NITROGEN DYNAMICS AS AN INDICATOR OF MINE WASTE REVEGETATION PROGRESS

by

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A Thesis Submitted to The Honors College

In Partial Fulfillment of the Bachelor's degree
With Honors in

Environmental Science

THE UNIVERSITY OF ARIZONA

MAY 2019

Approved by:

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Abstract

Hard rock mining is a crucial industry but causes massive land degradation due to mineral excavation and residual waste deposition. Reclamation is accomplished through revegetating mineral waste in an effort to return mine sites to productive ecosystems. In arid and semiarid regions, revegetation requires low-nutrient waste rock to transition into to a plant-sustaining soil/substrate. This process is mediated by the establishment of fertility islands comprised of pioneer plant species. The objective of this research is to evaluate potential biogeochemical indicators of soil health that can be used to track improvements in waste rock fertility as a metric of revegetation progress, with a focus on nitrogen dynamics. Bulk samples of unseeded waste rock, seeded waste rock, and natural desert soils; as well as targeted samples of waste rock associated with shrub and grass rhizospheres were analyzed for DNA biomass content, *amoA* gene abundance to quantify ammonia-oxidizing bacteria (AOB), total nitrogen content, and ammonium content. Strong positive correlations were observed between plant cover and DNA biomass content, *amoA* gene abundance, and total nitrogen content. Shrub rhizospheres demonstrated a greater fertility island effect than grasses. Understanding associations between pioneer species establishment and enhanced waste rock fertility is critical to managing revegetation.

1.0 Introduction

1.1 Degraded lands and mine reclamation

Hard rock mining produces significant amounts of waste material that disturbs the natural ecosystem and creates vast areas of degraded land. Mining fundamentally changes the land's topography: not only does the open pit leave a huge excavation scar on the landscape, but additional land is also disrupted and used to store mineral waste material. The area of impacted land from a single open pit mine is generally on the scale of tens to thousands of hectares (1). Prior to closure, mines must address this issue of waste to reduce their environmental impact for ecological, human health-related, and aesthetic reasons.

Revegetation is a practice that transforms degraded lands into soils that can maintain the abundant plant growth and diversity of a healthy ecosystem. This process is gradual because plants usually follow a succession of pioneer, intermediate, and late/climax species establishment as the soil slowly improves (2, 3). Revegetating waste piles is a valuable and widely accepted strategy to reintegrate mine sites into the surrounding environment (4, 5). Reestablishing native plants reduces sediment erosion from wind and water; controls dust emissions; stabilizes waste dumps; and transforms the land back into a productive ecosystem (4–6). Mines are often required to achieve a certain degree of plant cover over mineral waste dumps to comply with their environmental impact statements. However, revegetation usually requires direct human intervention because of the low fertility and challenging soil structure of the mineral waste material.

The primary types of mineral waste are tailings and waste rock. Tailings have been chemically stripped of their ore, leaving behind fine-grained mineral particles that may contain

metal contaminants and residues from reagents used to separate out the metal of interest. Tailings create a stressful environment for plants due to their high acidity or alkalinity, low levels of organic matter and nutrients, and potentially high levels of toxic metals (7). Waste rock, the second type of mineral waste, is also difficult to reclaim even though it is nontoxic. Waste rock is composed of unconsolidated mineral material that contains ore concentrations insufficient for economic extraction, and ranges from the size of clay particles to boulders. Waste rock is generated after overburden rock is blasted, removed, and discarded at the surface of a waste dump (1, 8). Unlike tailings, waste rock has not been chemically treated, so problems during revegetation arise from its physical and biological properties. Waste rock is nutrient-poor and has a low moisture-holding capacity: its coarse nature limits water retention, and low percentages of organic matter and fines reduce its cation exchange capacity and therefore ability to store nutrients (9).

Arid and semiarid regions are already constrained by limited water and nutrient content, making waste rock especially difficult to revegetate. The wide and frequent temperature fluctuations and sporadic precipitation in these areas further hinder plant establishment. These conditions cause revegetation to be patchy with exposed bare soil gaps between plants, rather than the continuous canopy cover achieved in temperate regions (10, 11).

This study focuses on Carlota Copper Mine, which is located in central Arizona and owned by KGHM International Ltd. Carlota's open pit mine operated from 2008 to 2014 and began reclamation efforts in 2009; the mine is scheduled to close in 2020. Carlota is currently in the process of revegetating their waste rock dump as required by the National Forest Service. Common methods for revegetating waste dumps include applying a soil cap or seeding the waste material directly (5, 6, 12). Soil caps promote revegetation better than seeding the waste rock

directly, since soil has a higher water-holding capacity and can supply more nutrients to the plants than waste rock; however, this technology is more expensive and environmentally destructive because the soil must be sourced from elsewhere (6, 12). Because Carlota has chosen to implement direct seeding without any amendments, the waste rock material must develop into a soil matrix with the potential to support plant growth. We hypothesize that the efficacy of revegetation efforts is associated with progressive development of the belowground substrate fertility, which can be quantified through comparisons to areas undisturbed by mining activity with naturally established vegetation. Progress in soil development can be determined by examining changes in belowground chemical and biological properties.

1.2 The soil microbiome and desert fertility islands

Mine reclamation success is often judged by what we can see above ground: plant growth and establishment, and canopy cover. Regulatory agencies monitoring mine cleanup are concerned with setting appropriate ground cover metrics that will properly reduce erosion and the detrimental visual impacts from waste piles. Thus, examination of belowground fertility is rarely considered, despite its direct effects on aboveground plant establishment (13). Without a healthy soil or substrate, waste piles are unable to maintain adequate vegetation. Because soil microorganisms play a decisive role in nutrient cycling, they are critical to soil fertility. Thus, the communities and abundance of belowground microbiota must be analyzed in order to best characterize the sustainability and success of revegetation (13, 14). Not only do soil microbes affect plant growth by directly converting many soil nutrients into plant-available forms, they also serve as good indicators of revegetation continuity and self-perpetuation because they are very responsive to ecosystem disturbance (13).

When revegetating degraded arid and semiarid lands, it is also important to understand plant development mechanisms specific to the desert, and how they differ from that of other climates. Because arid and semiarid areas receive little rainfall which is scattered and highly variable by location, microbial and plant activity is temporally sporadic and spatially dependent. The soil microbiome and plants mutually establish fertility islands, creating a patch-and-gap vegetation pattern (11, 15, 16). Initial plant establishment occurs in areas with nutrient concentrations high enough to nurture growth activities. These pioneer plants become fertility islands as they contribute leaf litter and organic matter to the soil, providing microbes with organic carbon that fuels metabolism (11). Elevated microbial activity increases nutrient cycling that benefits plants. A positive feedback loop occurs between the plant and microbe interactions, causing a larger distinction between the active plant understory and the bare soil gaps. In contrast to open spaces, fertility islands allow for higher soil moisture content, microbial biomass content, microbial functional diversity, and soil organic matter; together, these factors interact to improve soil structure and water infiltration (11, 15).

Shrubs in particular are often associated with fertility islands. When well-established, shrubs accumulate a thick layer of organic matter beneath their canopy not found in bare soil gaps, which supplies soil microbes with extremely valuable labile carbon (16). Although shrubs also provide physical protection from wind and sun beneath their canopy, Berg and Steinberger found that microbial activity was affected only by the additional organic matter inputs from the shrubs, suggesting that organic matter accumulation is more influential than constructing a physical barrier (11, 15).

The relationship between soil microbes and plants in establishing fertility islands is a central aspect of revegetation in arid and semiarid climates. Once a fertility island is formed, the

concentration of plant-essential nutrients increases and renders the soil more hospitable for a greater variety of plants. Identifying successful native pioneer species that can produce fertility island effects is significant, because pioneer species control the growth rate of early revegetation stages and are specific to climate and soil conditions (2, 3). These pioneer plants are used to prime the soil for additional native plants that do not grow under low-nutrient conditions but contribute to the benefits of ecosystem biodiversity. A robust array of native plant species is usually the goal of revegetation because they increase the ecosystem's sustainability and resilience to future environmental disturbances such as drought or fire. However, more research is needed to quantify the developmental effect of pioneer plant fertility islands on enhancing waste rock fertility and the capacity for colonization by an increased variety of native plant species.

At our study site, Carlota Copper Mine hydroseeded a portion of the west face of their waste rock dump in 2012 (Figure 1). The main plant species that dominate the seeded waste rock slope are two types of shrubs: fourwing saltbush (*Atriplex canescens*) and desertbroom (*Baccharis sarothroides*); and two types of grasses: sideoats grama (*Bouteloua curtipendula*) and purple threeawn (*Aristida purpurea*) (Figure 2). After seven years of growth, the area exhibits inconsistent vegetation in a patch-and-gap formation (Figure 3). One explanation for this incomplete canopy cover is that the waste rock currently has the capacity to only support fertility islands generated from pioneer species like shrubs and grasses, and is still too nutrient-poor and underdeveloped for late/climax species to develop. We contend that the belowground microbiome contributes significantly to availability of plant nutrients. Therefore, analysis of the substrate microbiome can predict the success of fertility islands, and by extension quantify revegetation progress.

