteriën inheems zijn aan de site waar ze getest werden en de historische bacteriën niet. Desondanks is er een groot potentieel voor succesvolle saneringdoor fytoremediatie van mijnsites.

Summary (English)

Introduction: Copper is a highly sought after metal owing to its use in green technology, unique antimicrobial surface, and highly conductive nature. Removing it from the environment, however, causes detriment to the Earth. This creates large devastated areas of land that are not only a risk to human health through groundwater contamination, but also a risk to the environment owing to high levels of soil contamination. Establishing plant life on these devastated sites is a key step in remediating them. Phytoremediation is the use of plant-microbe, plant-soil, and soil-microbe interactions to remove or stabilize contaminants on a site to encourage environment recovery. This thesis looks at establishing plant life on contaminated sites through exploiting local microbiomes of recovering mine sites (**Kamloops, Canada, Chapter 1**) and active mine sites (**Galicia, Spain, Chapter 2**).

Material & methods:A variety of techniques was employed to tell the complete story of the microorganisms and plants associated with these mine sites. Plant growth promotion (**PGP**) tests were performed on the culturable portion of bacteria, and **ICP-OES** was performed on soil and plant material to determine the level of contamination on all sites. **Ion-Torrent Sequencing** took place on samples from both sites, and shotgun sequencing was done on the Historic tailings samples. A **bioleaching test** was performed on the rhizosphere samples from Spain in order to assess their ability to make metals more bioavailable. Community changes were analyzed with **qPCR**. Analysis of community structure was performed on all samples to offer insight into what makes these communities thrive the way they do. Finally, two types of ***In plantae* tests** were performed: One using individual species of bacteria to determine the effect on root length, and the other using a consortium to determine the effect on germination, plant size, and copper uptake on contaminated soils.

Results: It was determined that using a consortium of bacteria from a contaminated site increases plant size, and has a significant germination effect (**$p<0.05$; $n=12$**). The rhizosphere from excluder species *Holcus lanatus* showed promise for phytoremediation, as it lower copper levels in the soil theoretically by making copper more bioavailable through leaching. The copper is then taken up in greater quantities into plants that are not excluders. Community assessment indicates that the structure changes in accordance to the environment, and that although a diverse community is a sign of a recovering environment, diversity is not a strength when it comes to decontamination.

Discussion & conclusion: Based on the results of this thesis, it can be concluded that mine site bacteria possess the traits necessary to assist in establishing plant life. Bacteria from accumulators and excluders hold more promise than historic mine site bacteria, but this is likely because these

bacteria were local to the site where they were tested and the historic bacteria were not. Regardless, there is high potential for successful site remediation through use of phytoremediation.

GENERAL INTRODUCTION AND OBJECTIVES

Throughout history, **mining** has been the foundation on which progress was built. This fact remains true today as precious metals are required to make advancements in clean energy, transportation, agriculture and medicine. **Copper** in particular has a leading role in the future, owing to its highly conductive nature, making it ideal for use in clean energies, such as wind and hydro(1). The metal is also sought after in the medicinal industry, as it has a highly antimicrobial surface, meaning it can be used to line doorknobs and working surface to lower the risk of spreading infection(2–4). Next to this, copper has also been used as a weed killer and fungicide in agriculture(5,6). As such, it cannot be denied that copper plays a much more active role in our lives than most people realize.

Acquiring copper for current and future technologies requires mining. Elemental copper, the element most searched for, exists within the earth in rock formations called **ore bodies**. These ore bodies contain copper iron sulphate, often called chalcopyrite or bornite, depending on the concentration of copper within the ore. Because the copper is locked in an ore body, the ore must be mined to extract the copper. Copper is normally mined through a type of mining called **open pit mining**. Open pit mines blast and excavate surface rock to access the ore body from the top, rather than tunneling in beneath and extracting from below.

The process of **open pit mining** has advantages and disadvantages, but one of the disadvantages that is consistent across all mine sites is the **waste products**, called **mine tailings**.Contaminants in mine tailings include but are not limited to, **heavy metals** such as Cd, As, and Cu, **organic contaminants** like toluene, benzene, and other effluents used during the purification process(7,8), not to mention **waste rock** from the blasting making the soil less arable(9,10). Because mine waste products are detrimental to the environment, and the mining process itself devastates the landscape through deforestation and pollution, mining companies are required by law to remediate the site after the mining process is complete.

In the past, **remediation techniques** have varied in regards to the type of contaminant affecting the site, but a few of the more popular ones included chemical techniques, such as remediation using actinide chelators and physical treatments, such as capping or cementitious waste forms(11). Chemical and physical remediation techniques offer a temporary solution to the problem at hand, but they possess the disadvantage of not removing the contaminants. Instead, they just remove the problem from sight. Adding chemicals to treat a tailings pond may work to remove and stabilize contaminants, but it has an unfortunate effect on pH, making the soil more alkaline, making it more difficult to establish plant life in the future, as most plants enjoy an acidic or neutral pH(12).

Establishing plant lifeon a recovering site is a key attribute to a successful remediation, as plants and their associated microorganisms play an interactive role in changing their surrounding soil and

removing contaminants through plant-soil interaction, plant-microbe interaction, microbe-microbe and microbe-soil interactions (13–16). Because these reactions play an integral role in removing contaminants and adjusting the soil to host more species, **phytoremediation** is becoming a more popular method for dealing with contaminated sites.Phytoremediation is a technique that involves exploiting the plant-microbe interactions by choosing select species in an attempt to detoxify a site through degradation or stabilization of contaminants(17).

When a mine site has achieved its objective of mining the ore body, the mine tailings are often left in the form of tailings pond, or slurry tailings, until an appropriate remediation technique can be decided upon. Sometimes this takes years and results in what is known as a **historic tailings pond**. Another form of historic tailings isa tailing that was treated to the standard required by the legislature in previous years, but does not meet the current standards and therefore needs further remediation efforts. This is the case of the **New Afton Historic Tailings in Kamloops (British Columbia, Canada)**. The historic tailings is the result of a mining operation that ended in the 1970's, and has had ownership passed between different mining companies for the past 40 years. Originally remediated to be a lake, the historic tailings was drained last year in an effort to reintroduce local plant species on the large acreage.

The activities at the **Touro mine in Galicia (Spain)** ended a bit later than the one in Canada, in the early 1980s. There is still a large area covered with tailings and areas used for cutting and mining operations that were exposed to rain, sun, freezing-thawing resulting in the biological and physicochemical erosion and transportation of metallic ions, sulfates, sulphuric acid towards rivers and the underground over a large area. This site has also only been partially restored or recovered. The site is currently undergoing phytoremediation to remove trace metals from the soil, through Gentle Soil Remediation Options (**GRO**). These techniques include research into selecting the right plant species for the remediation process, as well as topsoil analysis(18). Once appropriate plants have been selected, their potential to uptake trace metals on the mine site can be evaluated, making them potential candidates for full scale remediation (19).

However, as was stated previously, phytoremediation is not only about the plant-soil interactions, but also the plant-microbe interactions, the microbe-microbe and soil-microbe interactions. Microbial communities play an integral role in the remediation processof mine sites, but to date there has not been much research done on how they play a role, or what the community structure is. Previously, studies on total microbial community analysis of copper mine tailings have shown large numbers of Proteobacteria on the contaminated site, but have not linked function to these groups, or assessed the community in regards to revegetation and/or phytomining (20). Other papers addressed community changes due to bioleaching and acid mine drainage, but did not assess whether these communities could encourage the establishment of plant life (21,22). Again, some papers have addressed how microbial community changes in the soil led to the establishment of plant life, and how plant life can in turn change the bacterial communities, but did not contain whole shotgun metagenome data to

complete the story (23–26). This thesis completes the story through assessing microbial community structure, gene function and *in plantae* tests with microorganisminoculated seeds.

A main **impasse** in the phytoremediation and revegetation of mine sites is the efficiency, length of remediation, predictability together with the lack of insights in the microbiota associated with these 'extreme environments' which can help the plants to withstand toxicity (27). Before we can exploit microorganisms, we need to know which species are present. Once we possess that information, we can utilize these microorganisms to improve plant establishment, increase revegetation speed, and increase Cu-uptake from the soil. The key to successfully exploiting a bacterial community is understanding who is there and what roles they are playing.

In this thesis, I lookedinto the microbial communities of the New Afton Historic Tailing in Canada and the Touro copper mine in Spain to (a) assess the impact of high copper levels in soil (and other mine-site extreme conditions), on the microbial community structure and diversity in soil, plant rhizosphere and endosphere across a gradient of copper concentrations and different plant species, (b) isolate and characterize copper tolerant bacterial strains, (c) characterize the isolated strains for plant growth-promoting effects and copper translocation as well as Cu-bioleaching, and (d) to investigate whether transplanting a copper-tolerant rhizosphere microbiome from a hyperaccumulator or excluder to a cover crop species can improve plant copper uptake, translocation and/or tolerance.**It is hypothesized** that bacteria which live in contaminated, stressful environments possess the genes necessary to remediate the site as well as establish plant life there. In particular, that bacteria from the few vegetation hubs on the historic tailings will have a strong community of bacteria that support their plant growth and can be exploited to establish plant growth on other sites with similar contamination.

1.1 OBJECTIVES

1. Characterize the bacterial communities across a copper-gradient on the New Afton historic tailings, in order to determine community structure and diversity
2. Characterize and compare the microbial communities across the high-copper TouroArriba and lower-copper Touro Abajo in Spain.
3. Isolate and culture bacteria from the soils, rhizosphere and plant-endosphere in Canada and Spain.
4. Characterize the isolated bacteria for their *in vitro* PGP
5. Assess community bioleaching ability

1.2 OUTLINE OF THE THESIS

In chapter one, we describe the results and findings from the historic tailings from Kamloops, and in chapter two we assess the results from the Touro sites in Spain.

Chapter 1

CHAPTER 1: SOIL MICROBIOTAFROM HISTORIC WASTE TAILINGS HOLD FEATURES THAT CAN ASSIST IN COPPER PHYTOREMEDIATION

TORI LANGILL[1], JONATHAN VAN HAMME[2], BREANNE MCAMMOND[2], JAN D'HAEN[3],JACO VANGRONSVELD[1], SOFIE THIJS[1]

[1] Department of Biology, Centre for Environmental Sciences, Hasselt University, Belgium
[2] Department of Biological Sciences, Thompson Rivers University, Kamloops, British Columbia, Canada
[3]Institutefor Material Research (IMO-IMEC), HasseltUniversity, Diepenbeek, Belgium

ABSTRACT

Intro. Copper contamination on mine sites is an issue in establishing plant life on recovering mine sites. Bacteria from historic mine sites can assist in this endeavor, as it is hypothesized that they will possess unique genes and community structures tailored to remediating the soil.

Mat. And methods. Shotgun sequencing was performed as well as community fingerprinting, and culture-based isolation and phenotyping of bacterial strains for**plant growth promotion (PGP)**. Two types of *in plantae* tests were used to test the difference between the effect of adding a single enriched PGP microorganism, or adding an enriched community from a contaminated site. Statistically relevant bacteria were subsequently evaluated in a r**hizosphere grafting** experiment.

Results + conclusion. The historic tailings bacteria possess degradation traits, uniquely suited to their environment. Areas that have begun to revegetate show more diversity, but less ability to remediate as their community counterparts from unvegetated areas. Proteobacteria is the leading phylum of bacteria on copper mine sites, but in the event of higher contamination, Bacteroidetes becomes the prominent phyla.

Future outlook. Understanding soil bacterial communities and the role they play in phytoremediation will help in establishing plant life on contaminated sites. It will also assess a viable option for a passive remediation strategy for mine sites that is non-invasive to the environment and is economically feasible.

INTRODUCTION

Copper mining is essential to provide us with necessary ore for technology, but it comes at high environmental cost. Mining that occurs via open pit creates detriment in the form of air pollution and soil pollution. These types of pollution not only pose a health risk in the form of particulate matter and heavy metals introduced into groundwater, but also a large environmental hazard. Abandoned mine sites can be recognized by historical tailing ponds, which are a reservoir of mine waste, contained in a pond to prevent it from leaching further into the environment.

Remediation of mine sites is not an easy task, with toxicity and harsh, hostile conditions limiting seed germination and plant growth. Similar to most devastated sites, the seed bank of the soil containing the local plant species has been removed by the damaging activity (25).

Figure 1:Ongoing experiment using nurse plants and soil additives to encourage plant growth on alkaline tailings

One interesting mine site with history of copper mining and restoration is the New Afton Copper/Gold mine in Kamloops, BC, Canada. It has been used to access the local ore body through both open pit and underground mining. Restoration practices have been initiated with a dewatering and consolidation program (28), but in general, slow recovery is noticed, likely owing to soil contamination level and soil type limiting vegetative growth. Paul Antonelli previously attempted soil amendments using nurse crops to restore native grassland on tailings site (unpublished, personal communication) (**Figure 1**). Though the results of the study were positive, further advance in this field is only possibleby also looking at what is belowground, the associated microorganisms in soil and rhizosphere.

Microbial communities of extreme environments, such as mine sites, potentially have extraordinary properties. Culture-based surveys indicate lower levels of bacterial diversity in metal contaminated studies(29), but an increase in the level of metal tolerant species(30). This is because contaminated sites are a hotspot for **horizontal gene transfer**, allowing microorganisms to pick up the resistance and degradation genes they need to survive (31).

To date however, there are no studies on total microbial communities of copper mine sites and their functional characterization in respect to phytoremediation and plant establishment.Many of the open questions that are invoked are: how do community structures vary across a gradient of copper, how do communities change in regard to establishing plant life, can historic tailings associated bacteria be used to assist in removing heavy metal contaminants, and can these communities be exploited to speed up the process of establishing plant life.

In order to test the aforementioned hypothesis, three main objectives were developed. First, the level of contamination and general soil properties at each of the selected locations was determined. Second, community structure was determined, along with individual tests on a culturable portion of the bacteria, in order to assess their capability for phytoremediation. Finally, *in plantae* tests were used to assess the application value of this technology with test species alfalfa(*Medicago sativa*).

MATERIAL AND METHODS

Soil collection

Soil samples were taken from the Historic Afton Tailings on July 27th, 2016, from a depth of approximately 10cm. The temperature was 31°C, and 0% RH. GPS coordinates of each sample are summarized (**Table 1**). Soil was sampled from this depth to ensure maximum biological activity.The distance between samples was approximately 1 meter to give a distribution parameter of microbial life from areas on the tailings which contained plants towards the areas that did not. A total of eight samples were taken in duplicate, six from a vegetated hub towards non-vegetated area (**Figure 2**) and two samples 52 meters distant from this area, where the soil turned from loam clay to a reddish sandy clay (**Figure 2**). Alfalfa (*Medicago sativa*), a cover crop, and local plant species of crusted wheat grass, sage brush and mustard, were found growing in intermittent intervals and had rhizosphere samples taken.Samples were transferred to Thompson Rivers University, where they were sieved and homogenized. Aliquots were storedat 4 °C for bacterial isolation and at -80 °C for DNA-extractions.

Soil analysis

Soil was dried at 105°C for 12 hours with before and after weights determined for soil moisture. Soil pH was measured in water and in 0.1 M KCl in a 1:1.25 soil:solution ratio (32). Total metal extractions were performed according to the microwave digestion method (33), using 500 mg of soil material that was digested using 1mL *supra pur* HNO_3 and 3mL *supra pur* HCl in a microwave. Samples were measured on ICP-OES (Agilent Technologies, 700 Series, Belgium). Metal presence was also confirmed with scanning electron microscopy (FEI Quanta 200 FEG-SEM, Eindhoven, Netherlands). In brief, dried and < 1 mm sieved soils were fixed on a stub and observed under high vacuum conditions. The plant

available metal was extracted with $Ca(NO_3)_2$according to the paper by Camargo (34). In brief,five grams of soil was added to 25ml of 0.1M $Ca(NO_3)_2$, shaken overnight at 100rpm, filtered through Wattman 40 filter paper andacidified filtrate was analyzed by ICP-OES (Agilent Technologies, 700 Series, Belgium) against standards and negative controls. Total carbon and nitrogen content per dry mass soil was determined by a Vario MAX CNS elemental analyzer (ElementarAnalysensystemeGmbH, Hanau, Germany). Soil texture was determined by sieve-method and classified using the USDA-scheme (Soil Survey).

Bacteria isolation

Bacteria were isolated from the soil samples by spread plating soil suspension dilutions on different media and by *in situ* incubation using diffusion chambers. For the spread plating, aliquots of 5.0g of soil were added to 10.0 ml 1X PBS buffer, shaken overnight at 30°C and diluted to 10^{-1} and 10^{-3}. Each dilution was plated out in duplicate on four different selective agars: 869 (10.0g Tryptone, 5.0g Yeast Extract, 5.0g NaCl, 1.0g D-(+)-glucose, 0.345g $CaCl_2.2H_2O$), 284 selective (35), 79 minimal medium (5g sucrose, 5 g Mannitol, 100mg K_2PO_4, 400mg KH_2PO_4, 200mg $MgSO_4.7H_2O$, 100mg NaCl, 400mg Yeast, pH 6.8-7), and CYE (0.1g casamino acid, 0.1g yeast extract, 50mg $CaCl_2$, 500mg $MgSO_4$.7HO, 7.5g gellan gum, 1ml of filter sterilized soil extract)(36). After two weeks of growth, representative colonies were picked from the agar plates, re-streaked for purity on fresh media plates, and finally stored in a 15 % w/v glycerol solution at -45°C.

In an attempt to cultivate more interesting bacteria, diffusion chambers were used according to Nichols(37).Approximately 1.0g of soil was added to 5mL 0.1 M PBS, vortexed and shaking incubated for 2 hours. The mixture was then incubated at room temperature for 25 minutes until the soil had settled. From the supernatant, 3.0mL was mixed in a petri dish with 30°C 284 agar (1,5 %) using a sterile pipette tip. The sterile isolation chip was dipped in the hot agar and covered on both sides with sterile hydrophilic 0.05 µmpolycarbonatemembranes (Whatman® Nuclepore™ Track-Etched Membranes, Sigma-Aldrich, Overijse, Belgium). Subsequently the diffusion chambers were sealed in polypropylene holders and tightened with screws. The chambers were then buried in the Kamloops soils moistened to 70 % WHC and incubated in the greenhouse with conditions at 22/18°C, a photoperiod of 14:10h daylight and 67% air humidity.

After 30 days of growth, the diffusion chambers were removed from the soil and the agar trapped in the pores of the isolation chip were punched out with sterile toothpicks into each well of a masterblock containing 1.2 ml of 869 broth. After 48 hours of growth, 100µL of bacterial inoculate was transferred to a new master block containing 0.8mM Cu in 284 broth and left to grow for 2 weeks at 30°C at 120rpm agitation. Following the growth period, each well of the master block was plated for purity on 869 plates. Pellets of purified strains were suspended in 15 % w/v glycerol and frozen at -45°C.

Copper tolerance

Copper tolerance of the isolated strains was evaluated by growing the strains in minimal medium with different copper concentrations (0.4 mM and 0.8 mM as $CuSO_4$). First, the strains were pre-grown

overnight in 1.2 ml of 869 broth in sterile deep 96-well masterblocks (120 rpm, 30°C). Bacterial cells were pelleted by centrifugation (2000 g, 30 min), washed once with 1X PBS and 100 µL of bacteria sample was inoculated into 284 broth with 0.4mM Cu and 0.8mM Cu. Bacterial growth was assessed visually after 7 days of constant agitation at 120 rpm, 30°C.

In vitro plant growth promotion (PGP)

Plant growth promotion traits of the coppertolerant bacterial isolates was tested qualitatively.The bacteria were grown in 869 media at 30ºC for 24 hours, washed and resuspended in a 2 mL sterile 0.1M $MgSO_4$solution to obtain a suspension with OD_{600} of 1.20 µl of this suspension was used for the inoculation of 96-well plate assays (Greiner Bio-One, Wemmel, Belgium) for the detection of: auxin production using the Salkowski reagent method(38); 1-aminocyclopropane-1-carboxylate (ACC)-deaminase activity estimated by monitoring the amount of α-ketobutyrate generated by the enzymatic hydrolysis of ACC, a plant hormone ethylene precursor(39); and acetoin production, measured using the Voges-Proskauer assay (40). For siderophore testing, bacteria were subjected to 3 concentrations of iron in 284 media (0mM, 0.25mM and 3mM) and the evaluation method performed followed the CAS assay (41). For all the PGP-assays, the bacterial isolates were distributed into classes scored as '0' and '1' depending on their negative or positive responses. All tests were performed in triplicate.

