Microbial community structure and function in mine tailings-contaminated soils

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Microbial Community Structure and Function in
Mine Tailings-Contaminated Soils

by

October Frances Seastone
B.S. Bates College, 1994

presented in partial fulfillment of the requirements
for the degree of
Master of Science
The University of Montana
1996

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In natural environments, the geochemical, physical, macrobiological and microbiological systems are inextricably linked. The microbial players in the environment often serve as the primary mediators of cycling and transformations within and between those systems. Microbes are essential for many crucial processes such as nitrogen fixation, mineral alterations, and decomposition. Because the microbial community is extremely sensitive to environmental conditions and intricately involved with geochemical and biological processes, it should be an essential criterion for evaluating the state of a system.

This research considers the microbial community structure as part of a mine tailings-contaminated ecosystem that has been restored by various applications of liming and revegetation. This research begins with the crucial work focused on developing and improving procedures for microbial community studies in this uncharacterized environment, and culminates in the practical application of this work to an established mine tailings reclamation project.

The first chapter describes modifications made to improve epifluorescent staining of bacteria in these mine tailings-contaminated soils. Various chelators are used in combination with other modifications to enhance visibility of bacterial cells.

The second chapter investigates potential problems in using a recent method, carbon-utilization profiling, to assess the functional microbial community structure in mine tailings-contaminated soils. Because this method is relatively recent in microbial ecology studies, the suitability of using carbon-utilization profiles in harsh mine tailings-contaminated soils was explored. The effects of soil density, various methods of sample preparation, and possible interference of copper and tailings were investigated.

The third chapter uses plate counts and carbon-utilization profiles to assess the microbial community in a mine tailings reclamation project located outside of Butte, MT. By focusing on the interaction between the microbial community, the physical properties of the soil, and the plant success of each treatment, similarities and differences between intact (non-tailings-contaminated) sites, disturbed sites, and restored sites are exposed. This research may reveal valuable information that will assist in our efforts to quantify the impact of heavy metal contamination, the progression of restoration techniques, and their relationship to the intact environment.
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Improved Epifluorescent Staining in Heavy Metal-Contaminated Soils

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Epifluorescent staining procedures, using 4,6-diamidino-2-phenylindole (DAPI) and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), were modified for use in a disturbed soil environment. In addition to previously documented modifications, the introduction of chelators (NTA, Citrate, and HEDP) yielded significantly improved cell visibility and more efficient sample processing.

Hardrock mining activities have seriously impacted both surface and groundwater environments (1,3,5,6,7,11,13,15) in many geographical regions throughout the world. Floods have deposited metal-laden mine tailing from the historic Butte-Anaconda, Montana, mining complex down stream over 200 km of the Clark Fork River resulting in the largest superfund complex in the United States (7). Geochemical transformations resulting in the migration of heavy metals or metalloids into surface or groundwater are for the most part microbially driven. A well-known example of this is the oxidation of pyritic minerals by acidophilic iron-oxidizing bacteria that results in acid mine drainage (3). An understanding of the potential long-term environmental consequences of these pollutants is necessary to initiate reclamation and must stem from knowledge of the microbial ecology of these sites. Direct counts and biomass measurements set the foundation for such studies.

This paper focuses on procedures for two epifluorescent stains: DAPI, which is a nonintercalating DNA stain for total bacterial counts, and CTC, a red-fluorescing
tetrazolium salt used to detect viable bacteria (4,8,10,17,18). Using published procedures for these epiflorescent direct counts (8, 4) and ATP biomass (16) has proved difficult in metal-contaminated soils, presumably due to the heavy metal interferences and high background autofluorescence. Improved methods for epiflorescent staining were developed by modification of dilutions (12), settling time, incubation time and concentrations of DAPI (12, 4, 18) and/or CTC (10, 17, 18), adding chelators, and administering the stains with nutrient pads after sample filtration (9). These improvements enhanced visibility and countability of the stained cells and will eventually lead to more a complete understanding of the microbial community in this, and perhaps other, soil environments.

**Sample Processing and Staining Procedure.** Samples were collected from mine tailings-contaminated sediments on the banks of Silver Bow Creek, outside of Butte, Montana. Thirty centimeter deep cores were collected using sterile polycarbonate tubes (4.5 cm diameter), and stored at 4°C until processing (48h). This soil was a highly metal-contaminated (Fe>40,000 ppm, Mn>20,000 ppm, and Cu>8,000 ppm), acidic (pH of 4-5), heterogeneous mixture of fine- and coarse-grained sediment.

Two grams of soil were weighed, vortexed and sonicated for 10 mins in 9 ml of 0.2% sodium pyrophosphate (Sigma Chemicals Co., St. Louis, MO) and 1/4 strength R2A (Difco Laboratories, Detroit, MI), and the specific chelator for the sample. The chelator treatments included 0.34 mM citrate (Fisher Chemical, Fair Lawn, NJ), 1mM nitrilotriacetic acid (NTA, Sigma Chemical Co.), 2 mM ethylenediamine-tetraacetic acid (EDTA, Sigma Chemical Co.), and 2.5 mM 1-hydroxyethene-1,1-diphosphonic acid
(HEDP, Sigma Chemical Co). The slurries were allowed to settle for 2 hours at 23°C.