1.3 Nitrogen limitations

When characterizing soil fertility improvements, one of the most important nutrient cycles to study is that of nitrogen (N). Nitrogen is an essential plant nutrient crucial for plant growth and consequently, revegetation success. Carbon, hydrogen, and oxygen notwithstanding, plants require more nitrogen than any other nutrient (17). Plants use nitrogen to build amino acids and proteins, nucleic acids, and chlorophyll. Nitrogen-deficient plants exhibit stunted growth due to reduced photosynthesis rates and chlorophyll concentrations (18). Nitrogen is the most limiting nutrient in most terrestrial systems and controls net primary production; in semiarid and arid deserts, nitrogen limitations are second only to water scarcity (19). In degraded systems such as mine waste rock dumps, examining nitrogen dynamics can be especially informative for understanding stresses restricting plant development.

Soil microbes are central to providing plants with much-needed nitrogen. The majority of nitrogen enters the soil through nitrogen fixation, a microbial process wherein atmospheric N_2 gas is converted to ammonium (NH_4^+) that subsequently exists in soil solution and sorbed to colloid surfaces. In addition to nitrogen fixation, the process of ammonification releases NH_4^+ from organic compounds (20). Although plants can take up NH_4^+ directly through their roots, microbes usually outcompete plants over a short-term timescale and utilize the substrate before plants can absorb it, suggesting that plants can access NH_4^+ only after microbes are no longer nitrogen-limited (21–23).

Additionally, most plants can take up nitrogen in both the form of NH_4^+ and nitrate (NO_3^-). Unlike NH_4^+, plants have a greater competitive advantage for NO_3^- uptake over microbes (23). Plants receive most of their nitrogen through mass flow, which requires NH_4^+

and NO_3^- to be dissolved in the soil solution when taken up by plant roots. NO_3^- has greater mobility than NH_4^+ because it is an anion and less likely sorb to colloid surfaces. NO_3^- is generated in natural ecosystems by separate microbial taxa that perform nitrification, the conversion of NH_4^+ to NO_3^-, in two steps: ammonia oxidation and nitrite (NO_2^-) oxidation. Ammonia-oxidizing bacteria and archaea (AOB and AOA) carry out the first reaction, which occurs much slower than nitrite oxidation and is therefore the rate limiting step. Plants greatly depend on microbial nitrification to increase accessible nitrogen by producing NO_3^- that they can then assimilate (23).

Recent research shows plants with and without mycorrhizal associations can also take up organic nitrogen from amino acids and other small monomers (24, 25), implying that analyzing inorganic nitrogen pools alone may not be sufficient when quantifying the plant-available nitrogen present in a system. Schimel et al. presents a new paradigm wherein the depolymerization of soil organic matter (SOM) regulates nitrogen cycling, rather than mineralization processes: NH_4^+ is no longer the limiting substrate if both plants and microbes can transform organic monomers as well (26). However, to confirm this paradigm, research must conclusively demonstrate that plants can effectively compete against microbes for organic nitrogen, and that it contributes significantly to the plant's overall nitrogen uptake. This is a challenging task because it is difficult to accurately simulate and measure plant uptake of amino acids as well as competition between plants and microbes (22, 27). Even amino acids labeled with ^{15}N and ^{13}C can be difficult to track due to rapid transformations within the plant and soil, and results are further biased based on the amino acid chosen for measurement (27). Beyond plant-microbe interactions, this new paradigm must also integrate the effects of microsites and spatiotemporal variations on nitrogen availability, adding further complexity to the model (22,

26). Overall, it is clear that more research is needed in order to understand the ecological significance of plant uptake of organic nitrogen (24, 26, 27). Due to the current gaps in our understanding of plant organic nitrogen utilization, analyzing inorganic nitrogen transformations is still central to characterizing the nitrogen dynamics of a system.

1.4 Ammonia-oxidizing bacteria (AOB) versus ammonia-oxidizing archaea (AOA)

Ammonia oxidation, the first step of nitrification, is completed by both autotrophic archaea and bacteria containing the ammonia monooxygenase enzyme (AMO). The active site (A) of AMO is encoded for by the *amoA* gene (28), and thus real-time/quantitative PCR (qPCR) can be used to quantify gene abundance as an indicator of genetic capacity for ammonia oxidation in a given substrate. Gene sequences that encode for the AmoA protein are different for bacteria and archaea: AOB abundance is quantified using *amoA* primers, and AOA abundance is quantified using *archamoA* primers (29). As discussed, nitrification contributes significantly to the pool of plant-available nitrogen in soil, and consequently is instrumental in promoting plant growth. Hence, studying ammonia oxidation, the rate limiting step of the reaction, is of value when determining plant establishment and revegetation progress.

Although AOB and AOA provide the same nutrient cycling function, they do so under different soil conditions. For this study, we contend that measuring AOB abundance is more appropriate than measuring AOA abundance. AOA can access NH_4^+ in the soil at lower concentrations than AOB; therefore, AOB abundance better reflects NH_4^+ availability at fertility levels more relevant to plant growth. Studies have shown that AOB abundance increased when nitrogen fertilizer or urine was applied, whereas AOA abundance did not (30–32), suggesting that AOA are most competitive under oligotrophic conditions and that AOB responses to

nitrogen inputs are more similar to that of plants. Through revegetation and the formation of fertility islands, we anticipate seeing an increase of soil nitrogen in the seeded mine waste rock. Thus, we aim to identify a biological indicator of nitrogen cycling that could correlate with a chemical indicator and demonstrate how plant growth correlates with increases in nitrogen levels. In natural (non-amended) soils, AOB abundance responds positively to increased precipitation in arid and semiarid regions, whereas AOA abundance either responds negatively or is not affected (33, 34). These nutrient and moisture effects suggest that using AOB abundance as an indicator of improving soil fertility is preferable to using AOA abundance, because AOA survive better in stressed conditions. We want the waste rock to develop into soil that can support sustainable plant growth, so measuring AOB is more suitable since it is associated with soil fertility and AOA is not.

Additionally, AOB abundance is a better indicator of fertility island effects. One study found that under arid conditions AOB abundance was significantly higher under nitrogen-fixing shrubs than in open areas without vegetation, whereas AOA abundance was not microsite dependent and showed no significant differences between samples taken near vegetation or from open areas (29). Similarly, another study found that AOB abundance was significantly higher in areas vegetated with grass than areas of bare ground, and that nutrient factors significantly influenced AOB abundance more than aridity or abiotic factors (35). As described earlier, fertility islands have the potential to greatly assist with establishing soil conditions favorable for enhanced plant community development. The significance of vegetation and increased nutrient amounts on AOB abundance implies it is a promising indicator for measuring how the belowground microbiome develops during revegetation.

1.5 Biogeochemical indicators

Because soil is a dynamic and highly heterogeneous system, it is both impossible and impractical to measure all parameters related to soil quality for any given sample. Therefore, soil quality indicators are required to measure soil health and fertility improvements. Interrelationships between soil characteristics, such as statistically significant correlations, are used to develop these indicators. Soil quality indicators reduce the number of parameters that need to be quantified directly, and better inform us of the complex chemical and biological interactions taking place below ground.

The objective of this research is to evaluate the efficacy of four different biogeochemical indicators for tracking the development of waste rock from an incipient soil into a plant-sustaining matrix by analyzing correlations with plant establishment. These indicators can be applied to measuring the overall progress of mine waste revegetation. The four indicators are DNA biomass content, total nitrogen content, *amoA* gene abundance, and ammonium content.

DNA biomass content is a measurement of the total bacteria, archaea, fungi, and plant residues present, which gives an initial estimate of the microbial inputs into the soil. We are using DNA biomass as a proxy for organic matter, because microbial biomass represents a significant portion of the organic matter in unvegetated arid soils. DNA biomass content will allow us to compare the relative amounts of organic matter in our samples. Total nitrogen content (TN) is another way to characterize the overall belowground soil fertility, because it indicates the status of a vital nutrient in the soil. Additionally, when measuring other forms of nitrogen present in the soil, TN demonstrates the relative size of that nitrogen pool. *amoA* gene abundance is used to quantify the amount of AOB present in the soil. AOB are critical components of the nitrogen cycle because they determine nitrate availability through ammonia

oxidation. Lastly, ammonium content is measured because it is the substrate for AOB and the initial form of nitrogen in the nitrification process.

Examining the correlations between these indicators allows us to establish patterns of the waste rock's development and potential to support sustainable plant growth, thereby assisting Carlota Copper Mine in determining the effectiveness of their revegetation efforts.

2.0 Methods

2.1 Site description

This study was conducted at Carlota Copper Mine, which is located in Miami, Arizona, USA (North 33° 20’ 33”, West 110° 59’ 58”). The region is classified as a semiarid desert and receives an average of 14 inches of precipitation annually, but this varies from year to year. Carlota’s waste rock material is composed of friable dacite. The waste dump covers approximately 230 acres of land, and because Carlota has ceased open pit mining operations, no more waste rock will be added to the dump. The western side of the dump exists on National Forest Service land, so it must be revegetated to comply with Carlota’s environmental impact statement. Carlota intends to build a solar farm at the top of the dump, so revegetation efforts are currently focused on the sides/slopes of the dump.