*In Plantae*experiments

For plant germination, vertical agar plates were made using a basal medium (50ml 20X basal medium, 950mL dH_2O, 5 g/L sucrose, 10g/L agar) as described in (42). The medium was subjected to four concentrations of copper to induce stress in the germinating plant with $CuSO_4$ (0uM,50uM,100uM,150uM). The gradient was tested to determinewhich copper concentration had a visible effect on germination and root length.

The four bacteria that tested positive for all plant growth promotion traits had a 1.0mL aliquot grown overnight in 25.0mL 869 broth at 30ºC with constant agitation of 120rpm. The bacteria were centrifuged (5000 g, 10 min.) and washed twice with sterile 0.5M $MgSO_4$. The bacteria were resuspended and centrifuged at 2500 g for 10 min. The supernatant was placed into sterile beakers and diluted with 50.0 mL sterile 0.5M $MgSO_4$.

The test species, *Medicago sativa*sp., seeds were surface sterilized as described previously (43). The surface-sterilized seeds were placed in the bacterial supernatant solution for 1 min. 30 sec, followed by two washes in sterile dH_2O.

Seeds were sown on the agar with different copper levels, five seeds per plate, and were then incubated vertically with PAR provided at the rosette level of 170 mol m^{-2} s^{-1}, 22ºC/18ºC degrees, 12/12 day night cycle and 65% RH for 10 days. After 10 days, root length and number of side roots were measured with RootNav software(44).

Rhizosphere Grafting

M. sativa seeds were surface sterilized as described above and inoculated with an amplified rhizosphere microbial community from each of four Kamloops sample sites. The rhizosphere and soil associated bacteria were amplified by inoculating 10.0 mL of 869 broth with ~1.0 g of root associated soil. Growth was allowed to the log phase. The bacteria were collected by centrifugation at 5000 g for 20 min. and were resuspended in 5.0 mL 1X PBS. Sterile *M.sativa* seeds were placed into the PBS solution and shaken at 30ºC for 15 minutes. Following this, 20 seeds were sown on high concentration (Touro Arriba) and low concentration (Touro Abajo) copper contaminated soil. These soils were collected from the Touro mine site in Galicia (8° 20′ 12.06″ W/42° 52′ 46.18″ N; Touro, Galicia, NW Spain). Soils were sampled in the field in zipper bags and transported at 4°C to the lab in Belgium. For each condition, three pots with 20 seeds per pot were grown under controlled greenhouse conditions with 170 mol m^{-2} s^{-1}PAR, 22ºC/18ºC degrees, 12/12 day night cycle and 65% RH for 2 weeks. During this time, germination effect was measured on day 4, and plant area was determined through measuring leaf diameter and width on day 10. Plants, roots and soil were harvested after 2 weeks to determine levels of copper in each attribute.

DNA-extraction and ARISA fingerprinting

DNA was extracted from the eight sampled soils using the MoBio PowerSoil Kit(Qiagen, Venlo, Netherlands) according to manufacturer's instructions. Quality and quantity of the DNA was checked on a 1 % agarose gel and using nanodrop. DNA was subsequently subjected to an ARISA PCR using the ITSF Forward primer and the ITSReub reverse primer using PCR conditions as described previously (45) on the Biorad T100 (Biorad, Venlo, Netherlands). The amplified inter spacer regions were analyzed with the Agilent 2100 Bioanalyzer (Agilent 2100, Waldbronn, Germany) using DNA 1000 chips (Agilent 2100, Waldbronn Germany).

ARISA results were interpreted in two ways: Using R v2.13 and the package StatFingerprints(46) and using AdaptDB(47). R v3.4 was used to determine a bray-curtis index under a ward algorithm to create heatmaps of the species present. AdaptDB was used to create cladograms of potential species identified by the length of their interspacer region, and potential of pathogenicity according to matches within the SEED database and the NCBI database(47).

16S rDNA gene Sanger sequencing

Copper tolerant and PGP bacterial species of interest were sent for 16S rDNA gene sequencing using the universal prokaryotic1392R primer (5′ ACGGGCGGTGTGTRC 3′)and thebacteria-specific 27 F primer (5′ AGAGTTTGATCCTGGCTCAG 3′) with PCR-conditions as previously described (48). The raw sequence reads were quality trimmed and blasted against the Ribosomal Database (49).

Ion Torrent amplicon sequencing

16S rRNA gene amplification was performed on the DNA samples using the primer set 341f (5' TACGGGAGGCAGCAG 3') and 806r (5' GGACTACVSGGGTATCTAAT 3'), that target the V3-V4 variable region of bacteria as described previously(50).The library dilution factor of amplicon pools was determined using an Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific) prior to amplification and enrichment with an Ion PGM Hi-Q View OT2 400 Kit on an Ion OneTouch 2 System. The Enriched Ion Sphere Particles were analyzed using an Ion Sphere Quality Control Kit prior to sequencing with an Ion 316v2 Chip on an Ion Torrent PGM running Torrent Suite 5.2.2 using an Ion PGM Hi-Q View Sequencing Kit. Equimolar ratios of quality control checked libraries were sequenced on the Ion Torrent S5 platform. A total of 1.3 GB of data was generated.

Illumina shotgun DNA sequencing

Four soil DNA samples were subjected to Illumina shotgun sequencing using the Illumina Hiseq4000 platformwith 100-bp insert libraries prepared using the Nextera XT kit (Macrogen, Seoul, Korea). We generated about 8.6GB of data.

Data processing

Data pre-processing is necessary for both shotgunand amplicon data due to sequencing errors. For the shotgun reads, we trimmed trailing bases with quality score 2and discarded reads shorter than 30bp and with "N." The reads then were quality trimmed with fastq-mcf(version 1.04.662) (http://code.google.com/p/ea-utils) with "-l 50 -q 30-x 10 -max-ns 0 -X." The paired-end reads overlapping by more than10 bp were assembled into one long read by PEAR (version 0.9.8) (51). Quality controlled shotgun sequencing were subsequently assigned taxonomy using MetaPhlan2 (52), and functionally annotated using MG-RAST (53) and the Greengenes database(54) Function was also attributed using MG-RAST through the Kegg Orthologs and SEED database (55).Ion Torrent data were processed using the Qiime2 pipeline (56) according to the developers suggestions and with trimming of the first 15 bp in the dada2 noise removal algorithm.

Statistics

Statistics were performed using Rv3.14, QI Macros, and PAST. All statistically tested data was tested for normal distribution and homoscedasticity and then subjected to a one-way ANOVA with post-hoc Turkey's HSD test. Non-metric multidimensional scaling with Bray-Curtis was used to determine statistical differences in sample location.

RESULTS

Soil Properties

The soils were sampled across a gradient on the historic tailings (**Figure 2**). The collected soil samples were assessed according to the USDA system to determine soil type. Soils across the gradient were found to range from clay to sandy loam (**Table 1**).

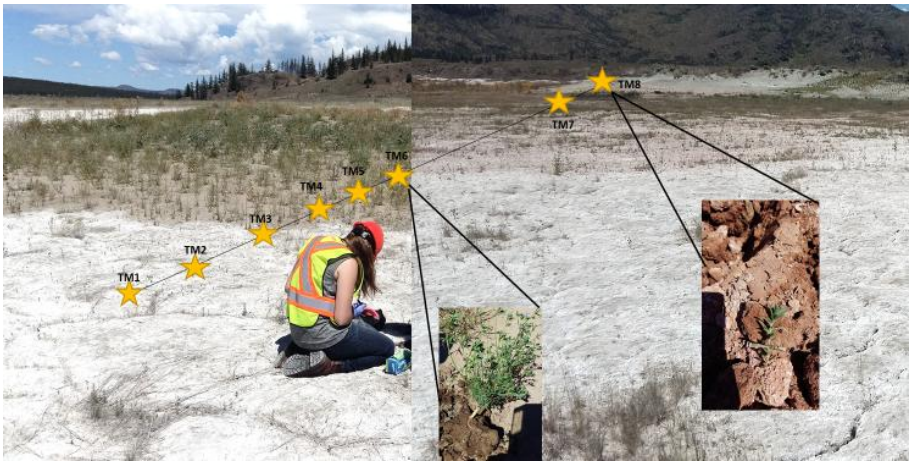


Figure 2: Sample gradient and soil types photographed by Sofie Thijs, 07/ 2016

Table 1: Soil Properties of sampled gradient taken across historic tailings pond in Kamloops, BC, Canada

Sample ID	Description	Coordinates	pH H_20	pH KCl	% C	% N	% moisture	USDA classification
TM1	Unvegetated tailing	50.651479, -120.536983	8.38	7.32	0.043	1.58	40.59	Clay
TM2	Unvegetated tailings	50.651479, -120.536992	7.92	6.89	0.049	1.47	22.28	Clay
TM3	Vegetation/unvegetation zone	50.651479, -120.536997	7.81	6.75	0.039	1.40	28.40	Sandy loam
TM4	vegetation	50.651479, -120.53702	8.00	6.94	0.038	1.44	24.20	Sandy loam
TM5	Alfalfa plants	50.651479, -120.53705	8.20	7.2	0.040	1.21	20.77	Sandy loam
TM6	Alfalfa rhizosphere	50.651479, -120.53705	7.97	7.28	0.039	1.22	20.64	Sandy loam
TM7	Red tailings unvegetated	50.650931, -120.53711	8.28	7.46	0.027	0.91	32.21	Clay
TM8	Red tailings rhizosphere	50.650931, -120.537012	8.28	7.46	0.027	0.81	32.78	Clay

SEM results of soil texture and metal analysis indicated an array metal contamination within the soil (**Figure 3**). High levels of silicon and carbon are observed and expected, due to the soil composition. High levels of iron were observed, as well as copper contamination in all tested samples.

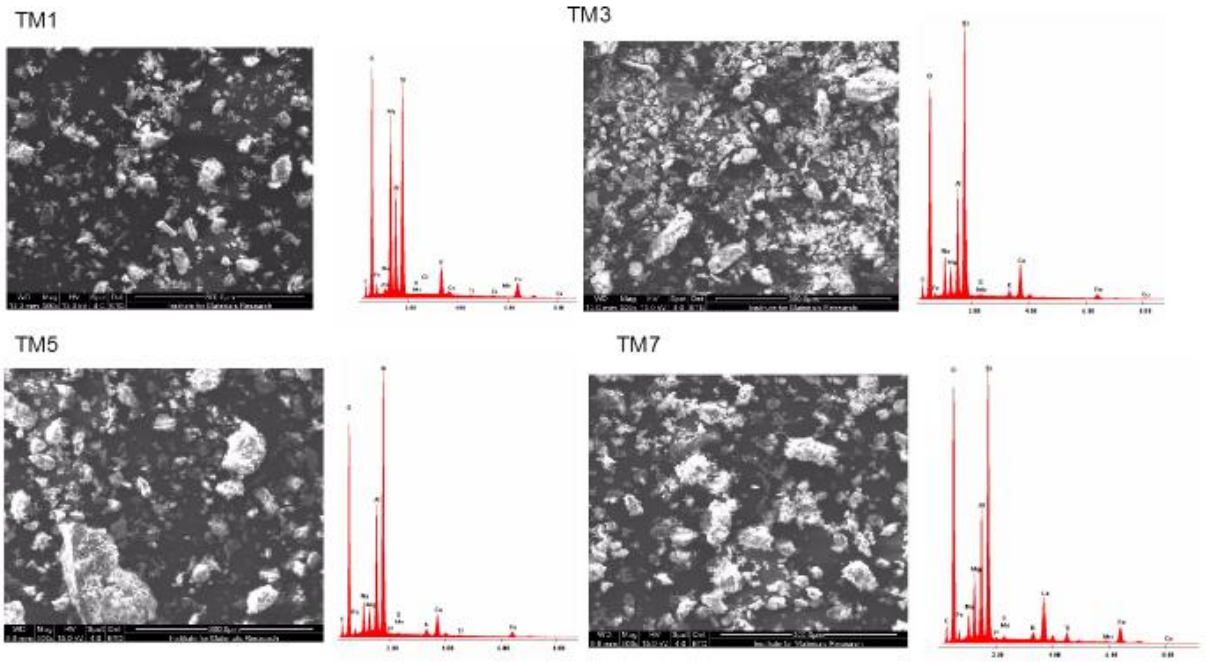


Figure 3: SEM results of soil representative of the sampled gradient. Highly conductive samples are indicative of metal contamination. EDX analysis confirms the presence of heavy metals within the historic site soil.

To assess levels of contamination across the sampled gradient, ICP-OES was performed. Higher levels of copper contamination were found in the red clay samples (TM7, TM8), located towards the barren part of the mine tailings (**Figure 4**). Within the plant island, copper contamination levels were lower, which is indicative of passive remediation through the plants and microorganisms in that area.A calcium nitrate extraction was used due to its correlation to plant available metals in soil (34).

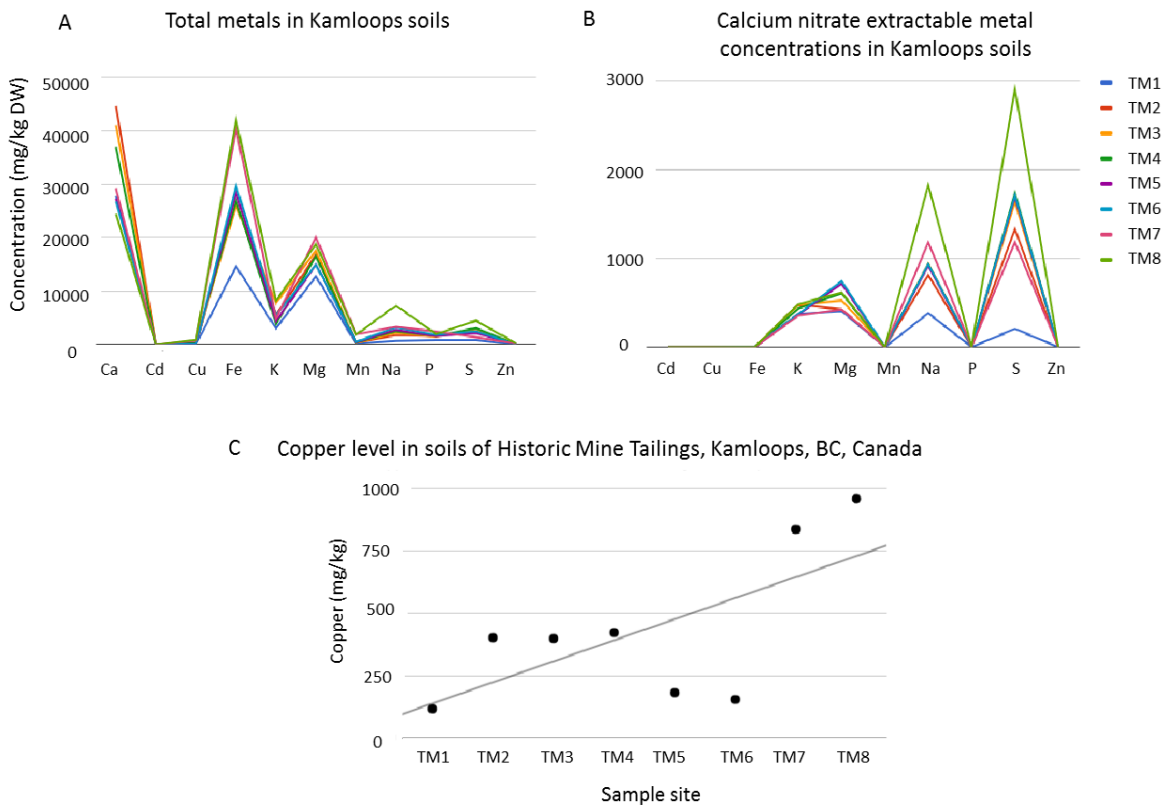


Figure 4: ICP-OES results of total metal extraction of soil samples from Kamloops, BC Canada. High levels of iron are shown, as well as high levels of calcium. The high calcium levels support the hypothesis that previous remediation was taken on this site to prevent acid mine drainage, as this is indicative of lime treatments.

Plant growth promotion tests

Bacteria isolated from the soil using selective media were subjected to copper resistance tests and plant growth promotion tests (**n=3**). Results from these tests indicated that all bacteria from the contaminated site, at all points on the gradient have the ability to produce siderophores to alleviate metal stress (**Figure 5**).

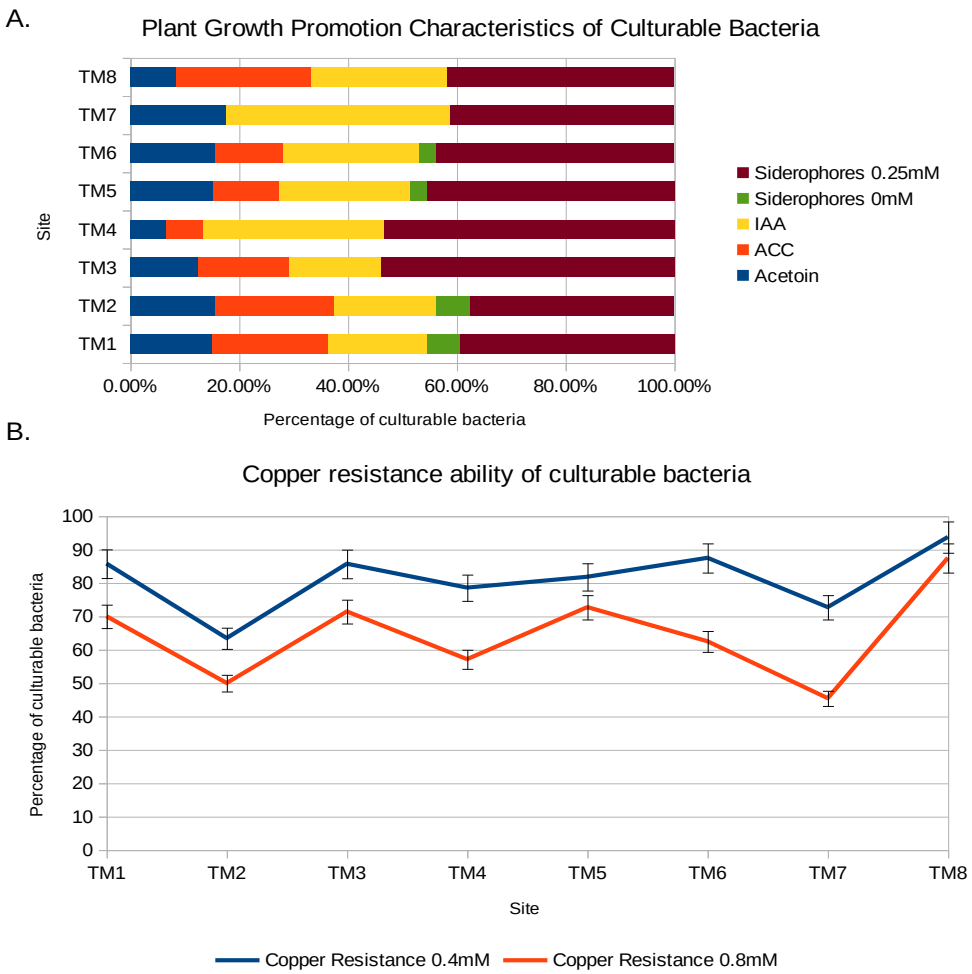


Figure 5: A) PGP Characteristics of culturable bacteria from Kamloops, BC, Canada, divided by site. B) Percentage of culturable bacteria which possess a copper resistance ability at high concentrations.

Of all the bacteria tested, only fourtested positive for all five plant growth promotion tests and both copper resistance tests. These bacteria were send for 16S sequencing (**Appendix1-A**)and then used to determine effect on root length of *M.sativa* under contaminated conditions.

VAPS test

Of the applied bacteria, none of the tested strains had a statistically significant result on the root length of the plants (**Figure 6**).