One ml of the supernatant was diluted (1:10 in 0.2% sodium pyrophosphate) and one ml of that dilution was filtered through a 25 mm 0.2µm black polycarbonate filter (Poretics Corporation, Livermore, CA), supported by a 0.45µm nitrocellulose backing filter (Millipore Corporation, Bedford, MA). The black filters were immediately removed from the filter apparatus and incubated for 4 h in the dark at 23°C on a nutrient pad containing 5 mM CTC (Improved Polysciences, Inc., Warrington, PA) and 1/4 strength R2A broth with a final volume of 2 ml on the nutrient pad. Filters were then transferred to a second nutrient pad containing 10µg/ml DAPI (Sigma Chemicals Co., St. Louis, MO) and 5% formalin, and incubated for 1 hour in the dark at 23°C. The sample filters were mounted on a glass slide with low-fluorescing immersion oil (Nikon, Bayshore, NY) and observed at 1000x. A minimum of 300 cells or 30 fields were counted. Cells were visualized on a Zeiss Axioskop 20 equipped with Chromatechnology Corp. filter set CZ902 (Chromatechnology Corp., Brattleboro, VT).

Variance between experiments was minimized by standardizing the data. The control means were used to additively transform the data of the untreated samples for each experiment according to the formula $t' = t - (u_\text{t} - u_\text{t,mean})$, where $t'$ is the adjusted treated sample, $t$ is the unadjusted treated sample, $u_\text{t}$ is the mean untreated sample, and $u_\text{t,mean}$ is the mean of all untreated samples. Because the hypothesis was independently tested multiple times by student's t-test, a Bonferroni-corrected level of significance was applied ($\alpha' = \alpha/n = 0.05/4 = 0.0125$) (14).
Results. As previously documented by Yu et al. (1995) and others (18, 4, 12, 10, 17), increased incubation time and stain concentration enhanced cell visibility. In this soil type, relatively high stain concentrations and long incubation times, 10μg/ml for 1 hour for DAPI and 5 mM for 4 hours for CTC, provided the most reproducible staining. The dilutions and settling time reduced background sediment and decreased three-dimensional distortions on the filter. By incubating samples on nutrient pads, more samples could be processed consistently and simultaneously (9).

The addition of the chelators citrate, NTA, and HEDP significantly increased DAPI-stained cell visibility (Figure 1). The increase in CTC-positive cells was statistically significant with citrate, but not with NTA, EDTA, or HEDP, due to the large error resulting from generally low CTC counts in these samples (Figure 2). Controlled experiments confirmed that this effect was neither due to pH change (a similar pattern was observed with varying pH), nor the possibility of chelators serving as carbon sources (the same effect was observed at different temperatures) (data not shown).

The described chelator effect not only provides a tool for improving direct counts, but also provokes inquiry to the physiological reason for the better cell staining. The work was initiated to address the possibility that stain uptake might be inhibited by divalent metal ions bound to the bacterial surface. Alternatively, the chelators may render inactive cells more susceptible to stain uptake by interacting with surface proteins, an interaction described in spore activation studies (2). Preliminary experimentation has indicated that cell counts may be similarly enhanced with NTA in forest soils, which do not exhibit high metal concentrations, but further research is needed to confirm the
breadth of applicability for this procedure. Although more detailed investigations are necessary to determine actual mechanisms for this effect, this work provides a simple method for improving direct microscopic quantification of total and respiring bacteria in these acidic, metal-rich soil environments.

This work was supported by the Montana Water Resources Center, and NSF EPSCoR Grant # OSR-955450.
FIG 1. Bacterial cell counts of DAPI-stained cells in untreated samples \((n=22)\), compared to samples treated with the chelators citrate \((n=24)\), EDTA \((n=5)\), NTA \((n=11)\), and HEDP \((n=5)\). Error bars represent the standard error of the mean.
FIG 2. Bacterial cell counts of CTC-stained cells in untreated samples ($n=22$), compared to samples treated with the chelators citrate ($n=24$), EDTA ($n=5$), NTA ($n=11$), and HEDP ($n=5$). Error bars represent the standard error of the mean.
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Biolog Carbon-Utilization Profiles for Soil Samples: Soil Matrix Effects

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ABSTRACT

Carbon-utilization profiling, with Biolog microtiter plates, has been used to characterize functional microbial community structures in soil and water samples. Use of this technology for soil samples requires careful consideration of the effects of sample preparation on the integrity and activity of the intact microbial community, and the resulting carbon-utilization profile. This research addresses three potentially problematic aspects of current Biolog methodology: 1) the effect of soil density, independent of inoculation density, on optical density readings; 2) the effect of several methods for extracting cells from soil (such as flocculation and sonication), and 3) the effect and possible interference of Cu and mine tailings on the development of the Biolog plates. Settling and flocculation decrease sample turbidity and reduce additional carbon sources, but may preferentially select for certain microbial populations and prevent accurate assessment of the complete microbial community. By characterizing the soil matrix effects on color development, it will be possible to improve methodology and interpretation of this technology for future use in explorations of intact microbial communities.

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INTRODUCTION

Carbon-Utilization Profiles have been used to characterize the microbial ecology of environmental soil samples (3,4,5,6,12,13). This method uses Biolog microplates, which consist of 96 wells containing 95 different carbon sources and a redox indicator dye, to determine carbon-utilization profiles that reflect the potential metabolic diversity of a sample (5). Because Biolog plates were developed for clinical rather than environmental settings, potential complications associated with environmental samples, especially soils, must be carefully considered. This research explores three aspects of the Biolog method as it is influenced by soil, specifically in a mine tailings contaminated site.

First, we tested the effect on well-color development of samples with varying sterile soil loads, while microbial density was held constant. While previous papers suggest concern about introducing soil carbon into the wells (5,13), we suspected a problematic masking effect in high density samples. Specifically, reduction of tetrazolium violet may not be detected due to opacity of dense soil particles.

The second problem addressed is the universal difficulty of bacterial extraction from soil. In preparing soil samples for Biolog carbon-utilization pattern analysis, methods such as sonication (12), diluting samples in a surfactant such as sodium pyrophosphate (4,7,8,11), settling (8), and flocculation (1,6,8) have been used to separate cells from soil. Surfactants and sonication are used to break up soil-microbe aggregates, freeing bacteria that might otherwise have settled out of solution while adhered to soil particles. Allowing settling and using flocculating salts causes larger soil particles to fall out, while cells remain suspended in the supernatant. Settling times, sonication, and
flocculation were each studied to determine their effect on total culturable cell numbers and Biolog patterns.