The southwest side of waste rock dump is divided into the N Slope and the D Slope. The N Slope consists of unseeded waste rock and is marked by the red pins in Figure 1. The N Slope has little vegetation (plant cover is less than 10%), which has not increased in the last five years since 2014 (Figure 4 and 5). The D Slope, marked by the purple pins in Figure 1, is located below the N Slope (Figure 3), and was hydroseeded in 2012. The D Slope is characterized by inconsistent vegetation, which is more abundant towards the bottom of the slope (the area that is further west) and has been increasing in the last five years, especially in perennial grasses and shrubs (Figure 4). A comprehensive plant cover analysis completed by Cedar Creek Associates in 2017 revealed that there are three types of vegetated sites along the D Slope: shrub-dominated, grass-dominated, and co-dominated with both shrubs and grasses. The major grass species are sideoats grama (*Bouteloua curtipendula*) and purple threeawn (*Aristida purpurea*); and the major

shrub species are fourwing saltbush (*Atriplex canescens*) and desertbroom (*Baccharis sarothroides*) (Figure 2).

The surrounding area undisturbed by mining activity is a natural desert ecosystem, referred to as OS for offsite (Figure 6). Major plant species include plains lovegrass (*Eragrostis intermedia*), broom snakeweed (*Gutierrezia sarothrae*), and velvet mesquite (*Prosopis velutina*). The soils from this area have a higher percentage of plant cover than the waste rock from the N and D Slopes (Figure 4, Table 1). Most notably, although the D Slope now has perennial grass cover similar to that of OS, it still has considerably fewer shrubs (Figure 4).

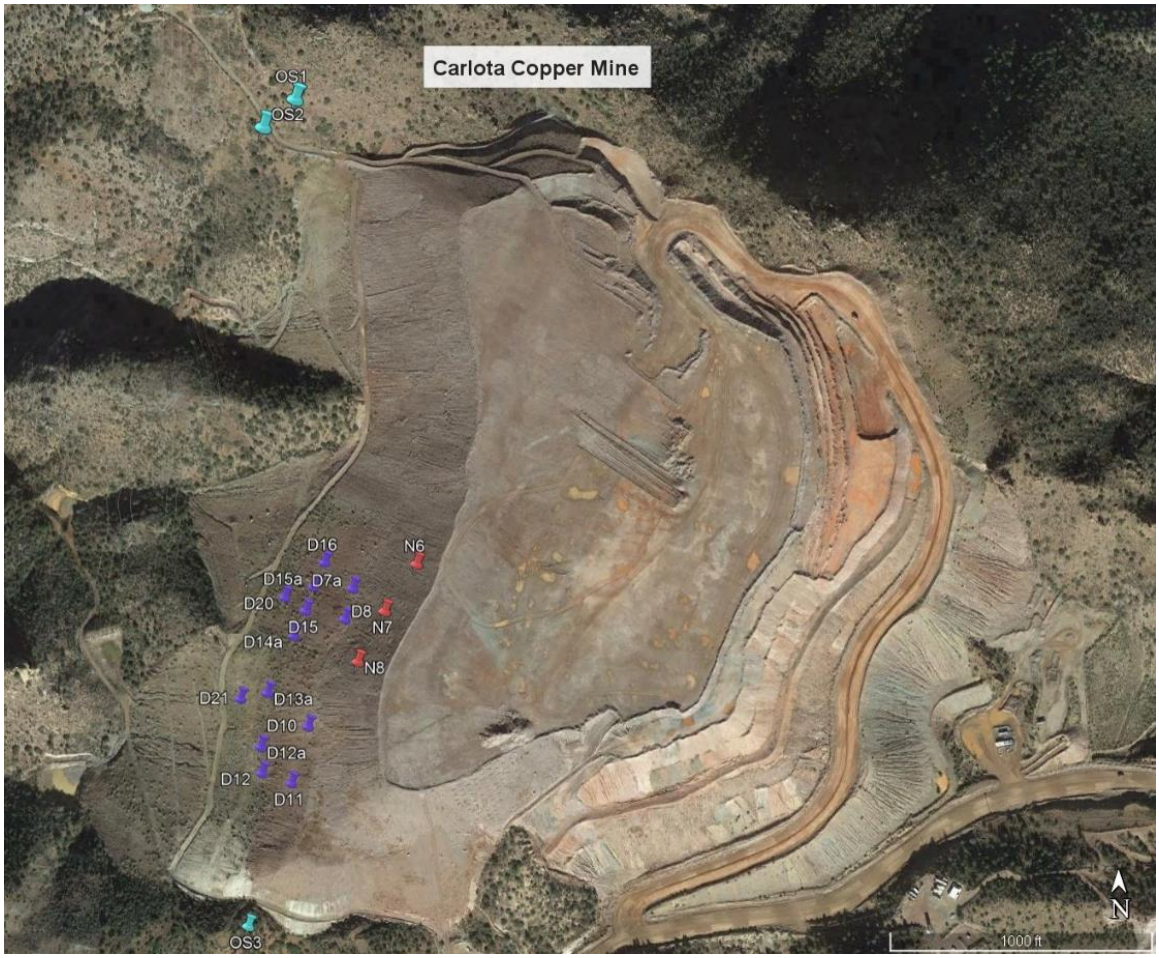

Figure 1. Map of sampling sites at Carlota's waste rock dump. OS undisturbed by mining is marked by blue pins (n = 3); seeded D Slope and targeted rhizosphere samples is marked by purple pins (n = 6 and n = 16, respectively); unseeded N Slope is marked by red pins (n = 3).

Figure 2. D Slope vegetation. The D Slope is dominated by shrubs and grasses. Left picture: desertbroom (*Baccharis sarothroides*) in foreground to the left; fourwing saltbush (*Atriplex canescens*) in background to the right. Middle picture: purple threeawn (*Aristida purpurea*). Right picture: sideoats grama (*Bouteloua curtipendula*)

Figure 3. N and D Slope. The D Slope, hydroseeded in 2012, is located beneath the N Slope, as indicated by the white dashed line. The D Slope exhibits inconsistent vegetation in a patch-and-gap structure, whereas the N Slope is mostly bare.

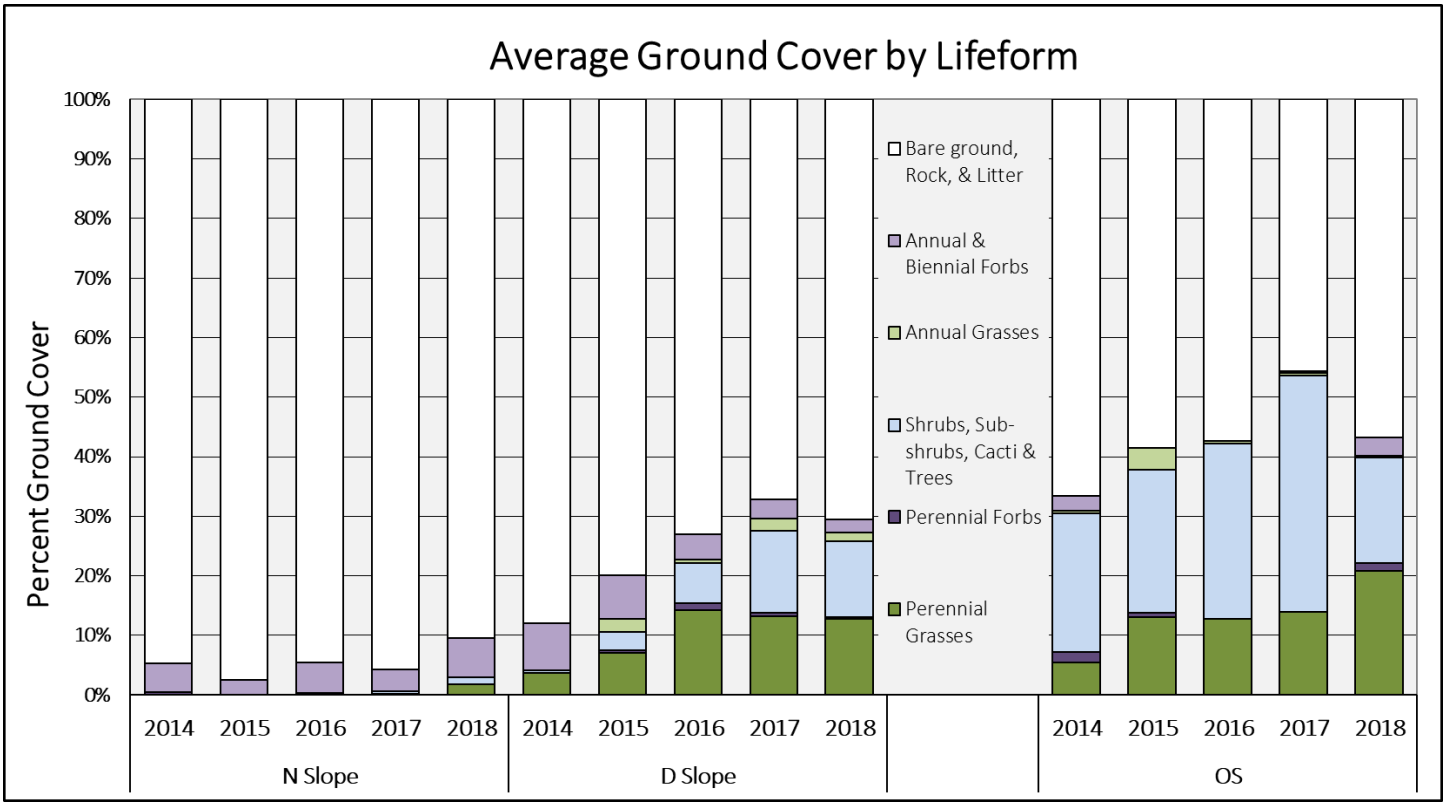

Figure 4: Average ground cover by lifeform. Plant cover analysis conducted by Cedar Creek Associates annually from 2014 to 2018. Plant cover averages include additional sampling sites that were not analyzed in this study but are located along the same transects and represent the slopes. N Slope and OS vegetation have remained stable; D Slope perennial grasses have increased and reached OS levels; D Slope shrubs have increased but not reached OS levels.