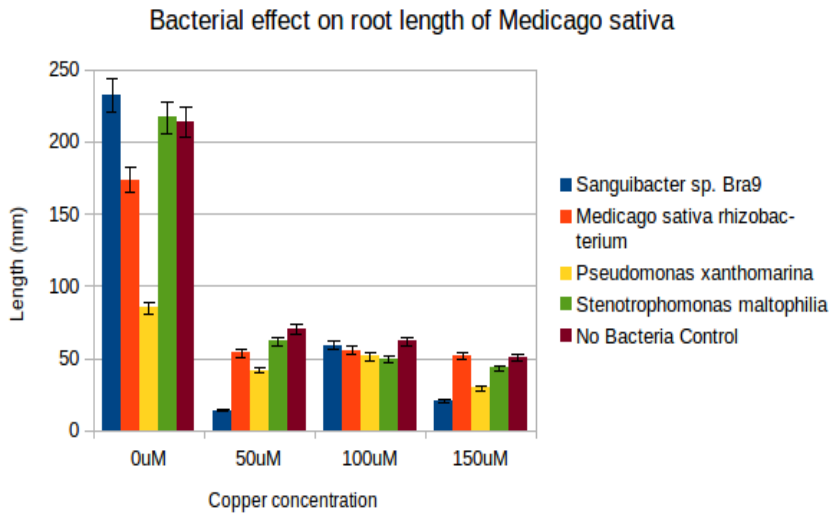


Figure 6: Applied PGPR bacteria from Historic Tailings had no visible or statistical impact on root length of Medicago sativa

Because enrichment with a single strain did not yield promising results, further analysis of microbial community structure and diversity, and how this relates to plant growth, is needed.

Community Structure

In order to determine the microbialstructure of the sample sites, an ARISA fingerprint was performed. The resulting heatmap shows an enrichment of one band across the vegetated zones and a distinct band for the red tailings rhizosphere (**Figure 7**).

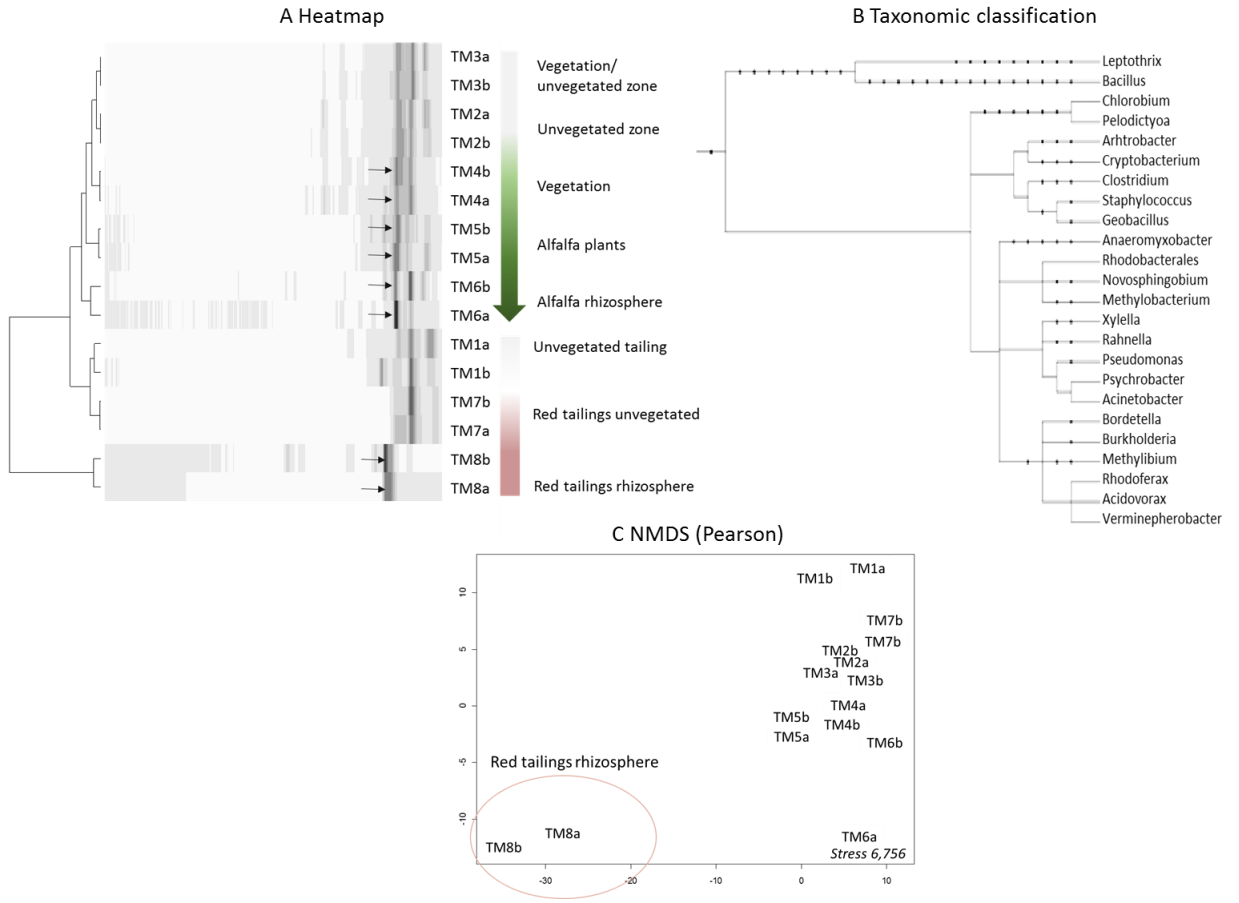


Figure 7: ARISA results presented as a heatmap using the Pearson method with Ward algorithm, supplemented by the NMDS, indicating the community structure differences between the sites, particularly the vegetation hub vs the red unvegetated dirt. A strong band, indicated by arrows on the heat map, shows an enrichment of a community across the vegetation hub, and is marked as an area of interest. The cladogram was generated using the AdaptDB Beta software to give a working identity to species within the rhizosphere

Communities which showed the strongest representative bands were then selected for shotgun sequencing in order to determine what bacteria were present and what genes they possessed.

Shotgun Sequencing

Shotgun sequencing results, analyzed with MG-RAST, allowed for characterization of community structure as well as identification of the functions those communities possess. From sample site TM2 to sample site TM5, we see a progressive increase in the number of Proteobacteriaexcept for at TM8, where Bacteroidetes have risen to be the dominant phyla(**Figure 8**). This is likely owing to the drastic difference in soil composition, as well as the increased metal contamination, which has been supported to lower community diversity in the soil. Lower-level classification shows the division of Proteobacteria into predominantly alpha- and gamma-Proteobacteriadepending on the location (**Figure 9**). At site

TM8, Flavobacteria are the predominant class, followed by gamma-Proteobacteria, making it the one site to have a class from a different phylum to have more influence.

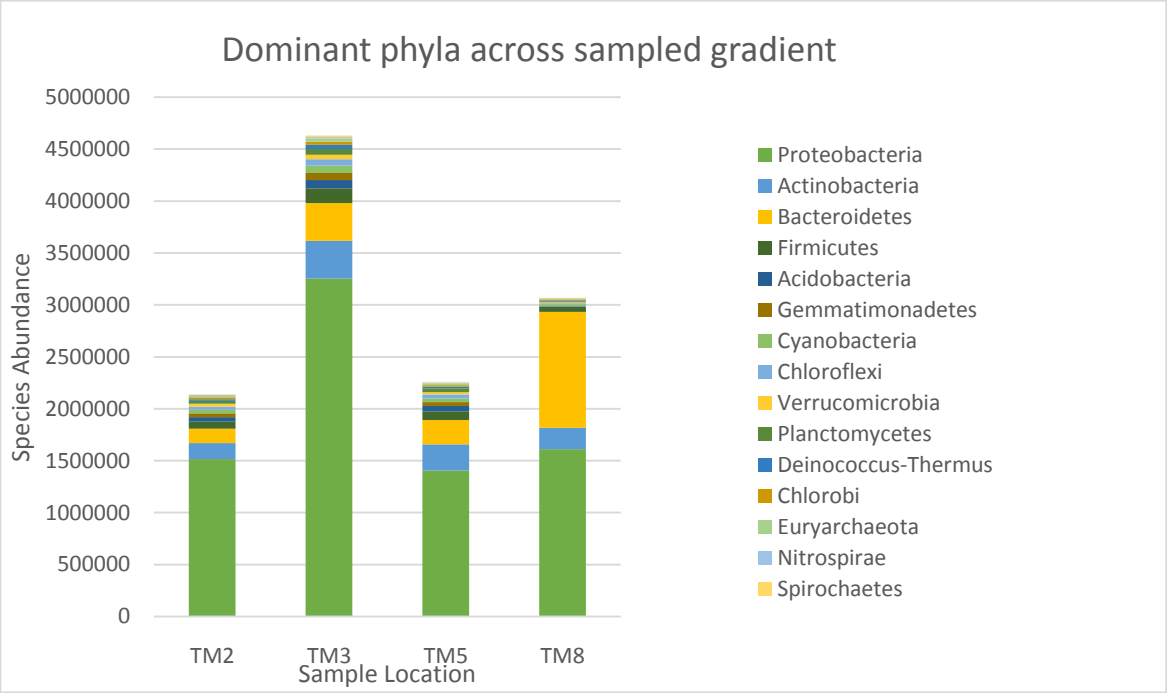


Figure 8: Site community compositions by phylum across the sequenced gradient.

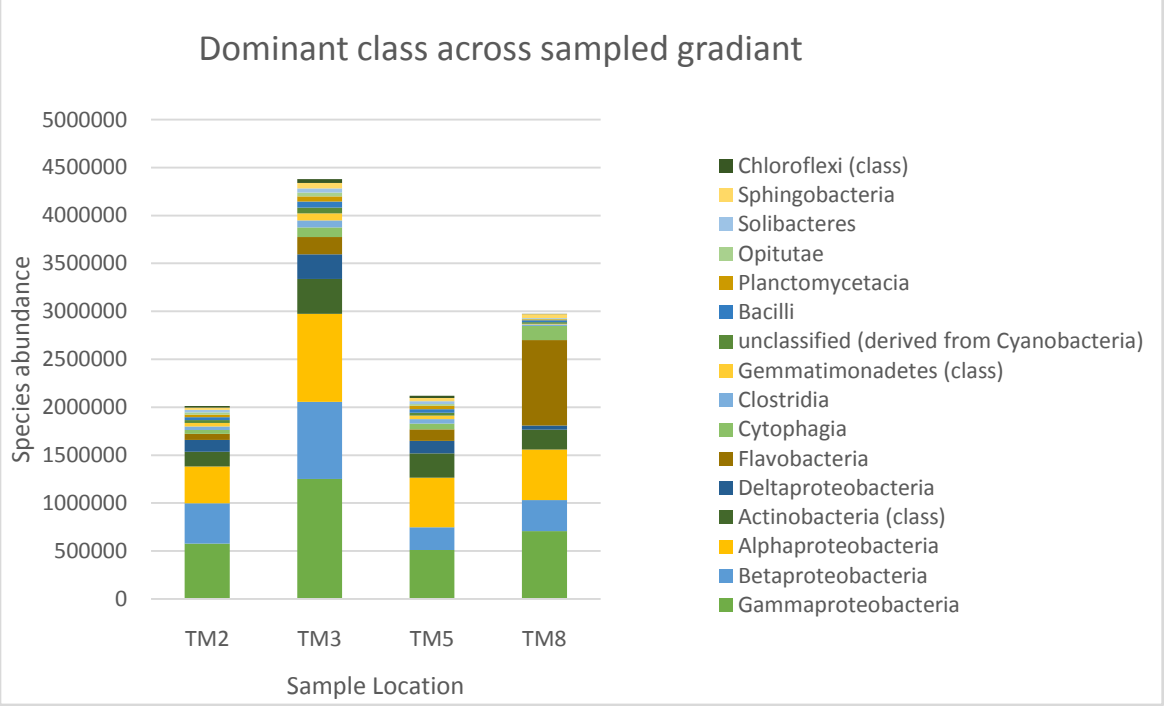


Figure 9:Community structure divided by class showing the dominance of alpha-and gamma-Proteobacteria.

Functions related to the sampled communities were assessed with MG-RAST. There were a large number of degradation genes and stress tolerance genes found within the samples (**Figure 10**).Areas with plant life already established (TM5) have lower levels of metal resistance and tolerance as the soil has already begun the process of phytoremediation.

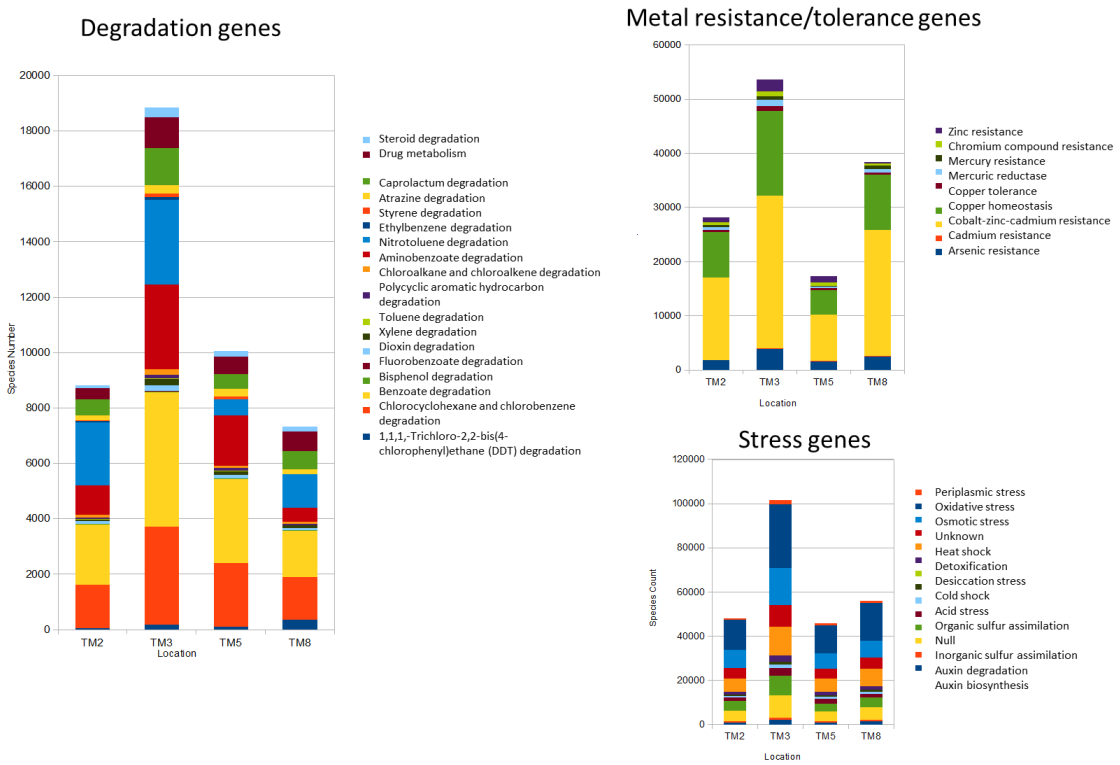


Figure 10:Genes of interest across the contaminated site show elevated levels of degradation, metal resistance, and stress tolerance features at the unvegetated/vegetation hub cross section (TM3).

With the community structure assessed, and the presence of degradation genes confirmed, an *in plantae* soil test was performed in an attempt to remediate a contaminated site.

Rhizosphere Grafting

In order to assess whether an enhanced community of bacteria had more effect of plant growth and remediation than a single enriched bacterium, *M.sativa* seeds were inoculated with enriched consortia from the historic tailings and sown on contaminated soil from an active contaminated site, which is discussed in detail in the following chapter. The two soils consist of one with a high concentration of copper (Touro Arriba **~2050 mg/kg**) and one with a lower concentration (Touro Abajo **~700mg/kg**). The site with the lower concentration is similar in contamination condition as the historic tailings site.
The number of seeds germinated out of the 20 seeds that were sown were counted after two days to offer insight into the germination effect (**Figure 11**). There was a statistical increase (**p<0.01; n=3**) in regard to germination, particularly with the addition of the enhanced consortium from rhizosphere TM2.Average leaf area of the plants was measured after five days to determine the effect

transplanting a rhizosphere would have on initial germination biomass (**Figure 11**). Rhizospheres from sites TM2 and TM7 showed a statistical increase in leaf area, when compared to the control plant on soil from site Touro Arriba. There was no statistical increase in plant size on soil from Touro Abajo.

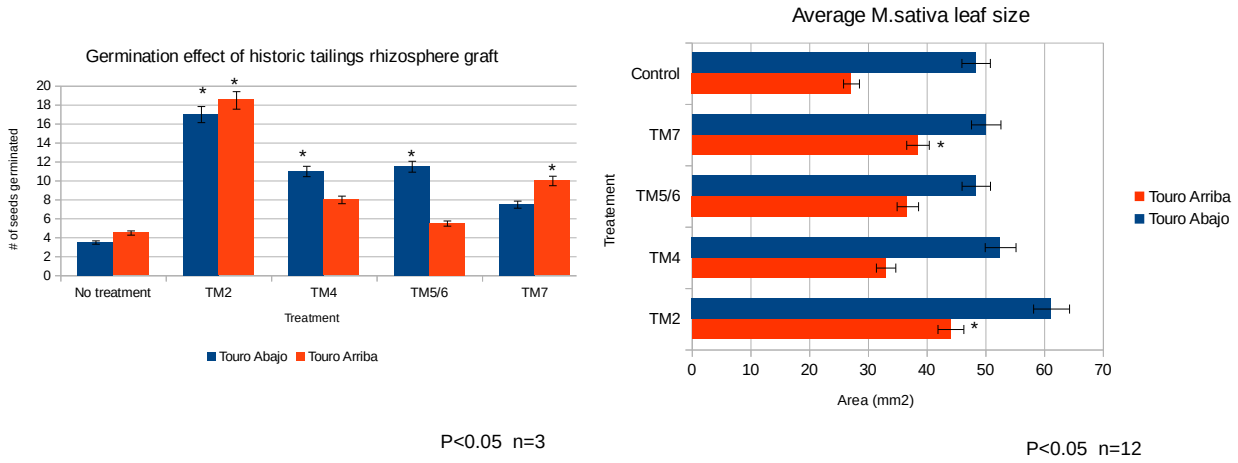


*Figure 11: M.sativa seeds inoculated with enriched bacterial consortia show a statistical increase in germination level, particularly on sites with similar levels of copper contamination (Touro Abajo). The treated seeds produce larger leaves on soils with high copper contamination when compared to the control plants. *: p < 0,005, n=3.*

In order to test the phytoremediation properties of the plants treated with an enriched rhizosphere, ICP-OES was performed on *M.sativa* roots and shoots, and the soil was also re-measured to observe and decrease in copper contamination (**Figure 12**).It was observed that plants treated with rhizosphere TM5/6, the rhizosphere from the alfalfa vegetation hub, had a significant increase in copper in the shoots of the plants (**p<0.05; n=12**) on site Touro Arriba, even though the plant size had not increased significantly, nor had the germination rate increased.

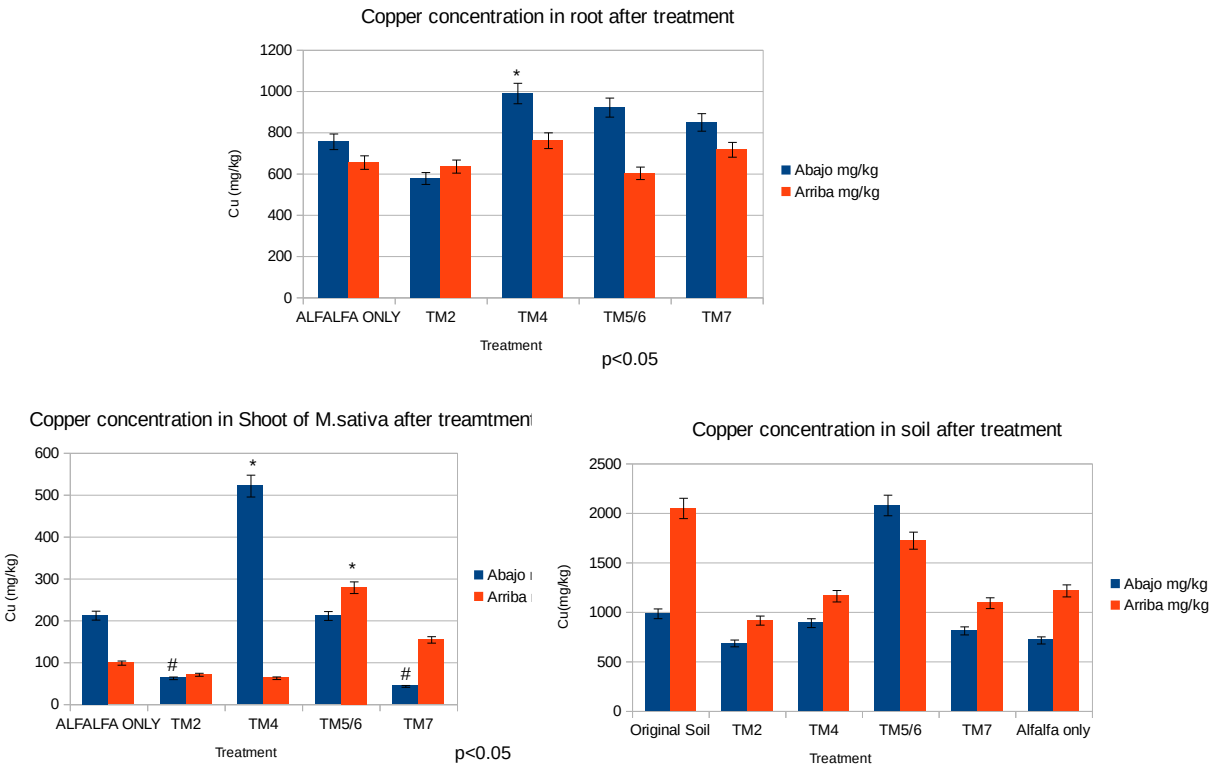


Figure 12:ICP-OES indicates increased levels of copper in M.sativa with the application of the enhanced rhizosphere from TM4. TM2 decreased the level of copper in the shoots of the plants significantly, as did TM7, but only in the on the Abajo soil.