The third soil effect examined is the impact of Cu on well-color development, as heavy metals (Cu in particular) and other mine-tailing components have been suspected of interfering with tetrazolium violet reduction (2). As this analysis is commonly used in environmental samples, often in disturbed environments, it is important to consider interaction of soil components with the assay reactants.

MATERIALS AND METHODS

Sample Collection. Soil samples were collected from a mine tailings-contaminated floodplain outside Butte, Montana. Soil for the color detection study was from a non-contaminated area just above the floodplain, soil for the cell extraction study was mine tailings mixed with topsoil, and soil for the assay inhibition study (involving copper or other tailings-associated compounds) was heavily contaminated tailing/soil. Five sample cores (2.2cm dia. x 10cm deep) were extracted using a sterilized soil probe (Forestry Suppliers, Inc., Jackson, MS) and composited for one sample. Soil samples were placed in sterile bags, transported on ice to the lab, and held at 4°C until processing.

Plate counts and microbial community structure analysis. Bacterial plate counts were determined by spread-plating samples on R2A agar (Difco, Labs, Detroit, MI) and incubating at 25°C for 72 h. Community structure analysis using carbon-utilization patterns were determined by using Biolog GN plates (Biolog, Inc., Hayward, CA), except where Biolog MT plates are specified. Each plate was inoculated with 150µl
of sample, prepared as described below. After incubation at 25°C, color development was monitored, using an ELISA microplate reader (Molecular Devices, Menlo Park CA), at approximately 24 hour intervals for up to 190 hours. Carbon-utilization patterns were analyzed after 190 hours by Principal Components Analysis (PCA) (PC-ORD), an indirect gradient analysis that simplifies complex data sets into a smaller number of axes (9). Using the values for the 95 carbon sources, the PCA separates data so that data points that are more similar cluster together.

**Effect of soil density on color detection.** Autoclaved soil was diluted to concentrations of $5 \times 10^{-2}$, $1 \times 10^{-2}$, $1 \times 10^{-3}$, and $1 \times 10^{-4}$ g of soil/ml in sterile PBS, pH 6.8. Eighteen ml of each of these solutions was inoculated with 2 ml of viable soil diluted to $1 \times 10^{-2}$. The final soil densities (sterile plus viable soil) in the samples to be distributed into Biolog plates were $5.1 \times 10^{-2}$, $1.1 \times 10^{-2}$, $2 \times 10^{-3}$, and $1.1 \times 10^{-3}$ g of soil/ml. Samples were prepared in duplicate.

**Effect of sonication, flocculation, and settling on cell numbers and carbon-utilization profiles.** For each treatment, 2 grams of soil was diluted in 18 ml of 0.1% sodium pyrophosphate (Sigma Chemicals Co., St. Louis, MO) in 50 ml conical centrifuge tubes, except for the non-surfactant controls which were diluted in PBS or sterile 0.85% NaCl. Samples were run in triplicate.

To monitor the effect of extended sonication times on viability of cells alone, samples were vortexed for 10 seconds, sonicated for 60 mins (Branson Ultrasonic Cleaner 3210, Danbury, CT), sampled at 0, 10, 30, and 60 mins, and spread-plated on R2A agar.
To examine the effect of both increasing sonication and varying settling times on cell numbers in the supernatant, samples were vortexed for 10 seconds, and sonicated for 0, 15 or 45 mins. After the designated sonication time, samples were vortexed and allowed to settle. The tubes were sampled and plated after settling for 0, 15, 30 minutes, and again after revortexing for 10 seconds. Microbial community structure was analyzed with these same treatments, but with an abbreviated sampling scheme: all samples were sonicated for 15 minutes, vortexed, and sampled after settling 0, and 15 minutes, and again after revortexing. One ml was removed and diluted 1:10 in PBS and dispensed into Biolog GN plates.

Investigations of flocculation procedures were done by sonicating samples for 15 minutes, vortexing for 10 seconds, and sampling at time 0. Then, 0.04 g of 8:5 (CaCl₂·2H₂O: MgCO₃·Mg(OH)₂·4H₂O) flocculation mixture was added, the tube vortexed, the soil allowed to settle for 15 minutes, and the supernatant was sampled. Finally, the tube was revortexed for a final sampling. Samples were standardized, according to indirect counts, to approximately 1x10⁴ cells/ml, and Biolog GN plates were prepared for each of the sample times, as described above.

Effect of CuSO₄ and tailings-soil on Biolog well-color development. Mine tailings-contaminated soils were plated on R2A agar containing 100ppm CuSO₄. A single Cu-tolerant isolate was selected and sub-cultured in 100ppm CuSO₄-R2A broth. For the Cu studies, filter-sterilized CuSO₄ (J.T. Baker Chemical Co., Phillipsburg, NJ) was diluted in R2A broth to concentrations of 100ppm, 10ppm, and 1ppm. Sterile tailings were diluted in R2A broth to concentrations of 10⁻¹ and 10⁻² g/ml. Each of these solutions were
inoculated with the Cu-tolerant isolate (to a final concentration of $1 \times 10^6$) and dispensed (150μl per well) into Biolog MT plates. Biolog MT plates contain the minimal salts for growth and tetrazolium indicator dye like the standard GN plates, but they contain no carbon source in the wells, allowing focus on the color development associated with Cu interference. Plates were incubated at 25°C and monitored at approximately 20 hour intervals for 101 hours.