Table 1: Average plant cover of sampling sites specific to this study from 2018.

	OS	N Slope	D Slope	Shrub	Grass
Plant cover (%)	50 ± 25	7 ± 3	25 ± 12	42 ± 16	29 ± 9

Values are averages from 2018 that include only the sample sites analyzed in this study.

D Slope samples refer to bulk waste rock, whereas Shrub and Grass samples are rhizosphere-influenced and taken from the root zone of plants on the D Slope.

Plant cover analysis conducted by Cedar Creek Associates in 2018. Area analyzed for plant cover was 9 ft^2 and contained the sampling site at the center. Shrub and Grass samples were taken from plants within the 9 ft^2 area analyzed.

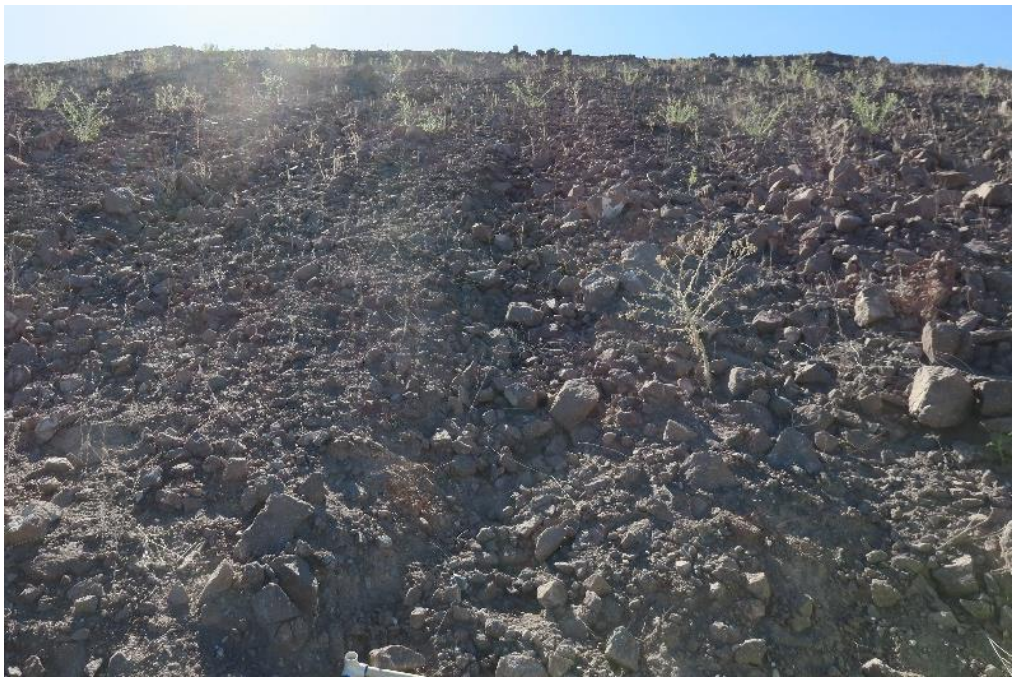

Figure 5. N Slope vegetation. The N Slope consists of unseeded waste rock and is mostly bare. The grasses present are from seeds that have blown in.

Figure 6. OS vegetation. OS (offsite) is undisturbed by mining activity and exhibits vegetation of a natural desert ecosystem.

2.2 Experimental design

Samples of unseeded waste rock (N Slope) and seeded waste rock (D Slope) were collected at sites along three transects on the west slope of Carlota's waste rock dump (Figure 1). Soil samples undisturbed by mining activity (OS) were taken near the waste rock dump (Figure 1) and used as a positive control. A 1-m^2 frame was placed at each sample site on the N Slope, D Slope, and OS; grab soil samples were collected to a depth of 6 inches at two opposite corners of the frame. The soil from the two holes within the frame was composited, sieved in the field to 2 mm, and stored for chemical analysis. The chemical analysis samples were dried at 45°C in the laboratory prior to analysis. Following chemical sample collection, samples for microbial analysis were collected from the same two holes within the frame along the 6-inch depth of the soil profile using sterile technique. Microbial samples were transported at 4°C and then stored at -80°C.

In addition to the bulk waste rock samples collected along the three transects, targeted samples were taken at the root zone of shrubs and grasses to examine the rhizosphere effect of these plants on the waste rock microbiome. These samples were taken from select plants located along the two D Slope transects, but differ from the general D Slope samples in that the samples were taken directly from the root zone of the plants. In the case of large shrubs with a thick canopy, targeted samples were taken as close to the plant stem as possible. The general D Slope sample sites incorporated both bare and vegetated areas, capturing the patch-and-gap layout of the slope. Targeted rhizosphere samples were also taken from two points at a 6-inch depth and composited, similar to the general samples. Targeted rhizosphere samples used for chemical analysis were sieved at 2 mm in the field, stored at 4°C, and then dried at 45°C. Targeted

rhizosphere samples for microbial analysis were collected using sterile technique along the 6-inch depth, transported at 4°C, and stored at -80°C.

Sampling for both the general and targeted rhizosphere samples occurred in May 2018; only samples collected in 2018 were analyzed for this study.

2.3 DNA extraction

DNA was extracted from samples for microbial analysis using the FastDNA Spin Kit for Soils (MP Biomedicals, Solon, OH) with modifications to increase DNA extraction yield (36). Deviations from the manufacturer's protocol are described below.

Prior to extraction, all materials and reagents except the MT buffer and binding matrix were sterilized via exposure to UV light for 30 minutes using a UVC-508 UV Crosslinker (Ultra Lum, Claremont, CA). Lysis of the 0.5 g soil sample was completed using a vortex for 15 minutes, rather than using a FastPrep Instrument. After adding the DNA extract to the binding matrix, the matrix was rinsed repeatedly with 500 µl of 6 M guanidine thiocyanate (Sigma-Aldrich, St. Louis, MO) to remove humic acids until the supernatant was clear. Once clean, the binding matrix was resuspended in SEWS-M, and the solution was transferred to the filter tubes. The amount of rinses with the SEWS-M wash solution was increased from one to two rinses of 500 µl. The filter tubes were dried at 37°C for 10 minutes to evaporate any remaining SEWS-M wash solution. Nuclease-free GeneMate water treated with DPEC (VWR, Radnor, PA) and prepared with two 30-minute sessions of UV exposure was used for eluting the DNA, rather than the DES water from the FastDNA Spin Kit. This nuclease-free water was preheated to 60°C before adding 50 µl to the spin filter with binding matrix beads and vortexing. The tubes were allowed to incubate for 10 minutes at 60°C, and then centrifuged for DNA elution. A second

aliquot of 50 µl of preheated water was added with another 10-minute incubation period at 60°C, and then centrifuged for DNA elution.

DNA extraction samples were quantified using a Qubit 2.0 Fluorometer (ThermoFisher Scientific, Waltham, MA) following the manufacturer's instructions, and stored at -20°C. A reagent blank was run with all DNA extractions, and only DNA extractions performed with a reagent blank that registered below the fluorometer DNA detection limit of 0.015 ng μl^{-1} were used in further analysis.

DNA biomass values are reported in µg DNA biomass g^{-1} dry soil or as µg DNA biomass g^{-1} dry waste rock substrate. Soil moisture was accounted for by measuring gravimetric moisture content from 10 g of sample dried at 105°C for over 24 hours. Moisture content measurements occurred immediately after field sampling.

2.4 Quantification of *amoA* gene abundance (AOB)

AOB abundance was quantified using qPCR to amplify the 491-base pair *amoA* functional gene. qPCR was carried out using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The 10 µl reaction consisted of 5 µl of SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA), 3.1 µl of nuclease-free water, 0.9 µl of *amoA*-1F/*amoA*-2R primers (28), and 1 µl of DNA template. The following protocol was used: initial denaturation at 90°C for 3 min; followed by 50 cycles of 95°C for 20 s, 62°C for 30 s, 60°C for 30 s; and then a melt curve generated from 65°C to 95°C at a 0.5°C increment for 5 s. This protocol was adapted from Nelson et al. (37) with increased denaturation and annealing times to improve efficiency. The annealing temperature was increased from 56°C to 62°C to reduce amplification of the nonspecific products evident from multiple peaks in the melt curve and confirmed after running

gel electrophoresis. After running a qPCR annealing gradient reaction from 55°C to 67° C, 62°C showed the greatest reduction of the nonspecific product band about 200 bp long while maintaining the brightness of the target *amoA* gene band at 491 bp (Figure 7). All samples were run in triplicate.