DISCUSSION

Soil Properties

The sampled tailings had an alkaline pH, which was contrary to the expected result. This could be the result of previous remediation attempts on the mine site, as alkaline reagents such as lime, limestone, sodium carbonate or sodium hydroxide are common reagents used to combat acid mine drainage of tailings(57). The addition of lime to tailings creates an alkaline pH, effectively prohibiting sulphur-oxidizing bacteria from leaching the ore and creating sulphuric acid as a by-product.Previous environmental assessments of this site have described the soil as "calcareous" and "unfit for reclamation purposes" (58).

Because of the results seen in Figure 4, we can conclude that in the Kamloops soils there is a high level of plant available sulphur. Sulphur has an important role in plants, and having a high level available in the soil can be a determining factor for plant growth. In the instance of copper contamination, plants are faced with extreme levels of oxidative stress, and having high sulphur can

alleviate that stress through increased proline, glutathione reductase and glutathione peroxidase production (59). Previously, it has been seen that plants that grow on sulphur rich sites are more resistant to heavy metal toxicity (60–62).

Although copper appears to be non-plant available according to the calcium nitrate extraction, this extraction is only a correlation and not a direct measurement of plant available metal. The contamination of the heavy metal, regardless of if it is plant available or not, continues to be a problem in establishing plant growth on the contaminated site.

Plant growth promotion tests

The high level of siderophore production observed in the plant growth promotion tests(**Figure 5**) relates back to the high concentration of iron on the site, as it is one of the most important macronutrients for bacteria to live successfully.

Siderophore producing bacteria (SPB) are necessary for successful phytoremediation as they alleviate metal stress for the plant, while making essential nutrients, such as iron, bioavailable. Because siderophores are metal chelators with an affinity for complexing iron, siderophores can also form stable complexes with metals such as Al, Cd, Cu, Ga, In, Pb, and Zn, as well as with certain radionuclides. When the siderophore creates a complex, it increases the soluble metal concentration, meaning more metals are in their bioavailable form, making them easier to extract through natural means.

Also, over 40% of bacteria from all sample sites were able to grow at contamination conditions of 0.8mM Cu. Copper toxicity in cells is directly related to the metals affinity for producing hydroperoxide radicals, as well as disruption of the cell membrane.

Although bacteria need copper in small amounts for certain protein functions, a resistance mechanism is needed if a microorganismis to survive in a high copper environment. Bacteria with these resistance mechanisms are of great interest, as it has previously been supported that these bacteria can potentially remove copper by adsorbing it, and accumulating it within their cell wall, effectively making the contaminant less bioavailable for plant life(63–65).

A large portion of our culturable bacteria also possessed the ability to produce Indole-3-acetic acid (IAA). IAA is a known plant auxin. IAA not only plays a role in the formation of roots and root hairs, but also in the signaling for stress tolerance(66). IAA is a secondary metabolite for bacteria as a result of the metabolism of L-tryptophan, and as it is a plant hormone, it seemingly has no role in bacteria, except for improving plant-bacteria interactions, resulting in increased fitness for both. It has also been hypothesized that because IAA improves root hair growth, the primary site for rhizosphere colonization, IAA producing bacteria can better colonize the plant (65).

Among having bacteria that produce IAA, all sites also possessed bacteria with acetoin producing ability, as well as 1-Aminocyclopropane-1-Carboxylate Deaminase (ACC-deaminase). Both bacterial

byproducts assist in alleviating plant stress, with ACC-deaminase lowering plant ethylene levels, whereas acetoin improves plant growth acting as a phytohormone.
On the plant enriched sites, culturable bacteria possessed similar levels of PGPR as on the unvegetated sites TM1 and TM2. This could suggest that colonization of plants on those sites is possible with the current microbiome.

VAPS test

No statistical trend was noticed when single bacteria were inoculated to plants, and this was contrary to expected results and the results of other papers (67–69). Although no statistical or visual trend was observed on the effect of *M.sativa* root length (**Figure 6**), this could be owing to the fact that plants prefer a consortium of bacteria in order to maintain optimal concentrations of PGPR products. IAA and acetoin at high concentrations can be detrimental to plant biomass, while increased concentrations of ACC-deaminase result in too low concentration of ethylene within the plant.
The negative results of the PGPR bacteria on the roots of the alfalfa led to the formation of a new hypothesis: plants under contaminated conditions prefer a consortium rather than an amplification of one single PGPR bacterium. Literature supports this, as tailored consortia and local consortia of bacteria have been used with great success in phytoremediation(22,70,71). Our hypothesis that a contaminated rhizosphere possesses bacteria which possess the necessary traits and genes to remediate the contaminated site led to the idea that an amplification of these bacteria as a consortium would have greater remediation success than a single amplified bacterium.

Community Structure

In order to successfully exploit and amplify a community though, it is first necessary to determine the structure, and which bacteria are present. Community analysis with ARISA resulted in a heatmap which showed an enriched band across the vegetated zones (**Figure 7**). This band is not enriched on the unvegetated zones, meaning that this phylum of bacteria potentially plays a leadership role in establishing healthy plant life. A cladogram was generated using the program AdaptDB, which identifies potential species through length of their interspacer (47).
The resulting NMDS with Pearson method supports the enrichment of one particular phylum, as the samples taken around the vegetation hub cluster together, with the red dirt samples showing drastically different community structures. The soils where revegetation had begun to occur show more diversity in their community structure, than those from the bare soils. The bare soils show enrichments of particular phyla. These phyla likely contain traits which allow for protection against the harsh conditions. This is consistent across the literature regarding bacterial communities from various soil locations (72–75).

Based on the results gathered in the ARISA, four samples of interest were selected for shotgun sequencing to assist in identifying the community of bacteria present in the sampled gradient, as well as what traits they possess that can assist with phytoremediation of the contaminated site.

Shotgun sequencing

Our site with the highest level of copper contamination (TM8) has Bacteroidetes as the dominant phyla (**Figure 8**). This is contrary to previous research results conducted on copper mines which place proteobacteria as the dominant phyla (20). However, this is likely because we sampled a gradient of soils resulting in various communities and contamination levels, rather than one particular spot on a mine site. Though the level of Proteobacteria is still high, the community diversity has decreased. The increased level of Bacteroidetes may be linked to the higher concentration of metal contamination on the site, or owing to the lower level of carbon and nitrogen.

Regarding class diversity, gamma-Proteobacteriais the dominant Proteobacterium, except in the case of TM3. At this site, alpha-Proteobacteria exist in greater abundance (**Figure 9**). This could be due to the fact that this is a transition area, meaning that a more complex rhizosphere is needed to combat against the more variable soil conditions.

Taking all factors into consideration, it can be assumed that across the gradient sampled, community structures change their environment, and then change their community structure. Site TM2 also had a high level of Bacteroidetes, but as it was closer to the vegetation hub than TM7, the community structure has begun to resemble that of the vegetation hub. Over time, it will be more likely that plants can be established on soil with a similar rhizosphere to those of TM3 and TM5, as those sites have already had success in establishing vegetative cover.

Community diversity is not always a strength when it comes to remediation. This is supported through characterization of gene function, in regard to degradation, metal resistance, and stress (**Figure 10**). It is observed that the site which possess the highest level of community diversity (TM5) does not possess a greater ability to remediate. Rather, TM3, the site where the desert soil meets the vegetation hub, has acquired the highest level of remediation genes. Site TM3 also possessed the highest level of Proteobacteria. Whether the elevated level of genes related to metal degradation and tolerance can be linked directly to the increased presence of Proteobacteria is unknown, but there is a strong correlation (Pearson coefficient 0.95). All tested sites had an elevated level of cobalt-zinc-cadmium resistance, and a high number of genes relating to copper homeostasis. The resistance to cobalt-zinc-cadmium is unexpected as no elevated levels of those metals were observed during the soil analysis.

In regard to stress genes, the bacteria possess high levels of oxidative stress genes, in order to help them cope with the reactive oxygen species (ROS) generated by the presence of copper.

Another set of elevated genes are those used for inorganic sulfur assimilation. In the soil analysis, it was observed that the correlated level of plant available sulfur was high, and that is again supported by the presence of these genes.

The difference in community structure speaks to the hypothesis that bacterial communities are tailored to survive and thrive in their habitat, and that they assemble phyla that can potentially lower

environmental stresses. The difference in community structure across the gradient is likely owing to external organic and inorganic factors, such as pH, total C, total N, amount of plant coverage, etc..

Each sample site having a distinctive community structure speaks towards the success of phytoremediation. From TM2 to TM5, we observe the steadily increasing level of Proteobacteria, along with the increasing level of vegetative cover. On site TM8 we observe a drastically different community, owing to the different soil type and external factors, but notice higher levels of cobalt-zinc-cadmium resistance.

It is also observed that there are a number of genes for inorganic sulfur assimilation. Inorganic sulfur in the form of hydrogen sulfide is biologically oxidized to sulfate through prokaryotes as an integral part of the sulfur cycle. This makes the sulfur bioavailable to be used in other needed compounds such as glutathione, an enzyme used to protect against oxidative stress. In regard to auxins, TM3 has over double the number of genes to produce and degrade these plant hormones when compared to all other sampled sites. Surprisingly, this is not evident in the culturable PGP tests, meaning that the majority of these bacteria are unculturable.

Because such a large portion of the bacteria are unculturable, it again supports the hypothesis that the best application of bacteria in phytoremediation is an enhanced community, rather than an enrichment of one single culturable species. This is also true in regard to community structure where there is a visible dominant phylum which would easily outcompete a single enriched added bacterium.

Rhizosphere Grafting

The statistical increase in plant germination (**$p<0.05$; $n=3$**) indicates that the application of an enriched rhizosphere has a positive effect on plant germination on sites with similar levels of contamination, but has no lasting effect on plant biomass. The opposite is true for treated seeds sown on soils with higher levels of contamination. Here, it is observed that fewer rhizospheres make an impact, but the ones that result in higher seed germination also result in larger plant biomass, as is the case with rhizospheres from TM2 and TM7.

However, the rhizosphere with the most significant impact (**$p>0.05$, $n=12$**) is TM2. Seeds treated with this rhizosphere were not only germinating at significantly higher rates, and producing bigger plants, but the level of copper contamination in the soil has also decreased, at both test sites. It is of interest to note that this rhizosphere appears to be keeping copper from entering the shoot and root of the plant, meaning that the decrease is occurring due to rhizosphere activity resulting in decontamination.

The additional rhizosphere appears to be remediating the soil in one of two ways: Introducing higher levels of copper into the plant, or by reducing the bioavailability of the copper. The first instance is seen with TM4, which has a higher level of copper in the roots and shoots of the test plant compared to the untreated control plant. The rhizosphere from TM2 appears to lower the amount of copper entering the plant. Comparatively, the amount of copper in the soil has decreased through the addition of an enhanced rhizosphere, particularly on the highly contaminated site of Touro Arriba.

The two mechanisms by which the soil is being remediated, plant extraction of metal and bacterial decontamination, relate back to the leaf size. Oxidative stress results in smaller plants, and when the copper is not bioavailable to the plant, the plant can grow bigger, and faster. In the instance of the plant extracting the metal from the soil, the plant species chosen must have the correct mechanisms in place to store and/or detoxify the metal within the plant. This will be discussed further in the following chapter.

These strategies are ideal for phytoremediation, as it is an ideal way to remove contaminants from the soil, either through phytoextraction or stabilization, as well as establish plant life on a contaminated site to assist in preparing soils to receive local plant species once again.

CONCLUSION & OUTLOOK

Phytoremediation is a rising technology, but there are many new avenues of application that are still to be researched. In the case of this experiment, it was discovered that applying an enriched rhizosphere to a seed resulted in better germination, larger plant size, and increased copper remediation. This avenue of application strays from the original ideals of identifying only PGPR bacteria and applying only those to the sterilized seed.

Community structure of contaminated sites was also revealed that diversity in community does not always parallel strength. A diverse community is a sign of a healthy environment, but a recovering environment assembles certain phyla with preference, likely because these bacteria can assist in the site remediation.

Future prospectives include assessing the community change of local soil bacterial communities, as well as monitoring the added community. Insight into the changing bacterial structure will allow a more detailed explanation for why enhancing a rhizosphere works better than only using PGPR species. One hypothesis is that an entire community is not outcompeted by local rhizosphere bacteria before they have a chance to assist in the remediation effort.

Future prospectives also include research into plasmid DNA of PGPR bacteria, and potential isolation of a copper resistance gene, which can be flagged and monitored throughout a community. Though much research remains to be done in this area of study, community structure and interaction play an integral role in phytoremediation, and rhizospheres from a historic mine site are a potential candidate to establishing vegetative cover on other contaminated sites.

ACKNOWLEDGEMENTS

We like to thank Miguel Portillo Estrada for total carbon and nitrogen analyses at the Centre of Excellence PLECO, Department of Biology, University of Antwerp (Belgium). Further we like to thank Carine put for the ICP-OES analyses and Johan Soogen for the excellent construction of the diffusion chambers.

Chapter 2

CHAPTER 2: PLANT AND SOIL MICROBIOTAFROM WASTE MINE TAILINGS HOLD FEATURES THAT CAN ASSIST IN COPPER PHYTOREMEDIATION

TORI LANGILL[1], SOFIE THIJS[1], PETRA KIDD[2], BREANNE MCAMMOND[3], JAN D'HAEN[4], JONATHAN VAN HAMME[3], JACO VANGRONSVELD[1]

[1] Department of Biology, Centre for Environmental Sciences, Hasselt University, Belgium
[2] Instituto de InvestigacionesAgrobiologicas de Galicia (IIAG), Santiago De Compostela, Spain
[3]Department of Biological Sciences, Thompson Rivers University, Kamloops, British Columbia, Canada
[4]Institutefor Material Research (IMO-IMEC), HasseltUniversity, Diepenbeek, Belgium

ABSTRACT

Intro. Copper contaminated sites contain microorganisms that have evolved to deal with the stresses of the environment. Because of this, they contain genes that can be exploited to assist in remediation efforts of active mine sites. Plants which grow on copper contaminated (**Accumulators and Excluders**) soil possess associated microbiomes that are uniquely suited to this purpose as well.

Mat. And methods. Ion torrent sequencing was performed as well as community fingerprinting, and culture-based isolation and phenotyping of bacterial strains for**plant growth promotion (PGP)**. Two types of *In plantae* tests were used to test the difference between the effect of adding a single enriched PGP microbe, or adding an enriched community from a contaminated site. Statistically relevant bacteria were subsequently evaluated in a**rhizosphere grafting** experiment.

Results + conclusion. Accumulators and Excluders have similar bacterial enrichment at the site of detoxification (shoots for accumulators, roots for excluders). Enriched rhizospheres from these plants can increase plant growth on contaminated sites, through PGPR interactions, copper tolerance, and bioleaching ability.

Future outlook. Understanding soil bacterial communities and the role they play in phytoremediation will help in establishing plant life on contaminated sites. It will also assess a viable option for a passive remediation strategy for mine sites that is non-invasive to the environment and is economically feasible. Further insight into accumulator and excluder plant-microbe interactions will assist in successfully choosing plant species for remediation

INTRODUCTION

As was established in the previous chapter, mine sites, both recovering and active, have a large amount of **metal contamination** present. Despite this contamination, there are some plant species that manage to grow and thrive in this contaminated environment. Plants have a variety of mechanisms for dealing with the stress caused from their environment, ranging from ethylene production to up-regulating stress response genes(76–78). The stress response systems vary between plants, meaning each plant has a unique way to handle the stress. Regardless of phylum, any plant that grows in a metal contaminated environment has evolved physiological mechanisms that help them detoxify their environment(79). These mechanisms do not prevent uptake, but rather allow for internal detoxification, resulting in a plant that is either an **accumulator** or an **excluder**.

Accumulators are plants which have their primary method of detoxification take place within the shoot of the plant. Up-taken metals are restricted to the cuticle and the trichomes of the plant, where endophytic bacteria assist in the removal and/or detoxification of the metal through volatilization, or conversion to a less harmful form (**Figure 1**) (80). Accumulators, particularly **hyperaccumulators**, have been considered for phytoremediation previously, but the efforts were abandoned owing to the typically small biomass of the plants. However, a recent study suggests that an enhanced rhizosphere can not only improve plant growth, but also increase biomass (81). Within accumulators, though the main function of metal removal is through root to shoot translocation, these plants can also accumulate a fair amount of metals within their roots. This is where the main difference between an accumulator and an excluder take place, as within the shoot of an excluder, there is significantly less metal than found in the roots. Recent research has indicated at a plants ability to "call" helpful bacteria though various forms of chemical signaling, meaning that it is likely that these plants recruit helpful bacteria to assist with the metal detoxification process(82–84).

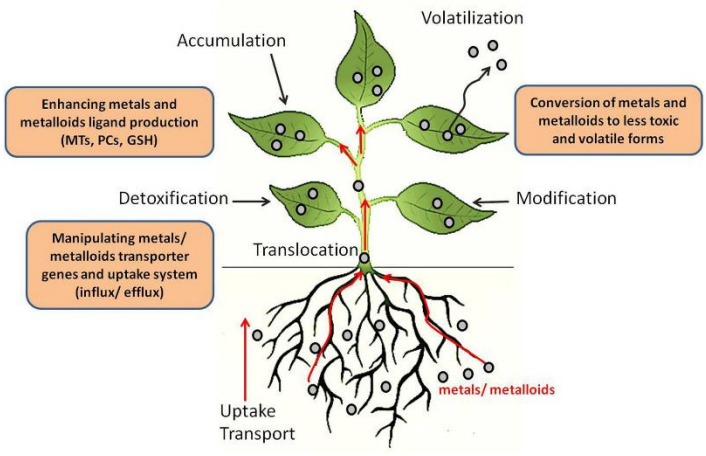


Figure 13: Accumulator plants uptake metal and metalloids from the soil and translocate them to the shoot. Here they are either accumulated, detoxified, or volatilized in an attempt to remove them from the environment (79).

Excluders, as the name suggests, restrict metal from entering the shoot of the plant, preferring to accumulate it within the roots. Unlike accumulators, excluders have no mechanism to regulate metal uptake. Instead they have a restriction mechanism which prevents root to shoot translocation (85). This mechanism results in low metal concentrations within the shoot across a large contamination gradient, until the contamination becomes too high and this mechanism breaks down. Because excluders limit the metal to the roots, root associated bacteria play an active role in the detoxificationand removal of the contaminant. The plant itself will normally possess a metal cycling ability, but the plants ability to recruit specific microorganisms to assist in dealing with the toxic compounds that allow it to grow under contaminated conditions (**Figure 2**) (86). These bacteria play an active role through biocontrol, promoting plant growth and metal cycling.

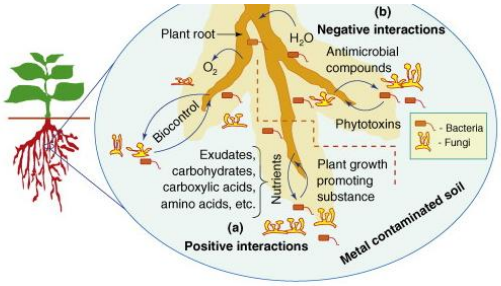


Figure 14: Root-microbe interactions resulting in decontamination of metal contaminated soil. Fungi and bacteria both play a role in the metal cycling, making the contaminant unavailable to the plant. (85)

The potential for **phytoremediation** with application of this technology is great, but in regard to knowledge of **microbe community structure and their function, still is much unknown**. In order to successfully apply this technology at an industrial level, first further steps must be taken to aid in understanding it.It has been established that plants play an instrumental role in the removal and decontamination of heavy metals from a mine site, but they would not be nearly as successful without their associated microbiota. As was shown in the previous chapter, microbiota from a historic mine site possess the ability to assist in establishing plant life. The diversity of communities across a gradient was also shown, indicating each aspect of a mine site contains uniquely suited microorganisms.