**RESULTS**

**Effect of soil density on color detection.** The results indicate that higher soil loads cause lower sensitivity in detection of color development, possibly due to a masking effect. The unadjusted absorbance readings clearly show higher optical density readings for more concentrated soil samples throughout the observation period (Figure 1A). After adjusting the absorbance readings for the initial soil differences (by subtracting time 0 readings), a drastic difference in well-color detection is evident (Figure 1B). Although the inoculation density was the same, the high-soil samples, those with $5.1 \times 10^{-2}$ and $1.1 \times 10^{-2}$ g/ml (with average initial optical densities of 2.540 and 0.955), show less color development across time. Specifically, the average optical density of the $5.1 \times 10^{-2}$ g/ml samples is 0.88 optical density units less than the $1.1 \times 10^{-2}$ g/ml sample.

**Effect of sonication, flocculation, and settling on cell numbers and carbon-utilization profiles.** The impact of extended sonication times on cell viability was determined by heterotrophic plate counts. Over a period of 60 minutes of low-level sonication at 4°C, there was no significant decrease in culturable cell numbers (Figure 2).
The combination of different sonication times with different settling times also showed no significant change in cell numbers in the sampled supernatant (Figure 3). The PCA shows each of these treatments distributed on the graph, with no discernible clustering within differently settled treatment groups (Figure 4).

After flocculating and settling for 15 minutes, a significant drop in CFUs in the supernatant was observed (Figure 5). When the sample was revortexed, the cell counts returned to approximately the original cell numbers. The PCA of carbon-utilization profiles shows the initial count (time 0) is plotted in a cluster with the other non-flocculated samples from the settling experiments, while both samples with flocculating agents added, those settled for 15 minutes and those revortexed, fall outside of the cluster, separated along principal component 2 (Figure 4). The PCA also provides a list of correlation coefficients indicating how strongly each of the 95 carbon-source contributes to the observed pattern. In an effort to confirm that this pattern results from differences in absorbance between treatments we compared the absorbance values of the primary carbon compounds contributing to the PCA pattern, those with the highest coefficient of correlation with principal component 2. A non-parametric Kruskal-Wallis one-way ANOVA shows that the variation in absorbance values for the primary carbon compounds is not significant between treatments (p values all greater than 0.05), indicating that there is no significant difference between carbon-utilization patterns contributing to this PCA diagram (10). The differences in absorbance readings were insignificant, so we do not detect meaningful differences in microbial community structure within or between flocculation and settling experiments.
Effect of CuSO₄ and tailings-soil on Biolog well-color development. Results indicate that color development was inhibited by high Cu concentrations, but not by tailings or Cu concentrations relevant to the environments of interest. The Biolog plate color development of all negative controls, tailings-containing wells, and low Cu-containing wells showed similar color development (Figure 7). The 100 ppm-Cu sample showed consistently low color development, while the 10 ppm-Cu sample showed a significantly different pattern of development across time, but eventually reached high color development similar to the control.

DISCUSSION

As carbon-utilization profiling has gained attention in recent microbial ecology studies, many potential complications, such as introduction of soil carbon into wells (5,13), differences in sample preparation, and the possibility of soil matrix interferences with assay constituents (2), have become apparent. When using Biolog-generated carbon-utilization patterns for environmental community structure analysis, the effects of sample preparation and soil characteristics must be carefully considered. This work has demonstrated ways in which soil density, bacterial extraction procedures, and soil components can influence the data generated, potentially leading to variance in interpretation of the microbial community structure.

In the first set of experiments, we demonstrated that equal microbial densities with higher soil loads actually result in lower well-color detection. Whether this phenomenon is the result of interference with the light-path by soil particles, decreased sensitivity due to
exceeding the range of the optical density reader, or possibly interference of a soil component with color development, it is a strong effect that alters absorbance readings. Although the increased carbon input to wells may potentially cause excess color development (5,13), the stronger influence demonstrated here is a masking effect, resulting in much lower color detection. This finding complicates this type of analysis in soil environments, especially environments in which microbial numbers are low and dilutions must be minimized. Although the magnitude may vary with different soils, sample preparation, and microplate plate readers, this masking effect should be considered as a potential impediment to precise measurement. In the effort to balance minimizing dilutions with minimizing the soil interference, the samples above should be diluted until initial absorbance readings are less than approximately 1.000.

Different bacterial desorption procedures had interestingly little impact on the final carbon-utilization patterns in these samples. First, the heterotrophic plates counts indicated that viable cells were not adversely affected by sonication, even for durations as long as 60 mins. The next experiments indicated that samples that were sonicated, vortexed, and allowed heavier soil particles to settle for up to 30 mins maintained equivalent heterotroph numbers in the supernatant, regardless of sonication or settling time. Carbon-utilization patterns of these samples also showed no difference between microbial communities in the supernatant after heavy soil particles settled. The final experiments of these procedural investigations considered changes in bacterial numbers and metabolic diversity before and after salts were used to flocculate soil particles. After flocculation and settling for 15 mins, the supernatant showed significant loss of
heterotrophic bacteria. In spite of this substantial loss of microbial numbers, significant
differences in carbon-utilization patterns were not detectable. Although this loss of cell
numbers did not result in an alteration of the metabolic diversity in this study, such a
selective reduction in microbes could potentially alter the microbial community structure
in samples with lower initial numbers or merely a different contingent of microorganisms.
These bacterial extraction procedures would be suitable in this soil, but these concerns
must be reconsidered with each new soil type encountered.

The high-Cu samples (100ppm and 10ppm) showed altered well color
development across time compared to the controls, tailings, and low-Cu samples. This
effect could be the result of slower growth of the organism in the high-Cu samples,
interference with tetrazolium reduction, or reaction with the reduced tetrazolium salt
resulting in a loss of purple color. There was no inhibition of color in the tailings-
containing samples, or in the low-Cu samples, which were most representative of the
estimated Cu content of the soil samples. This work suggests that no components of this
severely disturbed soil interfere with the Biolog plate development, and this assay may be
applied to this environment.