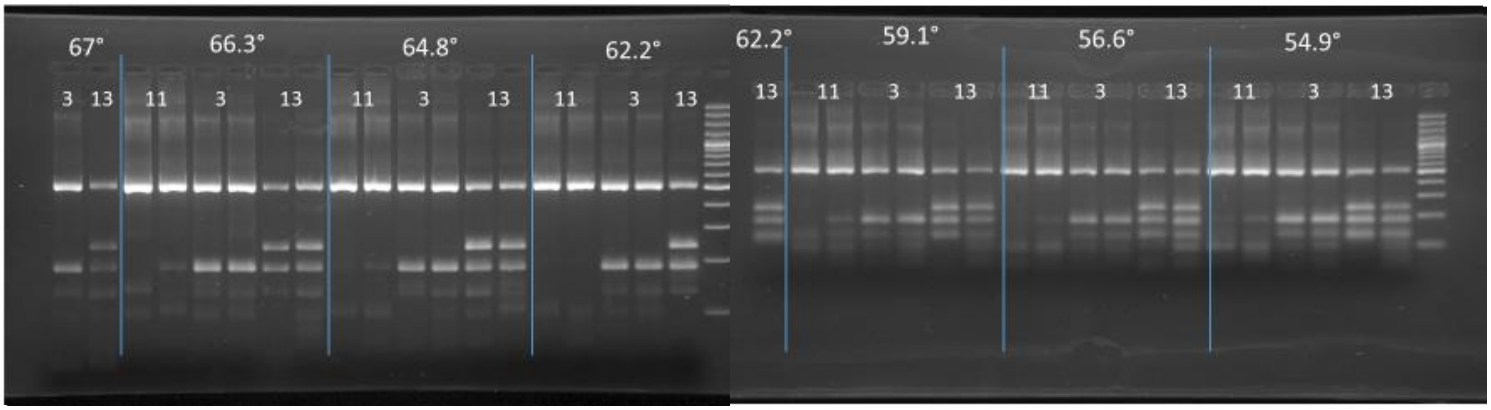

Figure 7. Gel electrophoresis for *amoA* qPCR annealing temperature gradient. The numbers 11, 3, and 13 indicate samples tested at the different temperatures, which are labeled at the top of the gels. 62.2°C was the temperature at which the greatest amount of nonspecific product bands was reduced (especially at 100-200 bp) while maintaining the brightness of the 491-bp *amoA* product band. A GeneRuler 100 bp Plus DNA ladder ranging from 3000 to 100 bp was run in the rightmost lanes, where the bright middle band is 500 bp (ThermoFisher Scientific, Waltham, MA).

Gels were made as 1.7% SeaKem agarose (Lonza Group, Basel, Switzerland) and TBE buffer. Gels were stained using GelRed (Biotium, Fremont, CA), and visualized using an AlphaImager camera and imaging software (Alpha Innotech, San Leandro, CA).

The *amoA* gene was quantified by qPCR using a standard curve generated from a 5x serial dilution with six standards run in triplicate, ranging from 10^6 to 320 *amoA* gene copies. The *amoA* standards were created for the curve by cloning the *amoA* gene into the Invitrogen pCR2.1-TOPO plasmid (37). Standard curve efficiencies ranged from 84% to 92%, with R^2 values greater than 0.99. The samples were quantified in a total of four separate qPCR runs, so C(t) values were normalized with a single standard curve using the manufacturer's interplate

calibration protocol and gene study program from the Bio-Rad CFX Manager software, v1.6 (Bio-Rad Laboratories, Hercules, CA).

Because the nonspecific product band at 200 bp could not be eliminated completely even after changing the annealing temperature, the detection limit had to be adjusted based on agarose gel results for each sample. Nonspecific products appeared during amplification of samples with extremely low DNA template concentrations. The nonspecific products added to the sample's total fluorescence, artificially enhancing the total *amoA* gene abundance, so samples with bright nonspecific product bands were considered below detection limit (BDL). Figure 8 shows examples of samples both above and below detection limit. Sample 5D7a – RS (leftmost) successfully amplified, with a bright 491-bp product band and only a very faint nonspecific product band at 200 bp; hence, it was above detection level. Sample 5D12 – RPT (middle) did not produce a specific *amoA* gene product; rather, the reaction amplified many faint bands of different lengths, so it was considered BDL. Samples 5D10 – RSG (right side) and 5D12a – RSG (rightmost) amplified the *amoA* gene well (the 491-bp band is evident), but also had prominent nonspecific product bands at 200 bp. Relative to the target 491-bp band, the 200-bp band is of equal brightness for both samples, which made them BDL. In contrast, sample 5D11 – RSG (middle) had a nonspecific product band at 200 bp equally as bright as that of 5D10 – RSG, but 5D11 – RSG's 491-bp product band is much brighter than 5D10 – RSG's. As a result, 5D11 – RSG's nonspecific product band contributed far less inaccuracy relative to 5D10 – RSG, so 5D11 – RSG was thus deemed above detection limit.

No other study to our knowledge has reported this problem. The nonspecific product bands are likely caused in part by the overall low DNA concentrations: the primers have so little target DNA to interact with that they are more likely to anneal in the wrong locations. Samples

with low DNA biomass content were more likely to amplify nonspecific products. Nonspecific product bands did not show up in any of the qPCR standards after running gels.

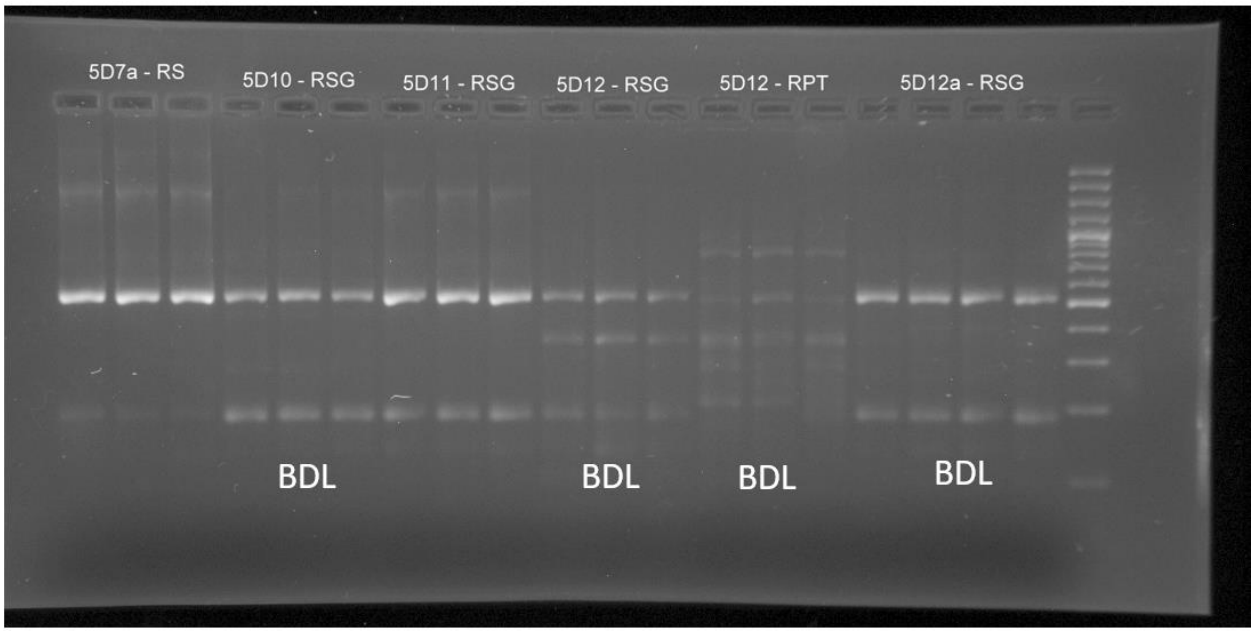

Figure 8: Gel electrophoresis for *amoA* qPCR showing samples categorized as above and below detection limit. The samples were run in triplicate, with the sample name above the wells. Samples with "BDL" marked below the middle lane were below detection limit. Sample 5D10 – RSG and 5D12a – RSG are examples of samples that amplifed the *amoA* gene well, but have too bright of a nonspecific product band at 200 bp relative to the 491-bp product band to be considered above detection limit.

2.5 Chemical analyses

Total nitrogen content (TN) was measured by dry combustion of solid samples concurrent with the Dumas method (38) using an Elemental Combustion System 4010 CHNSO Analyzer in accordance with the manufacturer's protocol (Costech Analytical Technologies, Valencia, CA). Prior to combustion, samples for TN analysis were milled using a rapid method of fine grinding soils for nitrogen analysis (39). The standard curve for TN analysis was generated using LECO soil standards (LECO Corporation, St. Joseph, MI) at seven concentrations. Standard curves

were used only when the error was less than ±15% for any one standard (standards with higher error were removed), and R^2 values were greater than 0.999. Each run had three LECO soil quality control samples to ensure accuracy of the instrument throughout the run. Samples were run in triplicate with a coefficient of variation (CV) less than 10%.

Ammonium (NH_4^+) content was measured using colormetric detection with ammonia salicylate and ammonia cyanurate HACH reagents (Hach Company, Loveland, CO) according to a protocol developed by Sinsabaugh Lab (personal communication). Absorbance readings were done with an Epoch 2 Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). Samples were extracted in duplicate using 2 M KCl, and each extract was analyzed in triplicate; triplicates with a CV less than 15% were used in further analysis. A third KCl extraction for a sample was used when the CV between the triplicates of both extracts exceeded 15%. The standard curve consisted of seven standards ranging from 0 to 0.15 mM NH_4^+, also run in triplicate. Standard curves were used only when the error was less than ±15% for any one standard (standards with higher error were removed), and R^2 values were 0.99 or greater.

2.6 Statistical analysis

Average means of biogeochemical indicators for each sampling location and plant type were compared using a one-way ANOVA and post-hoc Tukey HSD. Significant differences reported between sampling locations and plant type for means comparisons all have $P < 0.01$ significance levels. Pearson's correlations were determined between the four indicators, and reported at significance levels of $P < 0.01$, $P < 0.05$, and $P < 0.1$. All statistics were calculated using JMP 13 (SAS Institute, Cary, NC).