This **chapter** focuses on **plant and soil associated microorganisms** taken from an **active copper mine site** in Touro, Galicia, Spain. Two locations on the mine site were chosen: one with a high concentration of copper contamination (Touro Arriba) and one with a lower concentration (Touro Abajo). In order to assess plant associated microorganisms, fiveplants were chosen from the mine site: *Sedum brevifolium, Ericaceae sp., Trifolium sp., Festuca sp.* and *Holcus lanatus.***It was hypothesized that** rhizosphere bacteria associated with accumulators and excluders would possess the ability to improve plant growth while remediating the contaminated soil.

The **first research objective** was determining the level of copper contamination within the soil at the sites, and the amount of copper taken up by the shoots and roots of the plants. This would then lead to their determination of either accumulators or excluders. The **second objective** was to determine plant growth promotion properties of the isolated bacteria based on site. It was hypothesized that bacteria from site Arriba would have higher levels of PGPR, and copper resistance. Following this, bacteria that test positive for all PGPR traits and copper resistance would be subject to an *in plantae* test. The **third objective** focused on community structure between the two sites, as well as community differences between suspected accumulators and excluders. Soil community consortiums were then assessed for their ability to bioleach, for further insight into the industrial application of this technology.**Finally,** the tested soil communities were enhanced and added to *Medicago sativa,* to observe the effect of rhizosphere grafting on the plant.

Research questions for this chapter included, but were not limited to the following: (a) does contamination level at the site impact PGPR levels, (b) are there any community similarities between excluders and accumulators (c) if so, where are the similarities and what are they, (d) do communities from excluders or accumulators possess bioleaching bacteria, and (e) does the addition of these bacteria enhance plant growth on contaminated sites.

Research in this area not only increases knowledge about community structure of bacteria, but also increases the likelihood of this technology having an industrial application.

MATERIALS & METHODS

Soil and Plant Collection

Six plant species were selected from Touro Abajo and Touro Arriba (8° 20’ 12.06” W/42° 52’ 46.18” N; Touro, Galicia, NW Spain)and there were 11 samples in total. *Sedum brevifolium* was taken from both of the two sites, resulting in 4 replicates of this species. *Ericaceae sp* was only found on site Arriba. *Trifolium sp., Festuca sp.* and *Holcus lanatus* were only found on site Abajo. The plants were transferred to aerated containers along with their associated soils before they were transferred to Belgium for further analysis.

Soil and Plant analysis

Soil was dried at 105°C for 12 hours with before and after weights determined for soil moisture. Soil pH was measured in water and in 0.1 M KCl in a 1:1.25 soil: solution ratio (32). Total metal extractions were performed according to the microwave digestion method (33), using 500 mg of soil material that was digested using 1mL *supra pur* HNO_3 and 3mL *supra pur* HCl in a microwave. Samples were measured on ICP-OES (type, manufacturer, country).Total carbon and nitrogen content per dry mass soil was determined by a Vario MAX CNS elemental analyzer (ElementarAnalysensysteme GmbH, Hanau, Germany).

Plants had their roots andshoots harvested. Roots were rinsed with distilled water to remove soil. Plant material was dried at 80°C for 1 week, prior to acid digestion for ICP-OES. Plants and roots were digested in accordance to the method described by Zarcinas *et al*. (87).

Bacterial isolation

Bacteria were isolated from the soil samples by spread plating soil suspension dilutions on different media, and by *in situ* incubation using diffusion chambers. For the spread plating, aliquots of 5.0g of soil were added to 10.0 ml 1X PBS buffer, shaken overnight at 30°C and diluted to 10^{-1} and 10^{-3}. Each dilution was plated out in duplicate on four different selective agars: 869 (10.0g Tryptone, 5.0g Yeast Extract, 5.0g NaCl, 1.0g D-(+)-glucose, 0.345g $CaCl_2.2H_2O$), 284 selective (35), 79 minimal medium (5g sucrose, 5 g Mannitol, 100mg K_2PO_4, 400mg KH_2PO_4, 200mg $MgSO_4.7H_2O$, 100mg NaCl, 400mg Yeast, pH 6.8-7), and CYE (0.1g casamino acid, 0.1g yeast extract, 50mg $CaCl_2$, 500mg $MgSO_4$.7HO, 7.5g gellan gum, 1ml of filter sterilized soil extract). After two weeks of growth, representative colonies were picked from the agar plates, re-streaked for purity on fresh media plates, and finally stored in a 15 % w/v glycerol solution at -45°C.

Copper tolerance

Copper tolerance of the isolated strains was evaluated by growing the strains in minimal medium with different copper concentrations (0.4 mM and 0.8 mM as $CuSO_4$). First, the strains were pre-grown overnight in 1.2 ml of 869 broth in sterile deep 96-well masterblocks (120 rpm, 30°C). Bacterial cells were pelleted by centrifugation (2000 g, 30 min), washed once with 1X PBS and 100 µL of bacteria sample was inoculated into 284 broth with 0.4mM Cu and 0.8mM Cu. Bacterial growth was assessed visually after 7 days of constant agitation at 120 rpm, 30°C.

***In vitro* plant growth promotion (PGP)**

Plant growth promotion traits of the coppertolerant bacterial isolates was tested qualitatively.The bacteria were grown in 869 media at 30ºC for 24 hours, washed and resuspended in a 2 mL sterile 0.1M $MgSO_4$solution to obtain a suspension with OD_{600} of 1.20 µl of this suspension was used for the inoculation of 96-well plate assays (Greiner Bio-One, Wemmel, Belgium) for the detection of: auxin

production using the Salkowski reagent method(38); 1-aminocyclopropane-1-carboxylate (ACC)-deaminase activity estimated by monitoring the amount of α-ketobutyrate generated by the enzymatic hydrolysis of ACC, a plant hormone ethylene precursor(39); and acetoin production, measured using the Voges-Proskauer assay (40). For siderophore testing, bacteria were subjected to 3 concentrations of iron in 284 media (0mM, 0.25mM and 3mM) and the evaluation method performed followed the CAS assay (41). For all the PGP-assays, the bacterial isolates were distributed into classes scored as '0' and '1' depending on their negative or positive responses. All tests were performed in triplicate.

***In Plantae*experiments**

For plant germination, vertical agar plates were made using a basal medium (50ml 20X basal medium, 950mL dH_2O, 5 g/L sucrose, 10g/L agar) as described in (42). The medium was subjected to four concentrations of copper to induce stress in the germinating plant with $CuSO_4$ (0uM,50uM,100uM,150uM). The gradient was tested to determinewhich copper concentration had a visible effect on germination and root length.

The six bacteria that tested positive for all plant growth promotion traits had a 1.0mL aliquot grown overnight in 25.0mL 869 broth at 30ºC with constant agitation of 120rpm. The bacteria were centrifuged (5000 g, 10 min.) and washed twice with sterile 0.5M $MgSO_4$. The bacteria were resuspended and centrifuged at 2500 g for 10 min. The supernatant was placed into sterile beakers and diluted with 50.0 mL sterile 0.5M $MgSO_4$.

The test species, *Medicago sativasp.,* seeds were sterilized as described previously (43). The sterile seeds were placed in the bacterial supernatant solution for 1 min. 30 sec, followed by two washes in sterile dH_2O.

Seeds were sown on the agar with different copper levels, five seeds per plate, and were then incubated vertically with PAR provided at the rosette level of 170 mol m^{-2} s^{-1}, 22ºC/18ºC degrees, 12/12 day night cycle and 65% RH for 10 days. After 10 days, root length and number of side roots were measured with RootNav technology(44).

Rhizosphere Grafting

M. sativa seeds were sterilized and inoculated with an amplified rhizosphere from each of selected plants, excluders and accumulators alike. The rhizosphere and soil associated bacteria were amplified by inoculating 10.0 mL of 869 broth with ~1.0 g of root associated soil. Growth was allowed to the log phase. The bacteria were collected by centrifugation at 5000 g for 20 min. and were resuspended in 5.0 mL 1X PBS. Sterile *M.sativa* seeds were placed into the PBS solution and shaken at 30ºC for 15 minutes. Following this, 20 seeds were sown on high concentration (Touro Arriba) and low concentration (Touro Abajo) copper contaminated soil. These soils were collected from the Touro mine site in GaliciaSoils were sampled in the field in zipper bags and transported at 4°C to the lab in Belgium. For each condition, three pots with 20 seeds per pot were grown under controlled greenhouse conditions with 170 mol m^{-2} s^{-1}PAR, 22ºC/18ºC degrees, 12/12 day night cycle and 65% RH for 2 weeks. During this time, germination effect was measured on day 4, and plant area was

determined through measuring leaf diameter and width on day 10. Plants, roots and soil were harvested after 2 weeks to determine levels of copper in each attribute.

DNA-extraction and ARISA fingerprinting

DNA was extracted from the eight sampled soils using the MoBio PowerSoil Kit according to manufacturer's instructions. Quality and quantity of the DNA was checked on a 1 % agarose gel and using nanodrop. DNA was subsequently subjected to an ARISA PCR using the ITSF Forward primer and the ITSReub reverse primer using PCR conditions as described previously (45) on the Biorad T100 (Biorad, Venlo, Netherlands). The amplified inter spacer regions were analyzed with the Bioanalyzer (Agilent 2100, Waldbronn, Germany) using DNA 1000 chips (Agilent 2100, Waldbronn Germany).
ARISA results were interpreted in two ways: Using R v2.13 and the package StatFingerprints, and using AdaptDB(47). R was used to determine a bray-curtis index under a ward algorithm to create heatmaps of the species present. AdaptDB was used to create cladograms of potential species identified by the length of their interspacer region, and potential of pathogenicity according to matches within the SEED database and the NCBI database(47).

16S rDNA gene Sanger sequencing

Copper tolerant and PGP bacterial species of interest were sent for 16S rDNA gene sequencing using the universal prokaryotic1392R primer (5' ACGGGCGGTGTGTRC 3')and thebacteria-specific 27 F primer (5' AGAGTTTGATCCTGGCTCAG 3') with PCR-conditions as previously described (48). The raw sequence reads were quality trimmed and blasted against the Ribosomal Database (49).

Bioleaching Test

Soils had their rhizosphere amplified by shaking approximately 1.0g of sample with 5.0mL of 859 broth. Samples were left overnight shaking at 30°C at 100rpm. Following this, a 2.0mL aliquot was taken and centrifuged at 1500rpm, before being resuspended in 2.0mL 1X PBS. From this solution, 1.0mL of bacteria consortium from each plant was added to bioleaching broth as is described by Romo *et al.*(22). Approximately 1.0g of powdered bornite ore was added to each solution. The bioleaching reaction was carried out in a 25mL sealed Erlenmeyer flask, at 37°C, with constant agitation of 120 rpm. Samples were taken on day 0, and then intermittently every 10 days for analysis with ICP-OES. Solution pH was measured every 5 days. Samples were stored at -45 in glycerol to determine the change in bacterial community. This test was not a sterile test.
Following the bioleaching test, the bacterial community changes were assessed using qPCR. DNA was extracted using Qiagen DNeasy Blood & Tissue kits. qPCR primers designed for different phylogenetic groups were chosen from the paper of Pfeiffer(88). They were synthetized at IDT technologies (IDT, Leuven, Belgium). Standard curves were prepared by cloning the 16S gene of cultured strains in Pgem-T vector in E. coli JM109 cells, according to standard procedures. Purified plasmids were quantitated using picogreen, and diluted from 9 till 0 log copies per µl. In brief, qPCR reactions were

set-up in 10 µl volumes, using the SYBR green quantitect kit (Qiagen, Venlo, Netherlands), and 2 µl plasmid or DNA-template (1/10, 1/50 diluted), primer concentrations **(Table 1, Appendix 2-A**). Reactions were set-up in Applied Biosystems plate (AB, Bleiswijk, Netherlands), and run on the Applied Biosystems 7500 cycler (AB, Bleiswijk, Netherlands), using the cycling conditions outlined by Qiagen and PerfecTa SYBR green qPCR kits. Primer efficiency was close to -3.5 and final concentrations were calculated using the standard curves produced.

Ion Torrent amplicon sequencing

16S rRNA gene amplification was performed on the DNA samples using the primer set 341f (5' TACGGGAGGCAGCAG 3') and 806r (5' GGACTACVSGGGTATCTAAT 3'), that target the V3-V4 variable region of bacteria as described previously (50). The library dilution factor of amplicon pools was determined using an Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific) prior to amplification and enrichment with an Ion PGM Hi-Q View OT2 400 Kit on an Ion OneTouch 2 System. The Enriched Ion Sphere Particles were analyzed using an Ion Sphere Quality Control Kit prior to sequencing with an Ion 316v2 Chip on an Ion Torrent PGM running Torrent Suite 5.2.2 using an Ion PGM Hi-Q View Sequencing Kit. Equimolar ratios of quality control checked libraries were sequenced on the Ion Torrent S5 platform. A total of 1.3 GB of data was generated.

Data processing

Data pre-processing is necessary for both shotgunand amplicon data due to sequencing errors. For the shotgun reads, we trimmed trailing bases with quality score 2and discarded reads shorter than 30bp and with "N." The reads then were quality trimmed with fastq-mcf(version 1.04.662) (http://code.google.com/p/ea-utils) with "-l 50 -q 30-x 10 -max-ns 0 -X." The paired-end reads overlapping by more than10 bp were assembled into one long read by PEAR (version 0.9.8) (51). Quality controlled shotgun sequencing were subsequently assigned taxonomy using MetaPhlan2 (52), and functionally annotated using MG-RAST (53) and the Greengenes database. Function was also attributed using MG-RAST through the Kegg Orthologs and SEED database (55).
Ion Torrent data were processed using the Qiime2 pipeline (56) according to the developers suggestions and with trimming of the first 15 bp in the dada2 noise removal algorithm.

Statistics

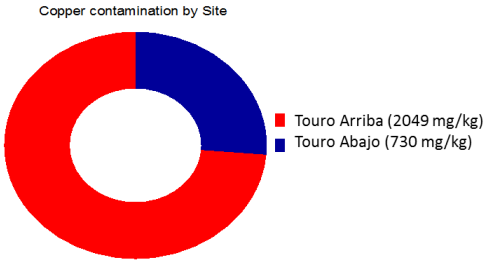
Statistics were performed using R 3.14, QI Macros, and PAST. All statistically tested data was tested for normal distribution and homoscedasticity and then subjected to a one-way ANOVA with post-hoc Turkey's HSD test. Non-metric multidimensional scaling with Bray-Curtis was used to determine statistical differences in sample location.

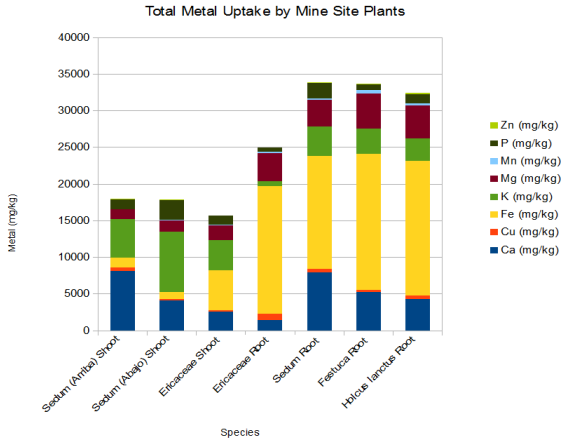
RESULTS

Soil and Plant Analysis

Of the six plant species collected ICP-OES was performed to assess whether the species were either accumulators or excluders (**Figure 3**). Soil around the roots was measured for total carbon and nitrogen, as well as measured with ICP-OES to determine total metal contamination of the sites, and plant available metal within the site. (**Figure3**) It was found that Touro Arriba was the higher contaminated site, and possessed less nitrogen than Touro Abajo.

Plant Type	Harvest Location	Total C	Total N
Ericaceae sp.	Touro Arriba	0.083742287	2.742794276
Sedum brevifolium	Touro Arriba	0.1010231127	2.898091713
Sedum brevifolium	Touro Abajo	0.148448199	4.397173882
Trifolium sp.	Touro Abajo	0.1666214094	5.438708067
Festuca sp.	Touro Abajo	0.0688213613	3.541659117
Holcus lanatus	Touro Abajo	0.060845051	2.508702517





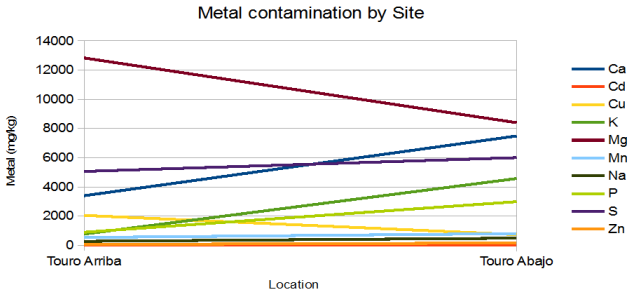


Figure 15: Plant species harvested from the two mine sites show variation in the level of copper and iron uptaken by the shoots and roots. Touro Arriba is the site which shows higher levels of contaminant.Roots take up a higher concentration of iron.

Plant Growth Promotion Traits

In total 96 Bacteria were cultured from Spain, and had a representative assortment selected randomly from each plant rhizosphere to test for plant growth producing factors, such as IAA and ACC-deaminase. It was found that bacteria from *Sedum* from the site with higher contamination possessed

a higher level of siderophore production at 0uM of Fe (**Figure 4**). It was also noted that Sedum from Arriba had a higher production level of Acetoin.

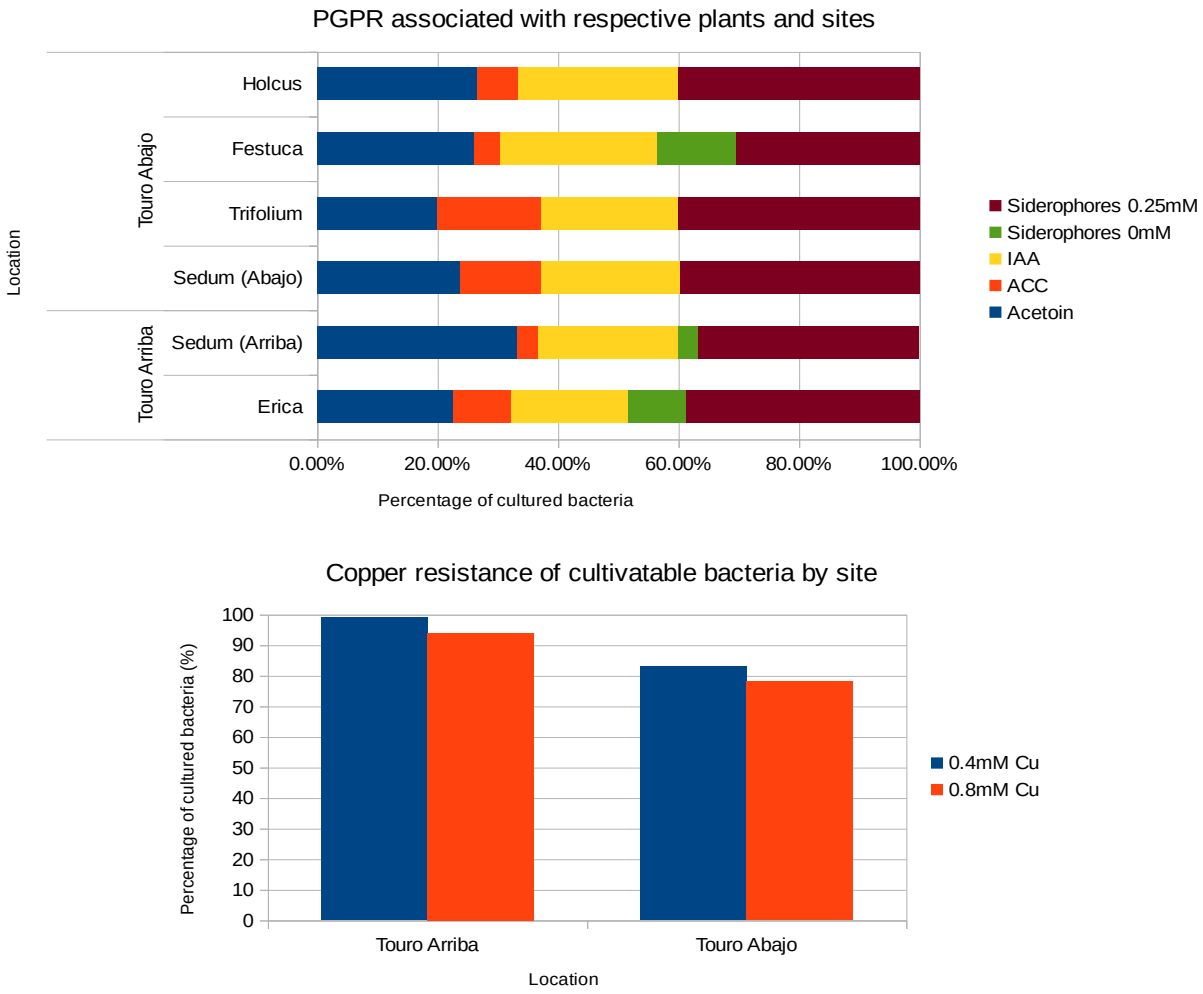


Figure 16: PGPR traits of the randomly selected representative bacteria of the plants, and sample sites. Both sites had a high level of culturable copper resistant bacteria, with over 70% of the bacteria from both sites able to grow at concentrations of 0.8mM

From this test, six bacteria were found that tested positive for all PGPR tests. These positive microorganisms were sent for 16S rRNA gene sequencing for identification (**Appendix 2-B**), and were then subjected to an *in plantae* VAPS test.