As environmental microbiologists continue to utilize and refine this valuable
method for metabolic characterization of microbial communities, it is necessary to pursue
and address the associated complications of this application. The results of this work
confirmed our confidence that Biolog Plate carbon-utilization profiles could appropriately
be used in this environment. When the effects of sample type and preparation are
understood, this method provides a valuable and informative tool for investigations of microbial community structure in soil environments.

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This work was supported by NSF EPSCoR Grant # OSR-955450, the Murdock Charitable Trust, the Montana Water Resources Center, and the National Mined Land Reclamation Center.
FIG. 1. Absorbance readings of Biolog plates with equal inoculation density, but varying sterile sediment density across time. Data represent duplicate samples diluted to 5.1x10^{-2}, 1.1x10^{-2}, 2.0x10^{-3}, and 1.1x10^{-3} g of soil/ml. Panel A shows unadjusted absorbance readings across time, while panel B shows color development, determined by absorbance with time 0 subtracted.
FIG. 2. Heterotrophic plate counts of samples treated with increasing sonication times. Error bars represent SEM, $n=3$. 
FIG. 3. Heterotrophic plate counts of samples treated with varying sonication and settling times. Data plots represent samples treated with no sonication, 15 mins. sonication, or 45 mins. sonication. Error bars represent SEM, n=3.
FIG. 4. Principal components analysis of soil samples before and after both settling and flocculation treatments. n=3.
FIG. 5. Heterotrophic plate counts of samples after flocculation and settling for 15 minutes. Error bars represent SEM, $n=3$. 
FIG. 6. Color development (optical density) across time of Biolog plates with varying levels of CuSO4 and tailings-sediment. Data points mark average optical densities, with error bars representing SEM, and n=3.
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Carbon-Utilization Patterns and Microbial Community Structure in a Mine Tailings Restoration Site

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ABSTRACT

This research investigates the microbial community of a mine tailings-contaminated environment, how that community differs from a nearby uncontaminated site, and how it is altered by proposed remediation strategies. We used carbon utilization patterns generated with Biolog microtiter plates (Biolog, Inc., Hayward, CA) to characterize functional microbial communities and detect subtle differences that may reflect the recovery of microbial communities. Differences in structure (determined by bacterial direct counts, heterotrophic plate counts, fungal plate counts, and spore plate counts) and function (determined by carbon-utilization profiles) of microbial communities were investigated in seven different treatments of mine tailings-contaminated soils. The research took place near Butte, MT on an established pilot-scale remediation project designed to test the efficacy of various mining waste remediation strategies such as shallow liming, deep liming, lime-slurry injection, and topsoil addition. All total culturable counts were highest in the uncontaminated and topsoil samples and lowest in the untreated mine tailings samples. Fungi and spore to bacteria ratios, however, differed from that ranking. The functional diversity of the areas varied substantially, with the untreated mine tailings plots

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demonstrating very low carbon-utilization activity, the topsoil and pristine plots with very high activity, and the lime-treated plots with varying levels of intermediate activity. This pattern of carbon-utilization was correlated with percent vegetative cover, but not with pH, soil moisture, or percent organic carbon. This research reveals crucial information that will assist efforts to quantify the impacts of mining wastes, measure the progression and long-term efficacy of remediation strategies, and relate these sites to the intact environment.

INTRODUCTION

The microbial community is recognized as a critical component of ecosystems in disturbed environments, such as the heavy metal-laden mine tailings-contaminated soils considered in this paper (15). Little is actually known about the microbial community structure of restored sites and its relation to long-term restoration success. Reasons for this are numerous, but are centered on the complexity of molecular methods and the selectivity of traditional methods.

As restoration efforts and assessments continue, there is a need for relatively inexpensive, simple methods for microbial community structure assessment. Traditional methods for microbial ecology studies, such as indirect counts, provide valuable information about the community structure, but are severely limited due to selectivity of culture media (15). Recent studies using molecular methods, such as denaturing gradient gel electrophoresis (11) and DNA fingerprinting (3,16), have been used to evaluate the genetic diversity within a microbial community. To achieve greater understanding of the functional capabilities of the microbial community, carbon utilization patterns, determined
by Biolog plates, have been used in a variety of environments (4,5,7,8,9,17,18,2,20).

These microplates consist of 96 wells, 95 that contain different sole carbon sources and a control well with no carbon. Minimal salts for growth and tetrazolium redox indicator dye are also included in each well. The carbon-utilization patterns that develop provide a metabolic profile for the sample community, identify metabolic capabilities that may be directly related to the disturbance or restoration being assessed, and serve as an indicator of the diversity of the microbial community.

The work described in this paper considers the microbial community structure in a mine tailings-contaminated ecosystem that has been experimentally restored by various applications of liming and revegetation (14). This research area is part of the mine tailings-contaminated Clark Fork Complex Superfund site that has resulted from over 100 years of mining operations conducted upstream (10). In an attempt to assess the feasibility and efficacy of various treatment strategies, the pilot-scale restoration phase of the Streambank Tailings and Revegetation Studies (STARS) project was initiated in 1989. Since that time, the progress of those treatments has been monitored in terms of vegetation, soil chemistry, and hydrologic activity (14). The microbial community, although a critical player in the ecology of the system, has been overlooked in the assessment of the site restoration. Our research compared microbial communities in mine tailings-contaminated soils with varying treatments and a nearby pristine site above the mine tailings-contaminated floodplain. Differences in microbial community structure and function were analyzed in relation to vegetative cover, soil pH, percent moisture, and percent organic carbon.
MATERIALS AND METHODS

Research Site Description. The STARS project (14) is comprised of six 16 by 20 ft. plots, each with a different treatment. These treatments include an untreated plot (X), a plowed and seeded unamended control plot (C), an agriculturally-tilled and limed plot (15-20cm deep) (A), a deep-tilled and limed plot (1.2m deep) (D), a pressure injected lime-slurry plot (I), a topsoil-amended plot (T), and a nearby pristine site above the contaminated floodplain (P) (Table 1).