3.0 Results

The four biogeochemical indicators, DNA biomass content, *amoA* gene abundance (AOB), total nitrogen content (TN), and ammonium (NH_4^+) content were analyzed for all samples from each sample location. Samples collected from the OS, N Slope, and D Slope that did not specifically target plant rhizospheres are referred to as bulk samples. These bulk samples represent composites of open space and rhizosphere-influenced soils; however, no plants were present at any N Slope sampling sites. Significant differences were observed between sample locations for all biogeochemical indicators evaluated.

3.1 DNA biomass content

In comparing the DNA biomass content between the bulk sampling locations (OS, N and D Slope), shrub rhizosphere-influenced, and grass rhizosphere-influenced, we found that the natural desert soils (OS) and shrub rhizosphere-influenced samples had significantly higher biomass than unseeded waste rock (N Slope), bulk seeded waste rock (D Slope), and grass rhizosphere-influenced waste rock (Figure 9). Natural desert soils (OS) averaged 6680 ± 237 ng DNA g^{-1} dry soil. Shrub rhizosphere-influenced waste rock averaged 4160 ± 2780 ng DNA g^{-1} dry waste rock. Shrub rhizosphere-influenced waste rock had higher sample variability than OS, as indicated by their 67% coefficient of variation (CV) and 4% CV, respectively. Bulk seeded waste rock (D Slope) and grass rhizosphere-influenced waste rock had higher DNA biomass contents than unseeded waste rock, but the difference was not significant (Figure 9).

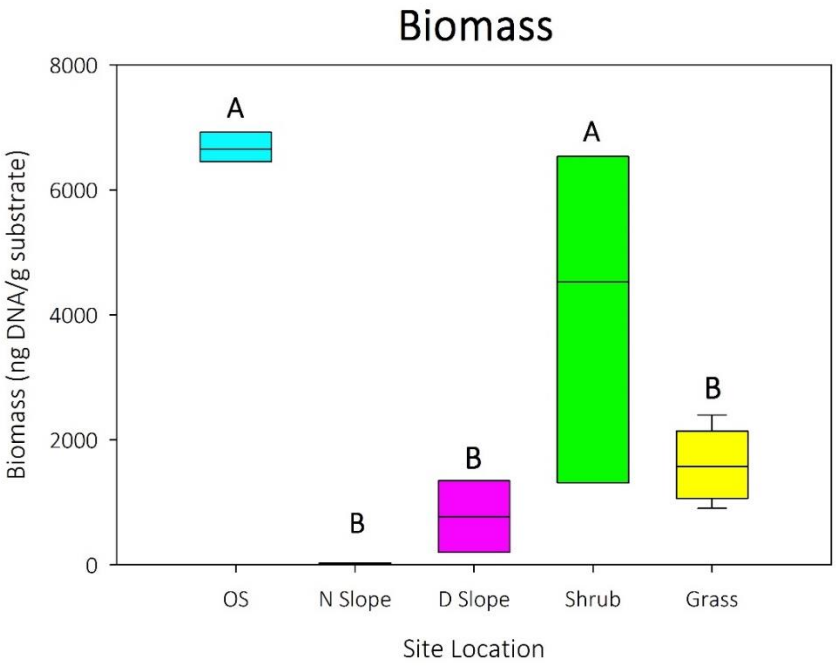

Figure 9: DNA biomass content comparison between sample locations. Natural desert soils (OS) and shrub rhizosphere-influenced waste rock had significantly higher DNA biomass content than unseeded waste rock (N Slope), bulk seeded waste rock (D Slope), and grass rhizosphere-influenced waste rock. Mean values for boxplots labeled with different letters are significantly different (ANOVA, $P < 0.01$; Tukey HSD).

3.2 *amoA* gene abundance (AOB)

AOB abundance, as measured by *amoA* gene abundance, exhibited trends similar to that of DNA biomass content. Shrub rhizosphere-influenced waste rock has significantly higher AOB abundance than unseeded waste rock (N Slope), bulk seeded waste rock (D Slope), and grass rhizosphere-influenced waste rock (Figure 10). Natural desert soils (OS) also have higher AOB abundance than unseeded waste rock (N Slope) and grass rhizosphere-influenced waste rock (Figure 10). *amoA* gene abundance for shrub rhizosphere-influenced waste rock samples ranged from 2.65×10^5 to 2.77×10^7 copies g^{-1} dry waste rock, while grass rhizosphere-influenced waste rock samples ranged from 0 (below detection) to 6.70×10^5 copies g^{-1} dry waste rock. All N Slope samples were below detection.

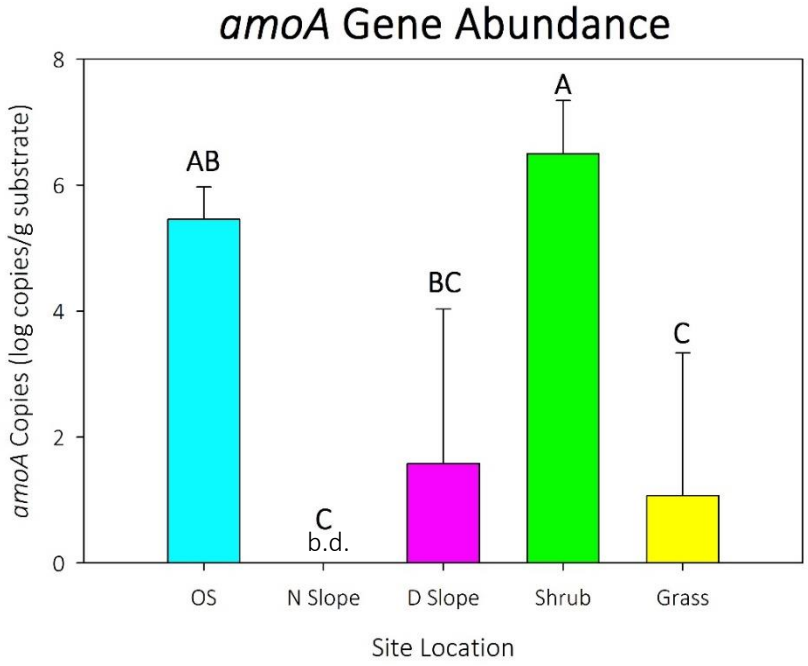

Figure 10: *amoA* gene abundance (AOB) comparison between sample locations. Shrub rhizosphere-influenced waste rock has significantly higher AOB abundance than unseeded waste rock (N Slope), bulk seeded waste rock (D Slope), and grass rhizosphere-influenced waste rock. Mean values for bars labeled with different letters are significantly different (ANOVA, $P < 0.01$; Tukey HSD). Error bars represent one standard deviation.

3.3 Total nitrogen content

Total nitrogen content (TN) also varied significantly by sample location. Natural desert soils (OS) had an average TN of 1.164 mg N g^{-1} dry soil, which was significantly higher than that of shrub rhizosphere-influenced waste rock at 0.586 mg N g^{-1} dry waste rock (Figure 11). Both natural desert soils (OS) and shrub rhizosphere-influenced waste rock had significantly higher TN than unseeded waste rock (N Slope), bulk seeded waste rock (D Slope), and grass rhizosphere-influenced waste rock. Thus, shrub growth has a significant positive influence on nitrogen accumulation in mine waste rock. Bulk seeded waste rock (D Slope) and grass

rhizosphere-influenced waste rock had higher TN levels than unseeded waste rock (N Slope), but the difference was not significant.

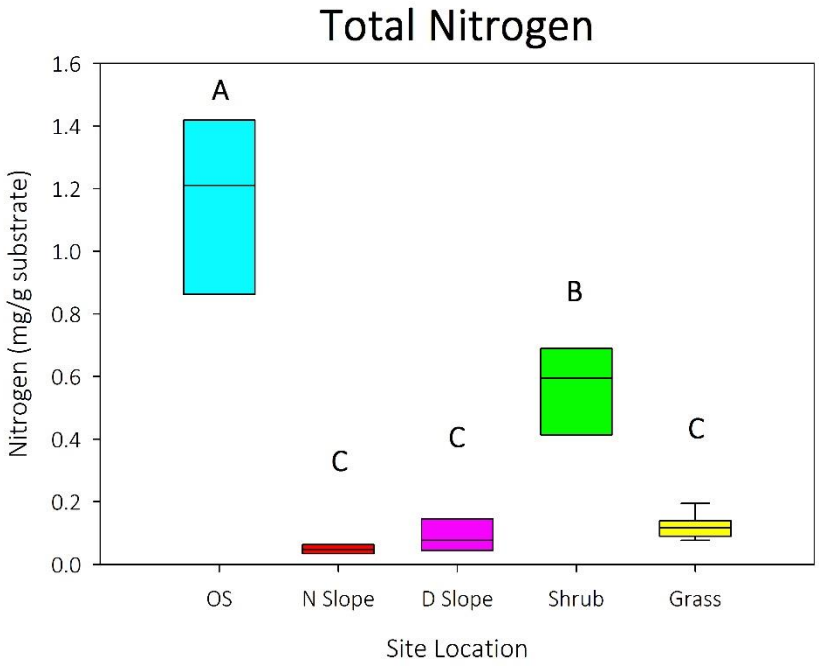

Figure 11: Total nitrogen content (TN) comparison between sample locations. Natural desert soils (OS) had significantly higher TN than shrub rhizosphere-influenced waste rock. Both natural desert soils (OS) and shrub rhizosphere-influenced waste rock had significantly higher TN than unseeded waste rock (N Slope), bulk seeded waste rock (D Slope), and grass rhizosphere-influenced waste rock. Mean values for boxplots labeled with different letters are significantly different (ANOVA, $P < 0.01$; Tukey HSD).