VAPS

With the enriched bacteria applied to the *M. sativa* seed, there was only one instance of significance noted across the copper gradient of 0uM, 50uM, 100uM, and 150uM. This instance was with the application of *Pseudomonas protegens* (**Figure 5**).

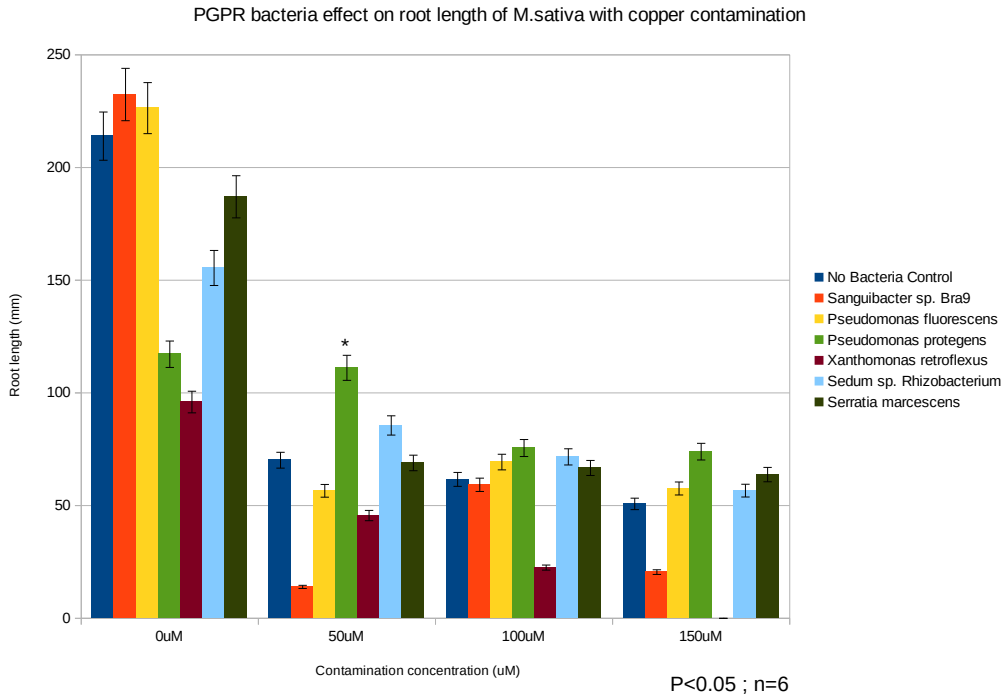
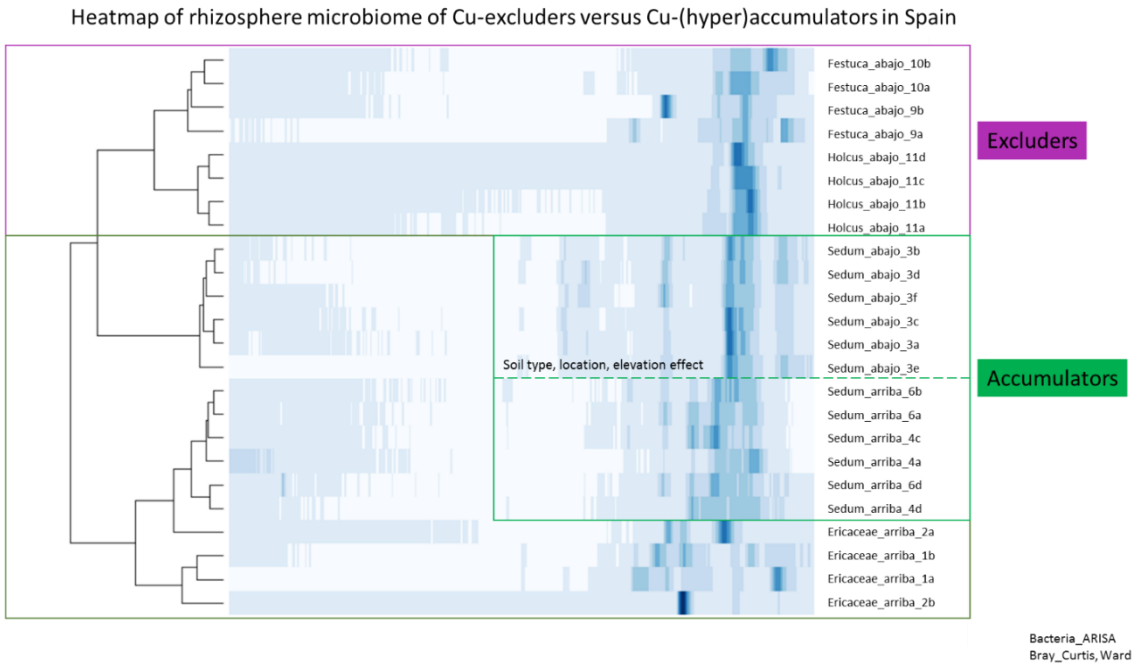


Figure 17: Root length of Medicago sativa after seed inoculation with PGPR bacteria. No levels of significance were found.

Again, there is no instance of significance when the *M. sativa* seeds are inoculated with the above bacteria. This supports the hypothesis that community interactions within a community are integral to promoting plant growth.

Microbial community structure

ARISA was performed on the three sets of samples to analyze the difference between shoot, root and rhizosphere colonies between accumulators and excluders. Based on the non-metric multidimensional scaling with bray curtis method, we can see that the rhizosphere of accumulators has a strong difference from the excluder rhizosphere based on clustering patterns (**Figure 6**). The observed stress value of 12.347 speaks to the level of difference between the communities. This is again supported by the heatmap, which shows that although there are high levels of similar phyla across both accumulators and excluders, the accumulators on average have a much higher level of diversity. This is also reflected by how they cluster on the nMDS test.



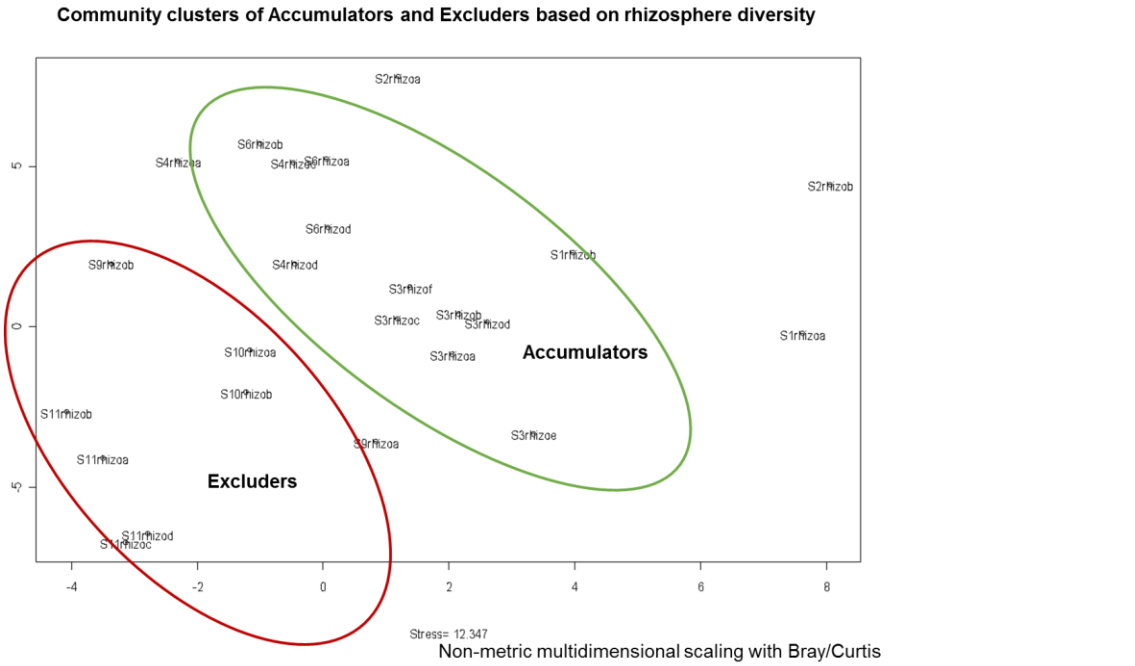


Figure 18: A heatmap representing the rhizosphere community structure between accumulators (Sedum) and excluders (Festuca and Holcus), along with Erica, which is not determined to be an excluder or accumulator. nMDS with bray curtis is used to determine relationship strength.

A comparison between roots, shoots, and soil of *Sedum* from Arriba, and *Sedum* from Abajo was done to observe if there were more community changes between sites (**Figure 7**).

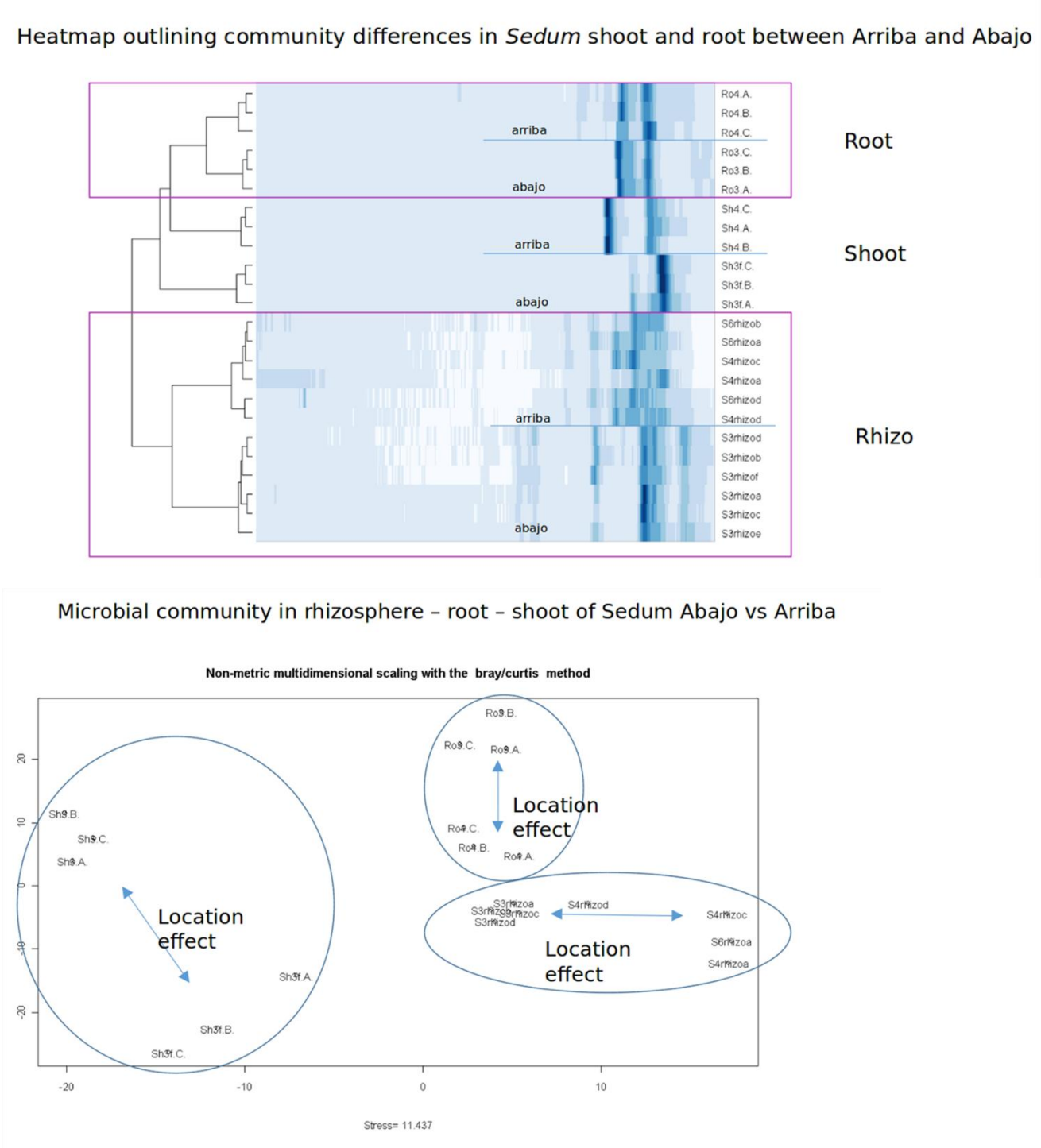


Figure 19:Heatmap comparison of Sedum species by site, addressing community structure of roots, shoots, and rhizosphere. nMDS clustering shows a clear location effect, and a stress of 11.437 indicates that the communities are very different from each other.

Referring to Figure 6, the rhizosphere community of *Ericaceae* is distinct from both the excluder and accumulator communities. However, based on the nMDS, it is more closely related to that of an

accumulator, even though the ICP-OES results show higher levels of metal in the roots than in the shoots of the plant. This is likely because rhizosphere bacteria are not the only important communities that assist a plant in its purpose, be it in accumulator, or exclusion. This is observedwhen we look at the *Ericaceae* in detail across root, rhizosphere, and shoot communities (**Figure 8**).

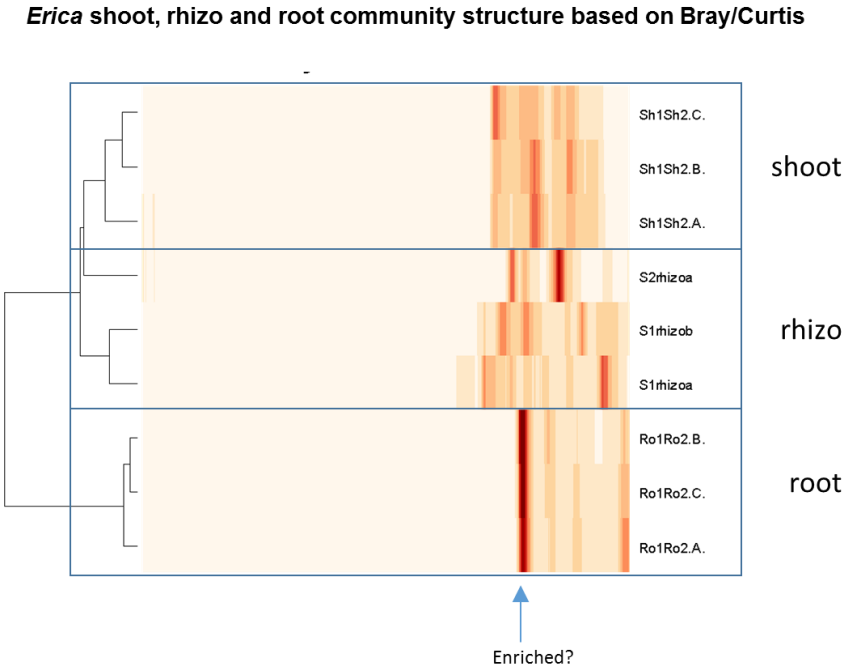


Figure 20: Erica community structure across shoot, root and rhizosphere. There is a phylum enrichment in the root of the plant, which could play a role in the exclusion process of containing metals to the root to be detoxified. The most diversity is seen within the rhizosphere.

There is an enrichment seen in the roots of the *Erica.* This enrichment also occurs in the same area as the enrichment on the *Sedum* shoot from Touro Arriba, strengthening the argument that this enrichment is key in detoxification and/or stabilization, as it occurs in both the root and shoot of plant species that have concentrated metals within the plant body (**Figure 9**).

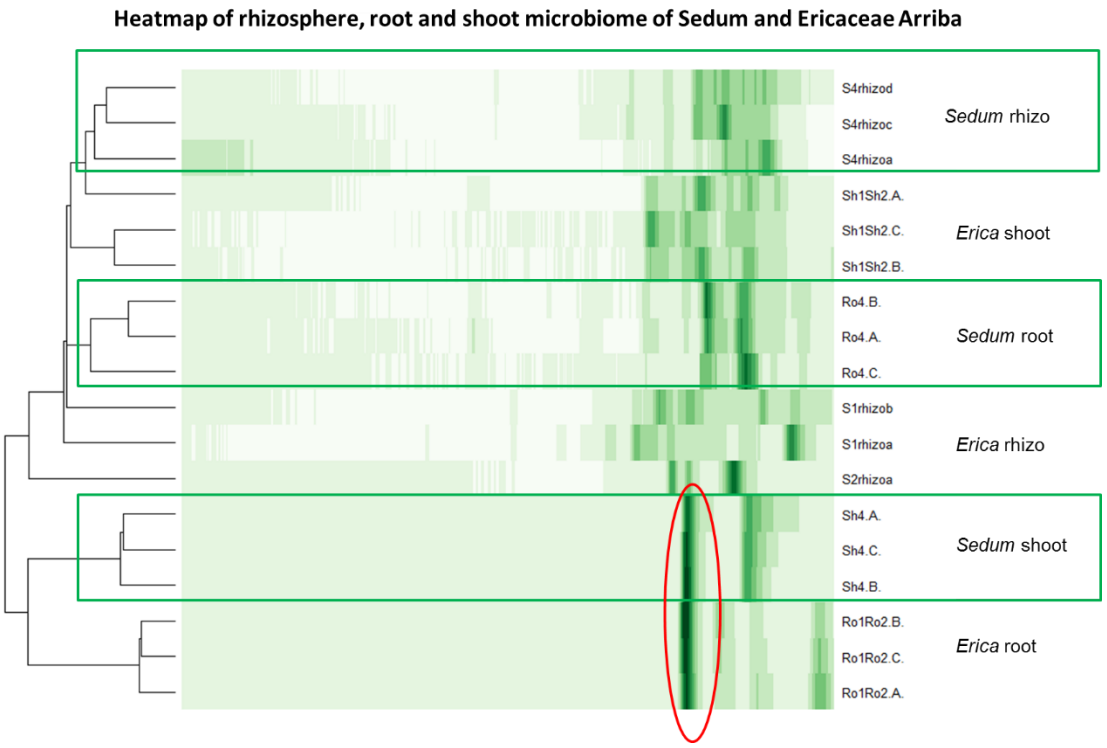


Figure 21:Community comparison between Sedum and Erica, in instances of shoot, root and rhizosphere. An enrichment is seen and shared between the Sedum shoot and the Erica root, indicating that this particular phylum of species plays a key role in the removal of metal contamination from soils.

Amplicon Sequencing

To gain further insight into the community structure, the samples were sent for amplicon sequencing. Results were processed with QIIME2, and online using MG-RAST and VAMPS. Based on the resulting amplicons, a Taxa plot was generated to visualize the dominant phyla inhabiting each sample site (**Figure 10**).