Sampling Method. Samples were collected from seven treatments of the STARS research project described above, in October, 1995. Each of the seven treatment plots was divided into fifteen equal sections. Five cores (from randomly selected sections) were pulled at varying distance from vegetation, and composited for one replicate. Three replicates were collected from each treatment plot. The cores were collected using a sterilized soil probe (2.2cm dia. x 10cm deep, Forestry Suppliers, Inc., Jackson, MS). Soil samples were placed in sterile bags and transported on ice to the lab for processing.

Culturable Bacteria and Fungi. The soil samples were homogenized, diluted 1:10 in 0.1% sodium pyrophosphate (Sigma Chemicals Co.), sonicated for ten minutes (Bransonic Ultrasonic Cleaner 3210, Danbury, CT), and used for the various microbiological assays. Heterotrophic plate counts were determined on R2A agar (Difco Laboratories), actinomycetes were enumerated on the same plates, detected by colony and microscopic morphology. Total bacterial endospore (free and cell-associated) counts were determined by plating on R2A agar after a 10 minute incubation at 80°C. Total
fungal counts were determined on Rose Bengal agar (Difco Laboratories) supplemented with 0.1g/L chloramphenicol (Sigma Chemicals Company, St. Louis, MO).

**Carbon-utilization Pattern Analysis.** After 72 hours, the heterotrophic plate count data were used to adjust the samples to a standard cell density for Biolog plate inoculation. A second soil slurry was prepared as described for the indirect counts (1:10 soil in sodium pyrophosphate, sonicated for 10 minutes). This slurry was further diluted in phosphate buffered saline to a standard density of \(10^4\) CFU/ml. 150µl of each sample was inoculated into Biolog GN microplates and incubated at 23 °C for 115 hours. The optical density of the wells was measured approximately every 24 hours with an ELISA microplate reader at 570nm (Molecular Devices, Menlo Park, CA).

These data were analyzed first by considering average well color development (AWCD) across time (5). For this, the absorbance readings for each well in a single sample plate were averaged, after first subtracting the time zero reading. Principal components analysis (PCA) was conducted to determine relative diversity of samples. The PCA is an indirect gradient analysis that simplifies this multivariate data set into a smaller number of axes. The correlations between patterns of activity in soil samples can be used to plot data points along principal component axes, clarifying relationships between samples (6,13). Because of the high number of variables incorporated into this PCA, it is necessary to confirm these patterns by returning to the raw data, as is done in Figure 10, a graphical representation of the carbon-utilization data. This analysis displays the absorbance response (y axis) of each sample type to each of the 95 carbon sources (x axis), clarifying overall patterns and importance of each carbon source.
Vegetation Estimates. Vegetative cover was estimated by analyzing three haphazardly placed Daubenmier plots within each treatment plot. A species list, and relative cover of each species and bare ground, were collected.

Soil Parameters. Soil moisture contents were determined by gravimetric difference between wet and dry weights of soil samples. Twenty-five grams of each sample was weighed, dried at 60°C for three days, and re-weighed to confirm dry weights.

For pH measurements, 7 grams of soil was combined with 7 ml of deionized water and allowed to equilibrate for 20 minutes.

For carbon analysis, soil samples were ground and passed through a #200 sieve. Soil organic carbon was determined by coulometry and calculated by the difference of the total and inorganic carbon.

The relationships between percent vegetative cover, soil moisture, pH, and percent organic carbon, and activity in the Biolog plates was analyzed by linear regression.

RESULTS

Culturable Bacteria and Fungi. The R2A plate counts revealed distinct differences in heterotrophic bacteria in each of the treatment plots (Fig. 1). The untreated (X) and unamended controls (C), and the ag-tilled (A) plots had 1-2 orders of magnitude fewer culturable heterotrophs than the other treatments. Soil from the pristine site (P) had the highest heterotroph numbers, followed by the topsoil (T), injected (I) and deep-tilled (D) treatment plots.

The actinomycete plate counts show lowest numbers, of less than $1 \times 10^3$, in the untreated control plot (X) (Fig. 2). The tilled, but unamended control site (C) showed
much higher actinomycete numbers (2.3x10^4), while all of the other treatments showed comparable higher numbers (approximately 1x10^6).

The endospore levels also varied greatly between treatments (Fig. 3). All treatments showed endospores at numbers between 1x10^3 and 1x10^4, except for the topsoil (T) and pristine (P) plots, which were several orders of magnitude greater. When considering the endospores in proportion to total bacteria, the ranking changes (Fig. 4). Each of the limed plots (A,D,I) had much lower ratios than both the disturbed control plots, and the topsoil (T) and pristine (P) plots.

Fungal counts followed a similar trend, but differences were much less pronounced (Fig. 5). The untreated plots (X) had particularly low numbers, the unamended control (C) and the ag-tilled (A) had higher intermediate numbers, and all of the remaining treatments had statistically equivalent high numbers. The fungi to bacteria ratios had a distinctly different pattern (Fig. 6). The unamended control (C) was the highest (0.123), the untreated control (X) also had a high ratio (0.0277), while the other samples all had ratios less than 0.005.