3.4 Ammonium content

In contrast, ammonium (NH_4^+) content showed trends opposite to the other indicators. The unseeded waste rock (N Slope) had the greatest amount of NH_4^+, which was significantly higher than the bulk seeded waste rock (D Slope), both shrub and grass rhizosphere-influenced waste rock, and natural desert soils (OS) (Figure 12). The percentage of nitrogen in the form of NH_4^+ is 4.29% for unseeded waste rock (N Slope); 1.10% for bulk seeded waste rock (D Slope);

0.682% for grass rhizosphere-influenced waste rock; 0.097% for natural desert soils (OS); and 0.082% for shrub rhizosphere-influenced waste rock. Interestingly, the NH_4^+ content of the natural desert soils (OS) was highly variable: most of the NH_4^+ levels were low except for an outlier with a very high level. Given the data from the other sampling locations, this may result from the fact the samples represented both vegetated fertility islands and open spaces. Further analysis should be done to examine this high variability.

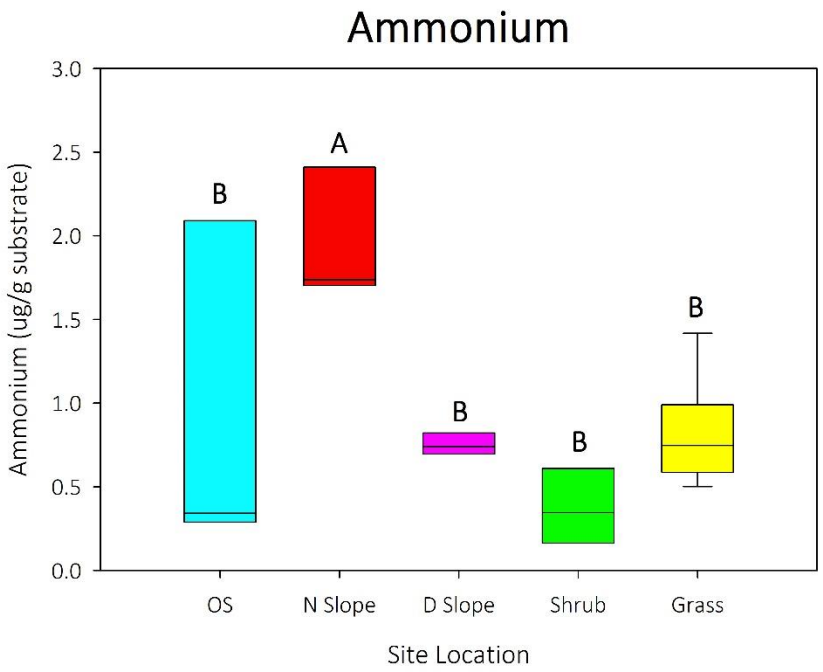

Figure 12: Ammonium (NH_4^+) content comparison between sample locations. Unseeded waste rock (N Slope) had the significantly higher NH_4^+ than bulk seeded waste rock (D Slope), both shrub and grass rhizosphere-influenced waste rock, and natural desert soils (OS). Mean values for boxplots labeled with different letters are significantly different ($P < 0.01$).

3.5 Correlations between indicators

Pearson's correlations were evaluated between each of the biogeochemical indicators and plant cover to quantify relationships between the different indicators examined. DNA biomass content

is strongly positively correlated with *amoA* gene abundance (AOB) and total nitrogen content (TN) (Table 2). TN was also strongly positively correlated with AOB abundance. Ammonium (NH_4^+) content is weakly negatively correlated with TN, DNA biomass, and AOB abundance. DNA biomass, TN, and AOB abundance have positive correlations with plant cover, whereas a strong negative correlation was observed between NH_4^+ and plant cover. The weak, negative correlation between NH_4^+ and TN indicates that nitrogen accumulates in an alternate form in this ecosystem. Nitrate (NO_3^-) content will be analyzed to determine whether the majority of the soil nitrogen is accumulating as organic nitrogen.

Table 2. Pearson's correlation values (R) between biogeochemical indicators and plant cover.

	Total Nitrogen	Ammonium	DNA Biomass	*amoA* (AOB)	Plant Cover
Total Nitrogen	1				
Ammonium	-0.328*	1			
DNA Biomass	0.893***	-0.328*	1		
amoA (AOB)	0.683***	-0.427**	0.665***	1	
Plant Cover	0.594***	-0.486***	0.411**	0.592***	1

* $P < 0.1$
** $P < 0.05$
*** $P < 0.01$

4.0 Discussion

4.1 Indicators and belowground microbiome development

This study evaluates the usefulness of DNA biomass content, *amoA* gene abundance (AOB), total nitrogen content (TN), and ammonium (NH_4^+) content as indicators of belowground substrate quality throughout incipient soil development. Incipient soils such as mine waste rock must establish a productive nitrogen cycle to provide plants critical nutrients and support growth. Results of this study suggest that DNA biomass, TN, and AOB abundance can be used as indicators to track waste rock fertility advancements during revegetation processes due to their strong correlations with each other and plant cover (Table 2). The correlations between all the indicators and plant cover illustrate the interconnectedness of chemical and biological soil properties, affirming that soil health is an important metric of revegetation progress and must include both chemical and biological metrics.

Biomass, TN, and AOB abundance are significantly higher in natural desert soils and beneath shrub canopies (Figures 9, 10,11), which are also the locations highest in plant cover (Table 1). Increased nutrient concentration, plant cover, and potential for microbial activity indicate the belowground substrate fertility of these areas is more developed than that of unseeded waste rock (N Slope), bulk seeded waste rock (D Slope), and grass rhizosphere-influenced waste rock.

Of the correlations between the biogeochemical indicators, the strongest exist between biomass and TN; biomass and AOB abundance; and TN and AOB abundance (Table 2). The positive correlation between biomass and AOB abundance indicates AOB abundance proportionally increases as the overall belowground microbiome develops and expands, and thus

AOB have a greater capacity for perform nitrogen cycling services as soil fertility improves. Together, these indicators can be used to estimate the microbial inputs into the belowground ecosystem and the potential for nitrogen cycling and increased plant-available nitrogen.

Due to both the weak correlation observed between ammonium and the other indicators (Table 2) and the technical limitations caused by the *amoA* gene quantification detection limit (Figure 8), TN is the best indicator of nitrogen cycling of the indicators evaluated in this study.

4.2 Nitrogen cycle inefficiency

Ammonium (NH_4^+) content shows weak negative correlations with the other indicators (Table 2) and follows an opposite trend: the unseeded waste rock (N Slope) has the highest NH_4^+ concentration (Figure 12). Combined with the lack of AOB established on the N Slope, this suggests that elevated levels of NH_4^+ exist in the waste rock because there are no AOB present to convert it to nitrite (NO_2^-). These results imply accordingly that unseeded waste rock (N Slope) has a severely underdeveloped microbiome with limited nutrient cycling capacity. Representatives from Carlota Copper Mine have indicated that the large amount of NH_4^+ present in the waste rock may result from industrial activities rather than biological N_2-fixation. More samples will be analyzed in the future to address this conclusion. Although plant-available nitrogen is present in the form of NH_4^+, the low DNA biomass content of the N Slope (Figure 9) indicates a probable scarcity of other nutrients that is likely restricting plant growth and preventing plant establishment.

Mummey et al. proposed the concept of nitrogen cycle inefficiency to explain the NH_4^+ and NO_3^- buildup in the soil cap of a uranium mine in Wyoming (13). In Mummey et al., one area of reclaimed soil seeded in 1982 showed no significant difference in NH_4^+ and NO_3^- content

when compared to the undisturbed control soil, whereas a reclaimed soil seeded in 1996 did have significantly higher inorganic nitrogen content than the undisturbed soil. Mummey et al. attributed the similar nitrogen levels between the undisturbed soil and soil seeded in 1982 to the possibility of a more efficient nitrogen cycle wherein no accumulation of inorganic nitrogen forms is observed. In contrast, the soil seeded in 1996 had less time to develop, which could be why inorganic nitrogen levels were higher due to an inefficient nitrogen cycling system. In a well-established healthy soil with abundant microbial populations, it is conceivable that nutrients do not accumulate because many organisms are present to utilize them immediately. Conversely, an incipient soil with an underdeveloped microbiome allows nutrients in an available form to amass and reach higher concentrations because few microbes exist to perform these nutrient conversions.

However, more research should be done on correlations between ammonium accumulation in low-nutrient arid soils and the status of the soil microbiome. Most research on nitrogen cycling efficiency is focused on preventing losses from agricultural systems or examining plant litter and nitrogen use efficiency. Due to fertilizers, agricultural systems are almost never deficient in nitrogen. Instead, the majority of nitrogen losses occur through ammonia volatilization or nitrate leaching, neither of which are relevant to semiarid degraded lands. Nitrogen losses can also occur through denitrification, but because denitrification is an anaerobic process in arid regions it is limited by water rather than nitrate availability (40).