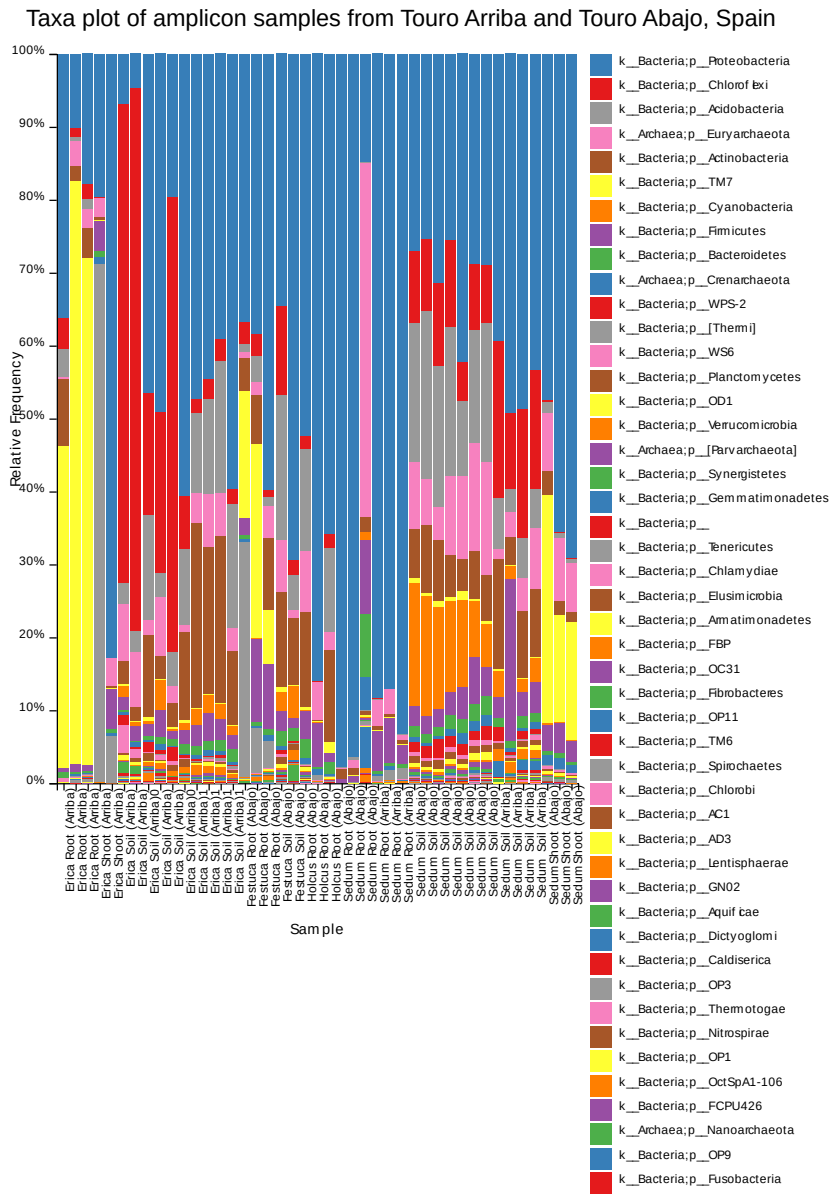


Figure 22:Taxa plot based on the 16S amplicon sequencing, processed using QIIME2. Proteobacteria play a dominant role across all samples, but are particularly dominant within the Sedum species, whereas Erica soil appears to possess a greater relative abundance of Chloroflexi when compared to other samples.

Based on the amplicon reads, a community comparison heatmap from the phylum level was generated, showing the similarities and differences between the soil samples at each site (**Figure 11**).

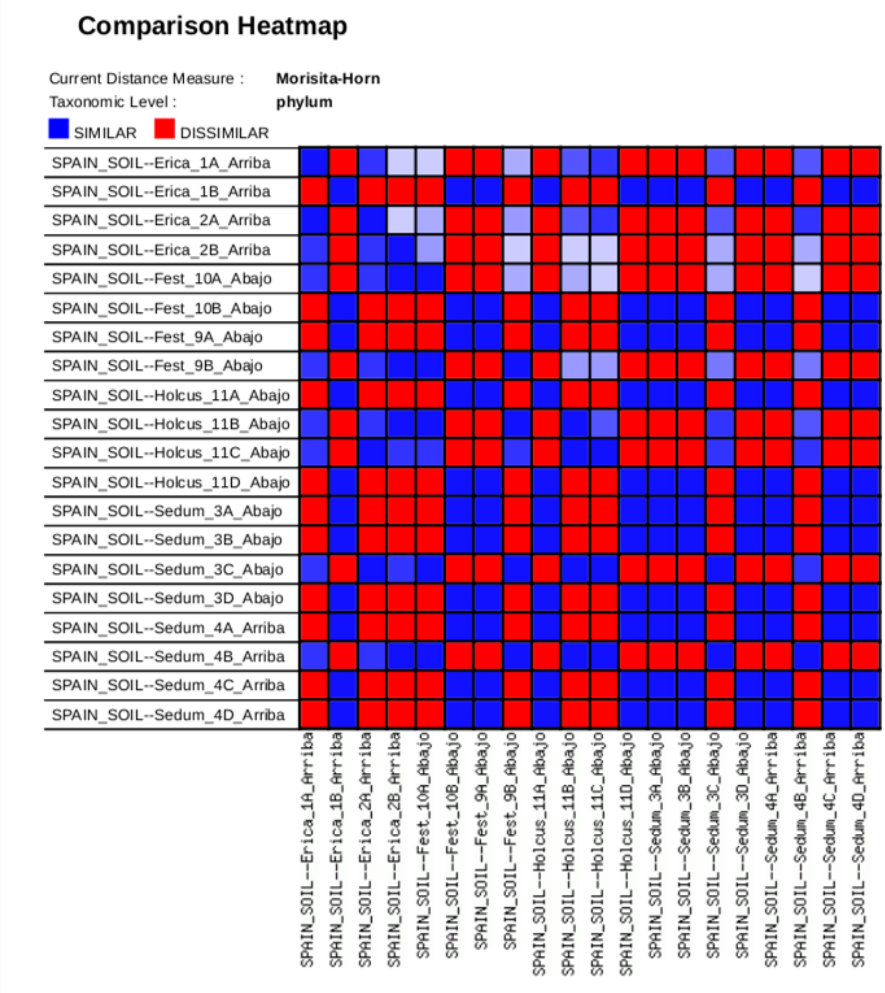


Figure 23: Heatmap comparison of total community similarity between rhizosphere samples at site Touro Arriba and Touro Abajo.

Bioleaching

Because of the community differences, soil bacteria were chosen to be amplified for the bioleaching test. Results show *Holcus*as a candidate possessing a bacterial community that leaches significantly (**p<0.05; n=3**). The *Sedum* species from site Arriba also leaches significantly, but only until Day 15. Following this, it is believed that the bioleaching bacteria were outcompeted, or died off due to rapidly changing environments (**Figure 12**).

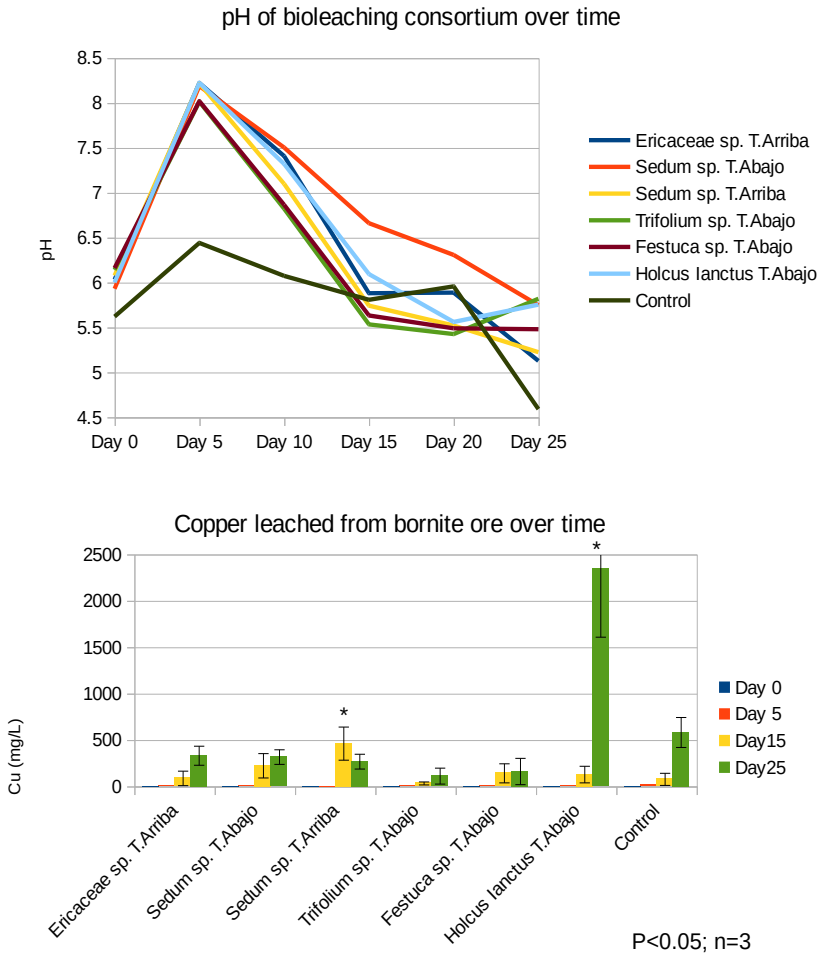


Figure 24: Bioleaching consortium pH over time and copper leached over time. The initial pH spike is due to sulfur reducing bacteria, though these SRBs appear to be outcompeted after 10 days, allowing for sulfur oxidizing bacteria and other acidophiles to enrich. One-way ANOVA on the copper leached over time shows two species which leached significant copper: Sedum and Holcus. Holcus rhizosphere consortium is proposed for further study.

SEM was performed at high vacuum to visualize whether the bioleaching bacteria were forming biofilms, or were leaching independently (**Figure 13**)

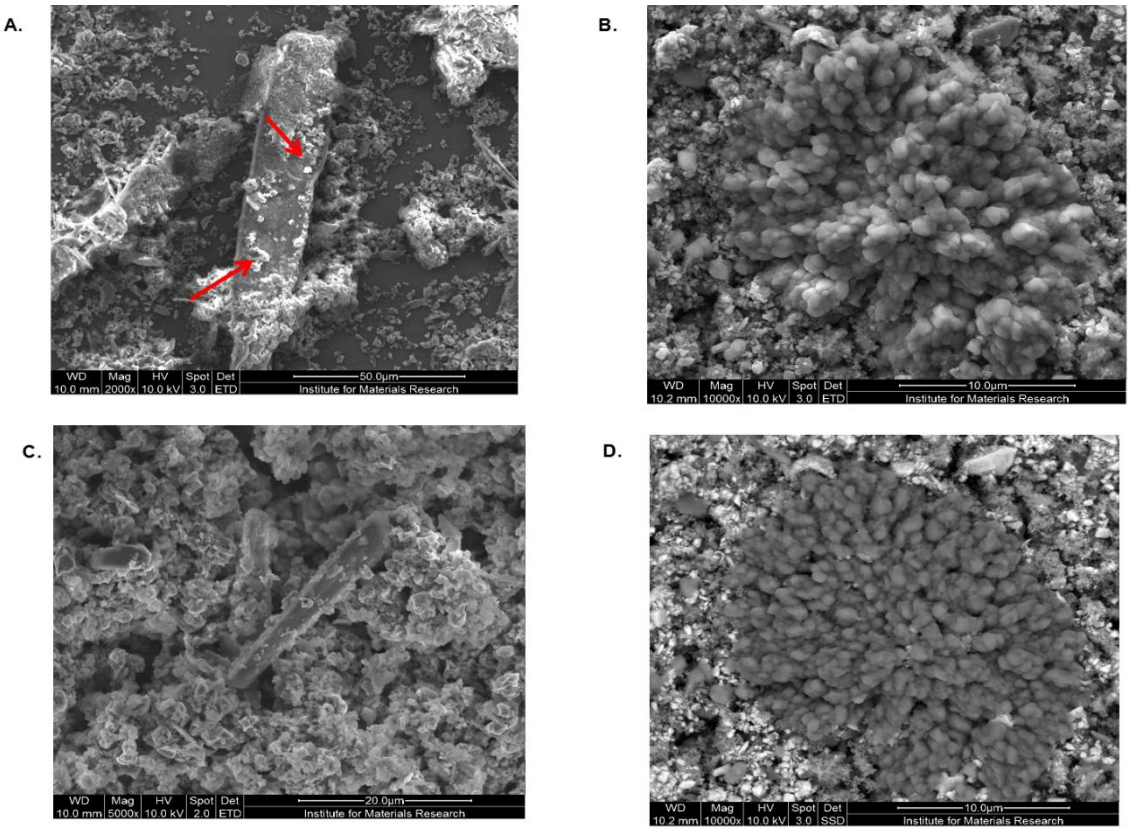


Figure 25:(A) Bacteria from Holcus consortium with copper on the surface, as established by EDX spectrum (B) Biofilm from control consortium (C) Various bacteria from Holcus consortium in bioleaching mixture (D) Control biofilm on top of copper, iron metals, measured with EDX spectrum.

Finally, in order to assess community change over the course of the bioleaching, the initial consortiums were compared to the final consortiums through qPCR 16S profiling (**Figure 14**).Holcus shows a large increase in phylum alpha-Proteobacteria, as does Festuca, Sedum (Arriba), and Erica. It is assumed that alpha-Proteobacteria play a key role in bioleaching communities.

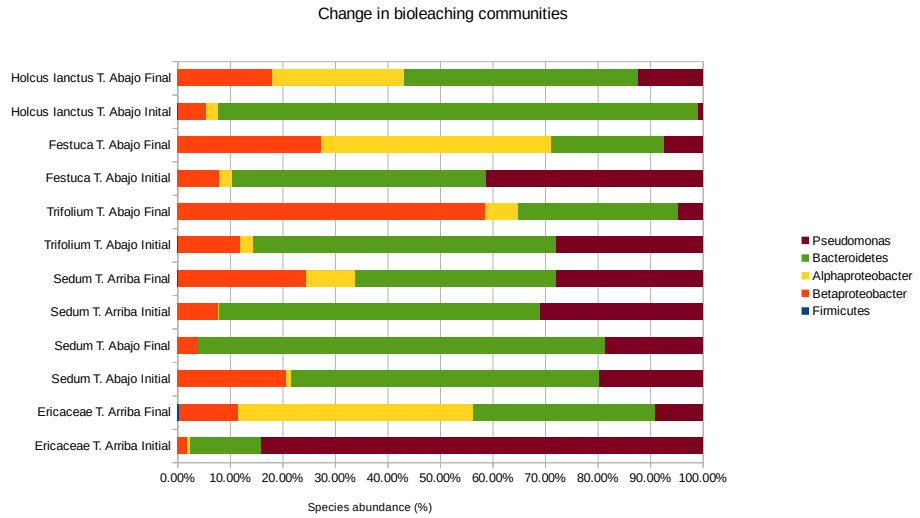


Figure 26: Bioleaching community structure before and after bioleaching treatment noticing the increase in alpha-Proteobacteria after leaching. Final: after leaching. Initial: at the start of bioleaching.

Rhizosphere Grafting

Inoculation of *M. sativa* seeds with the enriched consortiafrom the various plants resulted in significantly better seed germination in all instances, but particularly in *Holcus* (**Figure 15**). *Holcus* also significantly increased the leaf size of the plant on both soil types. *Sedum* from site Arriba also resulted in better growth, but it was only significant on the soil from site Abajo.

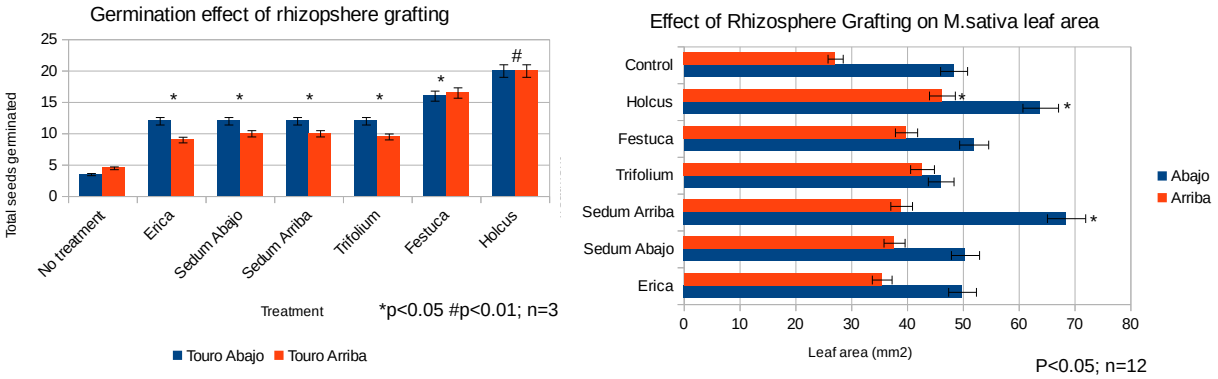


Figure 27:Germination effect of all added rhizospheres had a significant effect on all mine sites, with Holcus as the most significant. Holcus also had a significant effect on leaf area at both sites.

In regard to copper contamination, on site Arriba, copper decreased in the soil with the addition of *Festuca* rhizosphere, but appeared to have a stronger effect in lowering the amount of copper when no additional rhizosphere was added (**Figure 16**). However, given the time-frame of the experiment (**3**

weeks), it can be assumed that a longer incubation period is needed to see an effective copper decrease in the soil.

In the shoot, there is a significant decrease of copper uptaken by alfalfa with the addition of the enriched rhizosphere of *Sedum* from Abajo, *Sedum* from Arriba, and *Trifolium* (**p<0.05; n=6**).

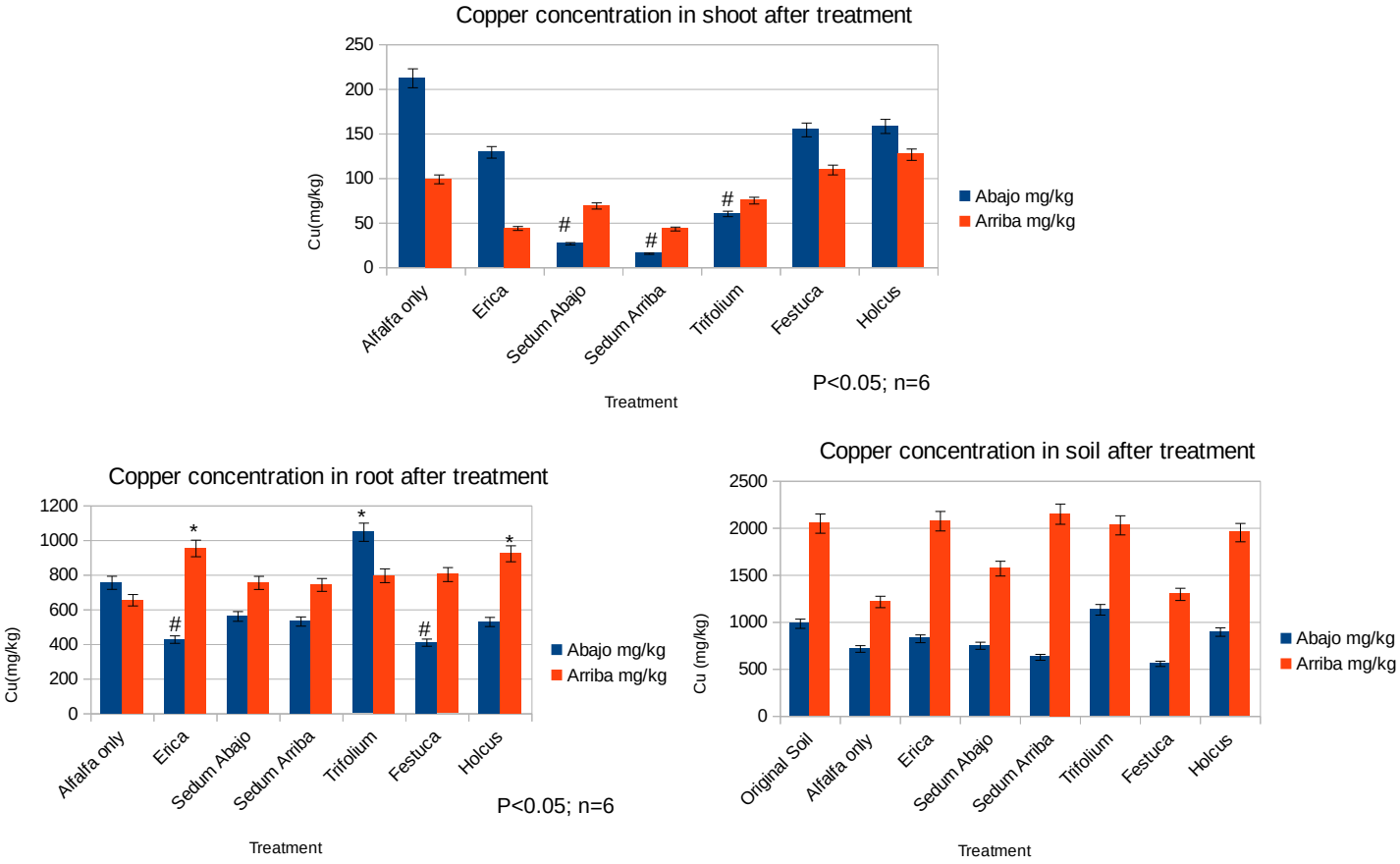


Figure 28: Copper concentration in the shoot decreased with the addition of Sedum rhizosphere's, contrary to what was expected from the addition of an accumulator. A significant decrease is shown with (#), while a significant increase is shown with (*).