Carbon-Utilization Patterns. The carbon-utilization patterns generated with Biolog GN plates were analyzed in several ways. First, the total activity of each sample across time was evaluated by calculating the AWCD of each sample (Fig. 7). Average Well Color Development was calculated by averaging the optical density readings for all wells for a given plate at a given time point. After 24 h of development, the samples show variation in overall activity levels. The samples fall into three groups according to activity, with the pristine (P) and topsoil (T) treated soils showing highest activity, the injected (I)
and deep-tilled (D) soils with intermediate activity (and greatest variance, suggesting heterogeneity), and the ag-tilled (A), unamended (C), and untreated (X) soils showing low activity.

In order to assess the distribution of this activity across carbon sources, we quantified the number of positive wells in each sample (Fig. 8). A positive response was considered to be a reading of at least 0.200 absorbance units over the time zero reading, because that was the general level at which a distinct purple color was visually detected. Plates were analyzed at 115 h because this was the time at which patterns were established and stabilized, but visible fungal biomass had not appeared in the plates (20). The number of carbon substrates utilized increased steadily from the untreated control (X, 5 carbons), to the tilled/unamended control (C, 9 carbons), ag-tilled (A, 23 carbons), deep-tilled (D, 41 carbons), injected (I, 61 carbons), topsoil (T, 67 carbons), and finally to the highest in the pristine soil (P, 77 carbons).

To examine relationships between carbon-utilization patterns of different treatments, the extent of color development for each carbon was analyzed by PCA (Fig. 9). All of the samples within each treatment cluster together, while treatment groups are separated primarily across the axis of principal component 1. The controls (X, C) and ag-till (A) are clustered on the right, the deep-tilled (D) and injected (I) plots are spread throughout the middle, and the topsoil (T) plot and pristine (P) site are on the left. Table 2 summarizes the carbon compounds responsible for the greatest contribution to the PCA pattern; those with high correlation coefficients and similar distribution of samples with
principal component 1. Seven of the top carbon compounds included adonitol, m-inositol, D-gluconic acid, D-trehalose, inosine, L-leucine, and bromosuccinic acid.

Because of the high number of variables incorporated into this PCA, it is necessary to confirm that these patterns are due to real differences in absorbance readings with the raw data graphical analysis (Fig. 10). Microbial communities from the untreated and unamended controls (X,C) had generally low activity levels for all carbon sources, the topsoil (T) and pristine (P) were variable, but displayed many high values, while the limed sites were intermediate. The primary carbon sources contributing to principal component 1 (listed in Table 2) are delineated with black lines. This graph, which shows clear differences in absorbance for different treatment groups, confirms the relationship between samples that has been simplified and displayed in the PCA (Fig. 9).

**Vegetation and Soil Parameters.** The relationships between activity in the Biolog plates and the vegetation and soil parameters were analyzed by linear regression (Fig. 11). The percent vegetative cover was correlated with AWCD ($r^2 = 0.70$), while none of the soil parameters showed a strong relationship with carbon-utilization activity.

**DISCUSSION**

**Culturable Bacteria and Fungi.** This research has revealed interesting differences in microbial numbers and diversity between variously treated mine tailings-contaminated soils. The heterotrophic plate counts showed lowest numbers in the control sites (X, C), and highest numbers in the pristine site (P) (Fig. 1). The heterotrophic bacteria levels fell into two statistically different groups: the controls (X,C) and the ag-
tilled (A) samples have low bacterial numbers, while the other treatments (D,I,T,P) have high numbers. This suggests that tilling alone and shallow lime incorporation were less effective at supporting culturable heterotrophic bacteria.

The actinomycetes may be an important component of this community because they are often associated with decomposition of complex organic materials and are commonly found in healthy soils (1). Our data indicate that the tilled/unamended control (C) had an increase in actinomycetes over the untreated control (X), presumably due to aeration of the soil during tilling (Fig. 2). The other treatment plots (A,D,I,T,P) show high numbers, possibly because of higher vegetative cover providing more dead litter matter, pH increase, or other changes in soil characteristics.

The total endospores were present in low numbers, except in the topsoil (T) and pristine (P) samples (Fig. 3). When considering these endospores as a portion of the total heterotrophic bacteria, a different pattern emerges (Fig. 4). The control samples (X,C) have a higher ratio of endospores to total bacteria than the lime treated sites (A,D,I). Perhaps these treatments have improved environmental conditions for heterotrophic growth, inducing endospore germination, and resulting in a lower proportion of endospores.

In fungal plate counts, all treatments show a great increase over the untreated control (X). As with the actinomycetes, the greater numbers in the unamended control (C) may be due to the aeration of soil during tilling. The fungi to bacteria ratios show a pattern highest in the controls (X,C), and lowest in the topsoil (T) and pristine (P) samples. A high fungi to bacteria ratio, as seen in the control samples (X,C) is commonly
observed in disturbed environments, probably because fungi are often more resistant to
drought, acidity, and high metal levels (12). The lower fungi to heterotroph ratios in
A,D,I,T, and P may reflect less harsh conditions, favoring other microbial growth.

**Carbon-utilization patterns.** Carbon-utilization profiles provide a simple,
inexpensive method for assessing functional microbial community structure. This
measurement of microbial community structure may be particularly relevant to assessment
and understanding of revegetation efforts, such as this reclamation project. The carbon-
utilization patterns provide specific information about potential metabolic activity and
diversity of the samples, beyond the structural information provided by the traditional
plate counts. In Figure 7, the total activity measured as average well color development,
results in three groups of treatments. The controls (X,C) and the ag-tilled (A) plots all
show low activity levels, the deep-tilled (D) and injected (I) plots show intermediate
activity (with large standard error), and the topsoil(T) plot and pristine (P) site show high
activity.