Nitrogen use efficiency (NUE) is the ratio of nitrogen incorporated into microbial biomass to nitrogen released into the environment in organic forms (41). The concept of NUE was developed to measure nitrogen losses in temperate forests when trees drop their leaves and to understand how leaf litter inputs affect partitioning between organic nitrogen (in the form of

biomass) and inorganic mineralized nitrogen (41, 42). Recent research has shown that when nitrogen exists in excess and carbon becomes limiting instead, microbes release nitrogen through mineralization as NH_4^+ (41). While these conclusions would suggest the opposite of what our study has found (that is, N-limited systems contain less NH_4^+ produced through mineralization), temperate forests have already established robust microbiomes, so presumably there are plenty of AOB present to immediately utilize the NH_4^+.

Other studies that have measured AOB abundance and NH_4^+ have found positive correlations, in contrast to our data (29, 34, 43). Sher et al. examined ammonia oxidation in semiarid and arid regions of the Negev Desert and found AOB abundance was positively correlated with NH_4^+, with significantly higher abundance beneath shrub canopies than in inter-shrub spaces (34). However, even in the inter-shrub spaces AOB abundance exceeded 3.2×10^6 *amoA* copies g^{-1} dry soil (34); none of their samples had low or nonexistent levels of AOB like our unseeded waste rock (N Slope).

In order to further explore the associations between NH_4^+ accumulation and nitrogen cycle inefficiency, research on incipient soils or degraded lands with nascent microbiomes must be conducted. Additionally, working to lower the detection limit when quantifying *amoA* genes would help us better understand how many AOB are truly present in soils of low biomass. The detection limit for this study was around 5×10^4 *amoA* copies g^{-1} dry soil, but Nelson et al. reported a detection limit of 1×10^4 *amoA* copies g^{-1} dry soil for quantifying AOB abundance (37), and detection limits on the order of 1×10^3 copies g^{-1} dry soil are possible for amplifying other genes such as 16S rRNA. Lowering the AOB quantification detection limit will allow us to take more measurements of AOB abundance and develop more accurate correlations between AOB abundance and NH_4^+. This will require further qPCR protocol optimization.

4.3 Fertility island effect of shrubs and grasses

The importance of shrub-induced fertility island effects is well documented in the literature (15, 16, 44, 45). Fertility islands increase the amount of organic matter beneath and near their canopy (15, 16, 44), improve soil structure and infiltration (44), increase nitrogen content (45), and provide physical protection from erosion and shade from the sun (15, 16, 44).

The indicators evaluated in this study suggest shrubs have a greater fertility island effect than grasses. Significantly higher DNA biomass content, AOB abundance, and total nitrogen content (Figures 9, 10, 11) indicate shrubs have more developed belowground microbiomes than grasses. Several of the fourwing saltbush (*Atriplex canescens*) shrubs we sampled had thick layers of organic matter approximately 2-5 cm thick and were over 1.7 m tall. The canopies of the saltbushes were too dense and low to the ground to allow any plant growth beneath them, so they did not provide physical protection to nearby plants, but certainly contributed nutrients and organic matter. Desertbroom (*Baccharis sarothroides*) are more upright-standing shrubs and do allow for other plants and grasses to grow at their base (Figure 4). At several sampling sites fallen branches and litter from the desertbroom covered parts of the ground, supplying a natural mulch.

Indeed, due to their size and abundance, once established shrubs can become quality fertility islands. However, based on the sequence of colonization documented by Cedar Creek Associates from 2014 to 2018 (Figure 4), it is likely shrubs are not the first pioneer species that begin to grow on low-nutrient degraded lands. Prior to seeing, the D Slope was probably similar to the N Slope and had very little vegetation growing. The seed mix spread over the D Slope in 2012 was comprised mostly of grass species including sideoats grama (*Bouteloua curtipendula*)

and purple threeawn (*Aristida purpurea*). Although sideoats grama and purple threeawn seeds were only about 13% of the mix, they were the only two grass species were present in abundance in 2018. Over 60% of the seed mix was *Secale* but it never established on the D Slope. The 7% of seed mix that were not grass species consisted of desert globemallow (*Sphaeralcea ambigua*) and fairyduster (*Calliandra eriophylla*), neither of which established well. The percentage of fourwing saltbush (*Atriplex canescens*) present in the seed mix is under investigation with Cedar Creek Associates at the current time.

Desertbroom (*Baccharis sarothroides*) was not part of the seed mix, and instead grew as a volunteer species. Figure 2 shows that sideoats grama and purple threeawn established on the D Slope before the shrubs; the amount of perennial grasses increased from 2014 to 2016 whereas the percent cover of shrubs did not begin to increase until 2016. This sequence of establishment indicates that sideoats grama and purple threeawn could have served as pioneer species for the shrubs. The shrubs are now pioneer species for native plants, and will hopefully help increase diversity on the D Slope beyond just four species of plants to better reflect a natural ecosystem and increase its resiliency.

4.4 Applications for the mining industry

It is clear that successful mine reclamation in arid and semiarid regions requires deliberate human intervention (4, 5, 9). Because hard rock mining generates such vast quantities of waste, it is nearly impossible for an ecosystem disturbed to such an intense degree to recover naturally within an acceptable timeframe. Hence, mining companies must implement practical and effective reclamation strategies to hasten rehabilitation and ensure public safety, in addition to improving environmental conditions and aesthetics.

This study demonstrates the necessity of seeding when undergoing revegetation efforts. Although the desertbroom shrubs established on the D Slope without seeding, they potentially required the sideoats grama and purple threeawn grasses to be seeded first. The N Slope remains unseeded and very few shrubs or grasses have grown in the unamended waste rock. If seeding were unnecessary, the N Slope would exhibit vegetation growth. However, it currently does not, nor does it show trends towards increasing plant cover (Figure 4). This suggests that the grasses cannot grow on their own without seeding, and although some shrubs can develop without seeding, they cannot grow without the grasses first altering the waste rock through plant-soil interactions.

After seeding, it is also critical for mines to monitor plant cover and belowground indicators annually. As discussed, focusing on aboveground seeding efforts alone is insufficient when measuring revegetation progress because microbial contributions to nutrient cycling are instrumental in facilitating plant growth. Further indicators should be examined to better understand the nitrogen cycle of the belowground microbiome. Quantifying nitrate (NO_3^-) concentration and dissolved organic nitrogen (DON) will assist in understanding the relative size of nitrogen pools in the soil. Similarly, quantifying the total bacteria population by amplifying the 16S rRNA gene will illustrate the relationship between AOB and bacteria present more accurately than what DNA biomass content can estimate.

Continual monitoring of belowground indicators allows the mines to determine if and how quickly waste rock material is developing into a soil. This data will advise mining companies in management decisions, such as determining if they need to re-seed, alter their seed mix, or add organic amendments to better promote plant growth. Because mine reclamation is a

lengthy process that often takes decades, the ability to make predictions about future plant growth based on current trends is highly valuable.

5.0 Conclusion

This research demonstrates that revegetation of mine waste rock material must be approached as a process of developing the system's soil fertility and potential to support plant growth. Cultivating soil fertility is a complex process that involves changes to chemical and biological properties which interact with each other to affect ecosystem development both above and below ground. Due to the heterogeneity of soils, biogeochemical indicators must be identified to measure soil health.

This study evaluated four indicators that correlate with each other and plant cover, suggesting they can be used to track improvements in substrate fertility and the expansion of the belowground microbiome. DNA biomass content, total nitrogen content, and AOB abundance quantified by amplifying the *amoA* functional gene are indicators that positively correlate with each other and plant cover, providing an estimate of the microbial inputs and nitrogen cycling potential. Combined with these three indicators, ammonium content provides further understanding of the nitrogen transformations and possibilities of nitrogen cycling efficiency.

The comparison of vegetation cover on the of N and D Slopes demonstrates that an active seeding program is required for plant establishment on mine waste rock within a 10-year timeframe. Successful revegetation management strategies for developing incipient soils and determining seed mix composition require an understanding of the plant types that drive ecosystem regeneration, including determining the successional patterns necessary to build a more fertile soil substrate. This study found that shrubs have a greater fertility island effect than grasses; however, the data suggest that grass establishment is required to facilitate subsequent shrub establishment. Utilization of belowground indicators of soil fertility enables proper

management and monitoring of mine waste rock revegetation and facilitates the creation of sustainable ecosystems from degraded lands.

Acknowledgements

I would like to express deep appreciation and gratitude towards my family, who has given me endless love and encouragement, always valued advancing my education, and worked very hard to give me the best opportunities possible. Without their unwavering support and confidence in me, I would have never been able to achieve my goals and accomplishments.

I am also extremely thankful for Dr. Julie Neilson, who is an incredibly inspiring role model and who always amazes me with her dedication to her work, students, and lab members; and Dr. Raina Maier, who has established and leads such a remarkable research group I am lucky to be a part of. I would like to thank all of my lab group who make this work so much fun, and especially those who helped me on this project: Ben Rivera, Juliana Gil-Loaiza, Lydia Jennings, Karen Serrano, and Priyanka Kushwaha. I also want to recognize Mary Kay Amistadi and Rachel Burnett, who worked with me extensively at ALEC; and to Jesse Dillon of Cedar Creek Associates, who conducted the plant cover analysis for this study and helped develop the sampling design.

Lastly, I would like to acknowledge the funding and support provided for this project by the UA Center for Environmentally Sustainable Mining, UA Copper Mine Industry Cooperative funded by KGHM Carlota Copper, and UA Superfund Research Program.

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