DISCUSSION

Soil and Plant Analysis

In the instance of copper, we have three excluder species, found in *Erica, Festuca*, and *Holcus lanatus*. These species concentrate the metal to the roots, rather than the shoots. In the event of *Festuca* and *Holcus*, there was not enough plant tissue left to determine shoot concentration, so this was established from literature (85,89). There is also an accumulator species present, found in *Sedum*. It was interesting to note that on the site with the higher level of contamination, the *Sedum* species was able to significantly accumulate over double the amount of copper (**$p<0.05$; $n=6$**). This supports the hypothesis that the associated microbiome plays a role in the accumulation and detoxification, as the plant species was the same.

Based on our gathered results, all tested plants possess the ability to hyperaccumulate and remediate magnesium from the soil, as they concentrate it in their roots and shoots, at levels of over 1000mg/kg.

The large amount of iron in the roots leads to the hypothesis that there is high siderophore activity in the associated rhizosphere. It is also predicted that there will be a higher level of copper resistant bacteria associated with plants from Touro Arriba.

Plant Growth Promotion Traits

As mentioned in the previous chapter, acetoin improves plant growth acting as a phytohormone (90). The acetoin is released as a volatile component which helps induce systematic resistance against plant and soil pathogens that would take advantage of stressed plant(91). In this instance, because of the high level of metal contamination in the soil at the site, it is reasonable to assume the plant is more stressed than the Sedum on site Abajo. Having a higher production of acetoin could be necessary to surviving on the site.

On site Abajo, the *Sedum* has a higher production of ACC-deaminase, than the *Sedum* from Arriba. This could be due to the ethylene lowering capability of ACC-deaminase, as a high level of ethylene is needed to induce acclimation processes with aid in plant tolerance and survival to stress (92,93). Since the environment at Arriba is more stressful, the benefits of having high ethylene within the plant may outweigh the need for lower stress levels.

In regard to accumulators vs excluders, the culturable community is similar in regard to plant growth production. From the tests, there were six bacteria found that tested positive for all PGPR and copper-resistance tests. These were selected for further testing with *In Plantae* methods.

VAPS

With the enriched bacteria applied to the *M. sativa* seed, there was only one instance of significance noted, in the case of bacteria *Pseudomonas protegens* (**Figure 5**). As the name implies*, P. protegens* has previously been identified for its ability to protect plants against a variety of pathogens. It has been shows to protect plant roots against phytopathogenic fungi, as well as against other pathogenic microorganisms(94–96). The bacteria also helps protect plants from insect damage through secretion of FitD, a toxin that acts as an insecticide (78).

Still, this is the first known recorded instance of *Pseudomonas protegens* to show protection against copper induced stress. It is also of interest to note that the bacteria are only beneficial when the plant is under stress, as at 0uM of copper, the root length of the plant is significantly lower than the no bacteria control (**$p<0.05$; $n=6$**).

In the case of *Xanthomonasretroflexus*, it can be seen that the bacteria is causing more harm to the plant than the copper. This is probably due to *X.retroflexus* being a plant pathogen, which is known to negatively affect tomatoes, rice and pepper plants(97,98). It is often one of the driving bacteria behind blight on citrus plants. Although this bacteria is a plant pathogen, it still exhibits plant growth promoting behavior, showing that not all PGPR are good candidates for remediation.

Because we only had one bacteria significantly help alfalfa growth improve, and only significantly at one condition, an enriched consortium of bacteria was proposed in an attempt to increase remediation and better establish plant life on contaminated soil.

Before testing the enriched consortium on the alfalfa, samples representative of accumulators and excluders were assessed to determine what was present in the community.

Again, we have seen that an enriched single microbe is not the most promising option for encouraging plant growth. Community structure and diversity must be analyzed in order for a strong consortium to be utilized.

Community Structure

Based on the generated heatmap (**Figure 6**), we can also see that there is a difference in community structure based on the location where the samples were collected. Contrary to expected, the rhizosphere of the *Sedum* from Touro Arriba possesses a higher level of diversity, even though the site is more contaminated. We believe that this is because the *Sedum* plant from Arriba has taken up more of the metal contamination, lowering the level of contamination around the roots, allowing for more soil diversity there(19).

Based on the heatmap of the *Sedum* species (**Figure 7**), it is observed that within the shoot of the plant, there is an enrichment of a phylum in the species from Touro Arriba. This enrichment is likely owing to the higher level of copper uptaken by this plant, when compared to its counterpart from site Abajo. From site Abajo, we see lower rhizosphere diversity, and although there is an enrichment in the shoot, it does not correlate to the enrichment from the more diverse, yet more contaminated site. Based on the nMDS with bray curtis, we can see that there is an obvious location effect between our samples, and that once again, the rhizosphere of *Sedum* from the higher contaminated site is the

most diverse. We also observe that the communities of the roots on both sites are the most similar, as the location effect is smaller, and the heatmap indicates similar community structure. This indicates that in the instance of *Sedum*, the root communities do not play much of a role in metal uptake of the plant.

In the case of *Erica*, the plant behaves like an excluder, as it clusters more closely to the other excluder species. It possesses the most diverse rhizosphere in comparison with all plant species tested, but interestingly possesses an enriched band within the roots of the plant. This enrichment could be related to the high level of metal witnessed in the roots of the *Erica* species. It is known that a contaminated rhizosphere limits the type of bacteria that can grow successfully there, meaning that in this case, because of the high metal concentration in the root, this enriched phyla not only grows successfully, but may be playing a key role in stabilization of the metals in the roots, a key trait of excluders .

This enrichment also occurs in the same area as the enrichment on the *Sedum* shoot from Touro Arriba, strengthening the argument that this enrichment is key in detoxification and/or stabilization, as it occurs in both the root and shoot of plant species that have concentrated metals within the plant body (**Figure 9**).

It is of interest to note the similarity of community structure between the *Sedum* rhizosphere and the *Erica* shoot, as well as the relationship between the *Sedum* shoot and the *Erica* root. The most diverse community from the *Sedum,* the rhizosphere, is owing to the removal of metals via translocation to the shoot of the plant. This in turn removes them from the surrounding soil, allowing for a diverse rhizospheric community. This is the mechanism through which accumulators operate.

Excluders, on the other hand, restrict metals to the roots of the plant, keeping the shoot of the plant stress free. This is supported by two factors: (a) the similarity between the *Sedum* rhizosphere and the *Erica* shoot indicate that these are the most diverse communities within the samples, and (b) the shared enrichment band between *Sedum* shoot, and *Erica* root indicate that this is where the detoxification and/or stabilization occurs within the plant, respectively resulting in the aforementioned diversity.

Previous remediation efforts on this site have focused on restoring microbial diversity as an endpoint for successful remediation, as a diverse microbiome increases biodiversity of the site (99). Using an enriched colony for remediation is an attractive option as the introduced community is outcompeted by the local microorganisms once the site becomes less contaminated, meaning there is no risk of invasive species (81).

Amplicon Sequencing

There is a high level of Proteobacteria in the *Sedum* root, as well as in the *Holcus* root. For the *Erica* root, and the *Sedum* shoot, we can see an enrichment of phylum TM7. Phylum TM7, also known as *CandidatusSaccharibacteria,* is a phylum of bacteria that to date has not been isolated in the lab. Because there are no pure cultures of any species from this phylum, it has only been studied through

metagenomic endeavors, making it a phylum of great interest. Already it has been identified as a key phylum in toluene and arsenic breakdown, meaning it likely plays a leading role in the metal detoxification within these plant species.

The resulting heatmap shows two excluder species, *Festuca* and *Holcus*, possess similar total bacteria communities in their soil, as the *Sedum* from Touro Arriba. *Erica* species are the most removed in terms of their rhizosphere community, except in one instance where it clusters with *Festuca*. These results once again show that the bacteria which play a key role in both accumulators and excluders are local to the plant, rather than the surrounding rhizosphere. This is expecting as the decontamination and/or stabilization does not take place in the soil, but rather in the roots and shoots themselves (100)

Bioleaching

Since it has been established that the key bacteria for metal remediation are of phyla TM7, and are yet to be cultured in the lab, we decided to test the soil bacteria of all the plant species for their ability to leach copper from ore bodies. This application is an industrialized form of remediation, as enriched colonies of bioleaching bacteria applied to soil can make copper more bioavailable in elemental form, meaning it will be easier to uptake by an accumulator or hyperaccumulator plant. The rhizosphere bacteria were chosen, as they are local to the soil, where the remaining ore and copper contamination exist. It is seen that the rhizosphere consortium from *Holcus* is the best candidate for enriched bioleaching (**Figure 12**).

Based on the pH increase during the first 5 days of the bioleaching, it is speculated that sulfur reducing bacteria are at work. As we saw with the historic mine site, sulfur is very predominant on a mine site, especially in the case of copper sulfide ores. Therefore, it is not surprising that SRBs exist within the bacterial consortiums. What is interesting is that they appear to only cause an initial pH spike before they are out competed by the other bacteria, and the pH begins to lower once more. This is an important consideration to take into account when considering the potential of SRBs as combatants against acid mine drainage.

Based on the level of copper leached from the added bornite ore, *Holcus* is the species with the rhizosphere that has the best potential for further research. It leached significantly more than the control on day 25 (**$p<0.05$; $n=3$**), and had a final pH that was not too acidic to support plant life.

Because the bioleaching done using unknown environmental consortiums, scanning electron microscopy was performed to assess bacterial structure within the consortium of *Holcus*, and to determine if biofilm formation was taking place (**Figure 13**).

Based on the SEM imagining, it does not appear that the *Holcus* rhizosphere leaches through film formation, unlike the control. Instead, various bacteria cycle and extract the copper themselves. In Figure 13-A, there is copper on the surface of the bacteria, compared to Figure 13-D, where the film resides on top of the metal(101).

This lack of biofilm formation could also attest to the slightly higher pH, as biofilms are more likely to form within an acid environment. Because the *Holcus* consortium pH does not go below 3.0, application on this consortium is less likely to result in acid mine drainage.

Because the consortium consists of unknown bacteria, qPCR was performed on the 16S region of the bacteria present to assist in establishing which phyla were present, and how the communities change in regard to bioleaching (**Figure 14**).

Both species that had significant levels of leaching, *Holcus* and *Sedum* (Arriba), show an increase in the level of alpha-Proteobacteria. The increase in *Holcus* is much greater than the increase in *Sedum*, and this is expected if alpha-Proteobacteria plays a role in bioleaching, as *Holcus* leached significantly more that *Sedum* (**$p<0.05$; $n=3$**). *Erica* had a large decrease in *Pseudomonas.* This decrease is consistent in all conditions except for *Holcus*, though no other decrease is as large as the one observed in *Erica*. It is also of interest that *Trifolium*, the species which leached the least amount of copper and had the highest final pH, had a large increase in beta-Proteobacteria, which is not observed in any other of the tested conditions.

Based on the community changes, we can conclude that bacterial communities change over time, with certain phyla out-competing others. It is proposed that alpha-Proteobacteria plays an active role in bioleaching communities, whereas an abundance of beta-Proteobacteria inhibits it. Unfortunately, gamma-Proteobacteria could not be measured due to primer issues.

Finally, the *In Plantae* application of all tested bioleaching consortium was tested using *M. sativa*, to observe the effect on shoot uptake, root uptake and removal of copper from the active mine site soil.

Rhizosphere Grafting

Sedum acted conversely to expectations, lowering the amount of copper uptaken by alfalfa rather than increasing it. *Holcus* performed opposite of expectations as well as it increased levels of copper uptaken, despite the added rhizosphere being from an excluder.

In regard to roots, *Holcus* also significantly increased the level of copper on site Arriba. *Trifolium* increased the copper significantly on soil from site Abajo, whereas *Erica* decreased the copper on this site, but increased it for site Arriba.

Observing the effects of the *Holcus* rhizosphere addition to alfalfa mark it as a species of interest to study further. The increase in copper in the shoot of alfalfa could be due to the added consortiums ability to bioleach ores, making copper more bioavailable within the soil, and therefore making plants more prone to root to shoot translocation in order to survive on contaminated sites. The increase of copper in the root is what is expected from the addition of an excluder rhizosphere.

The *Erica* rhizosphere causes different effects depending on the level of copper contamination. If the copper contamination is low (<1000mg/kg), such is the case with Touro Abajo, the rhizosphere appears to decrease contamination at the roots, but in the event of high contamination (>1000mg/kg), the rhizosphere encourages the plant to accumulate copper within its roots, and restrict it from the shoot of the plant. This action is consistent with the previous profiling of excluders.

Rhizosphere's from accumulators, such as *Sedum*, appear to be causing *M. sativa* to act as an excluder, contrary to expectations. This leads to the conclusion that the rhizosphere of accumulators and excluders are similar in species and function, with the true distinguishing microbial communities existing within the plant, either in shoots or roots. This is consistent with what was witnessed with the previous ARISAs.

This also leads to the hypothesis that the rhizospheres of accumulator plants restrict copper uptake into the plant, likely in an attempt to help regulate how much is uptaken, compared to how much is being detoxified within the shoots. Excluders work oppositely, making the copper in the ground more bioavailable, potentially through leaching, in order to increase uptake into the roots, until they are saturated.

CONCLUSIONS & OUTLOOK

Microorganismsthat exist on a contaminated site, whether plant or soil associated, possess traits necessary to assist in remediation of that site, and establish vegetation. This was seen consistently throughout the experiments performed both in Chapter 1 and Chapter 2 of this thesis.

Rhizosphere grafting is a new application of phytoremediation with much potential. A long term experiment is proposed to evaluate the effect on metal removal or stabilization within the soil. It is also proposed that the experiment be repeated with bacterial consortiums from the shoots and roots of the accumulator and excluder species. In regard to accumulators and excluders, it is seen that the key microorganisms exist in the area of metal detoxification, shoots for accumulators and roots for excluders, rather than the soil. These microorganisms are likely from phylum TM7, a phylum that has yet to be cultured in the laboratory. The rhizosphere associated with the plants is similar in regard to community structure, and does possess PGPR properties that aid in germination and biomass.

Plant species *Holcus lanatus* is proposed for further study, owing to the rhizospheric ability to bioleach without risk of acid mine drainage, and its ability to encourage copper accumulator in the roots and shoots of alfalfa.

This research shows the role of community structure in accumulators and excluders, as well as how community structure can change in regard to bioleaching, and which phyla evolve to be dominant on an active and historic mine site.

Application of this research will assist in establishing plant life on mine sites, both active and recovering, which will assist in stabilizing the soil, effectively phytoremediating the site.

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Appendix 1-A

16S sequencing results

SITE KAMLOOPS	
TM2	Sanguibacter sp. Bra9; JQ977071
	Stenotrophomonasmaltophilia; ZZ7; DQ113454
	Stenotrophomonasmaltophilia; LMG 11087; X95924
	Bacillus weihenstephanensis (T); DSM11821; AB021199
	Pseudomonas xanthomarina; 16; KF923426
TM4	Bacillus thuringiensis; ATCC10792; AF290545
	Bacillus thuringiensis; DQ286302
	Pseudomonas pseudoalcaligenes
	Pseudomonas pseudoalcaligenes; KF710; AB109888
	bacillus circulans
	clostridium bifermentans
	Stenotrophomonasmaltophilia; e-p3; AJ293464
TM5	Anoxybacillusflavithermus subsp. null; R-18839; AJ586357
	Citrobacter freundii
	Pseudomonas stutzeri; M16-9-4; HM030754
TM6	Anoxybacillusflavithermus subsp. null; R-18839; AJ586357
	Citrobacter freundii
	Pseudomonas stutzeri; M16-9-4; HM030754

Appendix 2-A

qPCR settings

Table 1: SILVA Primers used. Sequences available in paper (26)			
Phyla	SILVA primer	At (°C)	Reference
Alphaproteobacteria	aProt-0528-a-S-19 aProt-0689-a-A-21	61	(19) (26)
Actinoproteobacter	Acti-1154-a-S-19 Acti-1339-a-A-18	59	(19) (26)
Bacteroidetes	Bdet-0107-a-S-19 Bdet-0309-a-A-21	61	(19) (26)
Betaproteobacter	bProt-0972-a-S-18 bProt-1221-a-A-17	55	(19) (26)
Firmicutes	Firm-0352-a-S-18 Firm-0525-a-A-18	57	(19) (26)
Pseudomonas	Pse435F Pse686R	55	This study

Mastermix and Primer Concentration per sample (prepare 10% extra)

Each well contained 5.0 µLSYBR Green Master Mix, 0.3 µLForward primer (300 nM final)*, 0.3 µL Reverse primer (300 nM final)*, and 2.4 µL RNase-free water. 2.0 µL of the DNA was added, bringing the total reaction volume up to 10.0 µL.

Cycling Conditions

Table 2: Cycling conditions used for qPCR

Cycling Conditions	Qiagen 7500 Quanti Tect SYBR Green	Quanta PerfeCta Supermix SYBR Green
Initial Denaturing	15 min at 95°C	10 min at 95°C
40 Cycles*:		
Denaturation	1. 15s at 94°C	1. 15s at 94°C
Annealing	2. 30s at 50°C	2. 30s at 55°C
Elongation	3. 30s at 72°C	3. 30s at 72°C
Melting Curve stages	1. 15s at 95°C 2. 60s at 50°C 3. 15s at 95°C 4. 15s at 60°C	1. 15s at 95°C 2. 60s at 55°C 3. 15s at 95°C 4. 15s at 60°C

Annealing temperatures are optimized to obtain a slope -3,5 and an r^2 of > 0,999. Dissociation curves are used to check specificity, as well as to ensure no contamination in the NTC, or unspecific products being formed.

Appendix 2-B

16S Sequencing Results

SITE SPAIN	
BACTERIA CULTURED	
EricacaeaT.Arriba	Pseudomonas protegens
	pseudomonas protegens
	Brucellaceae bacterium PAOSE175; AY994315
	pseudomonas donghuensis
	pseudomonas cerasi
	Serratia marcescens; BNA; KT351729
	pseudomonas protegens
	Serratia rubidaea
	Citrobacter freundii
	Citrobacter freundii
	Pseudoalteromonashodoensis
	Lysinibacillus fusiformis
Sedum T.Arriba	Enterobacter aerogenes
	Bowmanelladokdonensis
	Planococcaceae bacterium NR93; DQ520805
	Bacillus thuringiensis;
	Serratia ureilytica
	sinobacaginghaiensis
	Bowmanelladokdonensis
	Acinetobacter calcoaceticus
	Pantoeaagglomerans; JCM1236; AB004691
Trifolium T. Abajo	Citrobacter freundii
	Lysinibacillussphaericus; B1; FJ009398
	Citrobacter freundii; 7; DQ294285
	Rhodococcusruber
	Citrobacter freundii
	clostridium bifermentans
	virgibacillusmarseillensi
	oceanisphaerapsychrotolerans
	serratia marcescens
	Comamonasterrigena; LMG 1249; AJ430343
	Clostridium bifermentans; TYR6; DSM 13560; AF320283
	Comamonas sp. V2M3; FN794215
Festuca T.Abajo	Lysinibacillus fusiformis
	Delftiaacidovorans
	Yersinia similis

	Pseudoalteromonashodoensis	
	Buttiauxellaagrestis (T); DSM 4586; AJ233400	
	pseudomonas chlororaphis	
	Citrobacter braakii	
	Halomonasloinensis	
	Bacillus thuringiensis; WS 2618; Z84586	
	raoultellaplanticola	
	Enterobacter ludwigii (T); type strain: EN-119	
	Xanthomonasretroflexus; AY841369	
	Citrobacter freundii	

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Mine site remediation enhanced through exploitation of local microbiome

Richting: **Master of Biomedical Sciences-Environmental Health Sciences**
Jaar: **2017**

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