To get an indication of how that activity was distributed in terms of metabolic
diversity, we compared the number of positive wells (with absorbance ≥ 0.200) in each
treatment. There were great differences in the number of carbon sources the different
microbial communities were capable of using. The fewest active wells were observed in
the untreated control (X) and numbers increased steadily in the unamended control (C),
ag-tilled (A), deep-tilled (D), injected (I), topsoil (T) and pristine (P) treatment plots. The
metabolic versatility of microbial communities steadily increased with the more intensive
treatments.
The PCA (Fig. 9) indicates that the metabolic diversity of each treatment relative to the others results in the same pattern. The controls (X,C) are clustered tightly to the right with the ag-tilled samples (A). The deep-tilled and injected samples are spread across the middle area, while the topsoil (T) and pristine (P) samples are towards the left. The pattern from right to left along principal component 1 seems to represent a gradient of increased metabolic diversity in the treatment plots. Again, the deep-tilled (D) and injected (I) treatment samples are widely distributed, suggesting heterogeneity.

The carbon compounds that contributed most significantly to this pattern may be key factors in understanding the relationship between disturbed soil microbial communities and those in successful revegetation. Interestingly, two of these carbons (m-inositol and D-gluconic acid) were also important in similar research investigating anthropogenic disturbance of forest soils (19). As indicated by Table 2, the control plots (X,C) and the ag-tilled plot (A), to a lesser extent, lack the capacity to metabolize these key carbon sources. Contributing to the goal of understanding plant-microbe linkages, the carbon-utilization patterns clearly indicate a lack of functional diversity in these plots (X,C,A) and therefore a decline in ecological amplitude.

This research reveals important differences in microbial community structure occurring with different reclamation strategies in mine tailings-contaminated soils. As measured in this study, the tilled/unamended control (C) and ag-tilled treatment (A) did not result in substantial increases in heterotrophic plate counts, potential metabolic activity, or metabolic diversity. However, aeration from tilling may cause increases in actinomycetes and fungi to heterotroph ratios. The deep-tilled (D) and injected (I) lime
applications resulted in higher numbers, activity and diversity, but these numbers were associated with high standard error, suggesting heterogeneity and possibly inconsistent amendment incorporation. Soil from the topsoil plot and pristine site consistently showed the highest microbial numbers, activity and diversity. It is especially interesting to note that treatment differences observed in structural studies did not match differences observed in functional studies. Carbon-utilization profiles provided greater resolution in distinguishing between treatments and presented a great deal of information about the metabolic capabilities of the microbial communities in different samples.

These patterns may be driven by, or may drive, differences in many environmental variables associated with the reclamation procedures. Of the parameters measured, vegetative cover was the most closely correlated with microbial activity. This may result from contribution of carbon sources from root exudates and sloughed plant material to sustaining the soil microbial community.

The profound differences in microbial community structure detected by this research opens many questions of organismal interactions and physiological adaptations that establish these differences in microbial ecology. Other investigations of vegetation and soil parameters, combined with controlled laboratory studies of specific carbons will lead to a better understanding of the dynamic interactions associated with successful reclamation.

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<table>
<thead>
<tr>
<th>Treatment</th>
<th>Description</th>
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<tbody>
<tr>
<td>UNUSED (X)</td>
<td>UNTREATED CONTROL</td>
</tr>
<tr>
<td>CONTROL (C)</td>
<td>ROTO-TILLED AND SEEDED; NOT LIMED</td>
</tr>
<tr>
<td>AG TILL (A)</td>
<td>SHALLOW-TILLED LIMING (15-20cm)</td>
</tr>
<tr>
<td>DEEP TILL (D)</td>
<td>DEEP-TILLED LIMING (1.2 m)</td>
</tr>
<tr>
<td>INJECTION (I)</td>
<td>LIME-SLURRY INJECTED TO A DEPTH OF 1.2m</td>
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<tr>
<td>TOP SOIL (T)</td>
<td>AG TILL AND COVERSOIL ADDITION</td>
</tr>
<tr>
<td>PRISTINE (P)</td>
<td>NEARBY AREA, ABOVE THE MINE TAILINGS-CONTAMINATED FLOODPLAIN</td>
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Table 1. Description of treatment plots, mostly from the STARS project, examined in this research.
Table 2. Primary carbon sources contributing to the principal components analysis pattern. These carbons had high correlation coefficients ($r^2$) with principal component 1 and support the pattern observed in the principal components analysis graph.
FIG 1. Heterotrophic bacteria plate counts in STARS treatment plots. 

\( n=3 \). Error bars represent ± SE.
FIG. 2. Actinomycete plate counts in STARS treatment plots.

n=3. Error bars represent ± SE.
FIG. 3. Bacterial endospore counts in STARS treatment plots. 
$n = 3$. Error bars represent ± SE.
FIG. 4. Bacterial endospore to heterotrophic bacteria ratios in STARS treatment plots. $n=3$. Error bars represent ± SE.
FIG. 5. Fungal plate counts in STARS treatment plots. \( n=3 \).
Error bars represent ± SE.
FIG. 6. Fungi to bacteria ratios in STARS treatment plots. $n=3$. Error bars represent ± SE.
FIG. 7. Total activity across time. Average Well Color Development was calculated by subtracting the time zero reading and averaging the optical density readings at 115 hrs. Data points represent the mean ± SE. n=3.
FIG. 8. Number of carbon sources used. Positive responses were wells with absorbance readings ≥ 0.200 at 115 hrs. n=3. Error bars represent ± SE.
FIG. 9. Principal components analysis of absorbance values for soils collected from STARS plots and the Pristine site. n=3.
FIG. 10. Graphical representation of raw data. Absorbance is plotted for each of the 95 carbon sources. Each line represents the mean for each treatment group. n=3.
FIG. 11. Analysis of environmental parameters vs. absorbance. Linear regressions have been calculated and plotted for each graph. Triplicate AWCD’s have been plotted against the means of (A) percent vegetative cover, n=3, (B) pH, n=3, (C) % moisture, n=3, and (D) % organic Carbon, n=2.
REFERENCES


