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The Effects of Heavy Metal Contamination on Bacterial Activities and Species Richness in Soils from Montana’s Clark Fork River Valley

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The Effects of Heavy Metal Contamination on Bacterial Activities and Species Richness in Soils from Montana’s Clark Fork River Valley

Submitted in Partial Fulfillment of the Requirements for Graduation With Honors to the Department of Natural Sciences at Carroll College, Helena, Montana

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April 5, 2004
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Date

Date

Date
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ABSTRACT

Bacteria play an essential role in nutrient cycling processes essential to the survival of plant and animal life. These processes may be adversely affected by the degradation of soil quality by heavy metal pollution. Heavy metal contamination is a significant problem in southwestern Montana’s Clark Fork River Valley due to the activities of the Anaconda Copper Mining Company during the years 1884-1980. This study focused on the effects of heavy metal contamination on bacterial activities, population density, and species richness. Using dehydrogenase activity, pH, ICP-AES analysis, plate counts, and ribosomal intergenic spacer analysis (RISA), I determined the following: (i) Availability of the heavy metals (Zn and Cu in particular) was pH-dependent; (ii) bacterial activity was pH- and metal concentration-dependent; (iii) tolerant bacterial populations were more prevalent as pH decreased and metal concentration increased; (iv) species “richness” decreased as pH decreased and concentration of metals increased; (v) RISA analysis suggested that soils contaminated with metals had lower species richness as determined by the presence of dominant bacterial communities.
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INTRODUCTION

Though not readily apparent, microorganisms constitute the major portion of Earth’s biomass (Madigan et al., 2003). Within the prokaryotes, the domain *Bacteria* is largely responsible for many essential ecologic functions. Bacterial communities in soil are involved in nutrient cycling and the process of nitrogen fixation (Madigan et al., 2003). Degradation of soils by metal pollution may limit these processes, and thereby adversely affect plants and animals.

Degradation of soils by heavy metal pollution has been shown to cause less efficient nutrient cycling (Pennanen et al., 1996). Several studies have demonstrated changes in soil microbial communities due to the presence of heavy metal contamination (Pennanen et al., 1996). Specifically, increases in the numbers of actinomycetes, fungi, and, most importantly, metal-tolerant bacteria as a result of the presence of heavy metals have been documented (Pennanen et al., 1996).

Heavy metal pollution of both soil and water is a significant problem in Montana; in fact, the nation’s largest SuperFund site is a 200-km section of the Upper Clark Fork River Valley in southwestern Montana. This site was contaminated with a variety of heavy metals including copper and zinc as a result of the operation of the Anaconda Copper Mining Company from 1884-1980 (Axtmann et al., 1997; Davis et al., 1999). Dull (2003) began a characterization of soil within the polluted region of the Clark Fork River Valley by examining the effects of copper on bacterial communities and concluded that bacterial activity was adversely affected by increased available levels of copper and that soils with
increased concentrations of copper and decreased pH had smaller populations of copper-tolerant bacteria. Along the same lines, Pennanen et al. (1998) found that lower pH caused a lower growth rate of bacteria. Dull (2003) also found a decrease in the metal tolerant bacterial population with elevated soil copper concentrations. Though this finding is consistent with the work of Yin et al. (2000), it does not agree with numerous previous studies, including that of Díaz-Raviña and Báath (1996).

In addition to measurements involving pH and metal concentration, biodiversity of a community is also valuable in determining the effects of heavy metals on bacteria in soil. Stability and productivity of ecosystems, in processes like nutrient cycling, are related to biodiversity (Yin et al., 2000). It has been hypothesized that higher levels of diversity create functional redundancy in an ecosystem thus creating stability (Doran et al., 1994). Biodiversity, measured as the number of bacterial species present in a soil, in this case can be quantified using a community fingerprinting method such as ribosomal intergenic spacer region analysis (RISA). This technique provides bands representative of the genetic structure of the community and is valuable in characterizing bacterial communities and detecting shifts following environmental perturbations, such as pollution by heavy metals (Ranjard et al., 2000).

Thus, this study sought to further the characterization of soil contaminated by heavy metals in the Clark Fork River Valley of Montana using measurements of bacterial activity, community fingerprinting, and population density. Previous characterization of this soil had employed an unrelated soil as a control; for the
present analysis, uncontaminated soil taken from a downstream site on the Clark Fork River was used. Further, the effects of two heavy metals, copper and zinc, were analyzed, while previously, only copper had been studied (Dull, 2003). I hypothesized that heavy metal pollution would negatively impact bacterial activity and metal contamination would cause a decrease in species richness (measured by community fingerprinting).
MATERIALS AND METHODS

Soil Sampling. Once in early June and once in mid-July 2003, four bulked soil samples consisting of four cores each were taken from each of two heavy metal-contaminated sites in the Upper Clark Fork River Valley near Galen, Montana. Four bulked soil samples consisting of four cores each were also taken from an uncontaminated site in the Lower Clark Fork River Valley near East Missoula, Montana. Sampling was conducted using a stainless steel soil corer to a depth of 8 cm. Cores were 2 cm in diameter. Samples were placed on ice during travel and were homogenized in plastic bags upon return to the laboratory. Samples were stored in a refrigerator at 4°C when not being used for testing.

Dehydrogenase Activity Assay. Soil dehydrogenase activity was measured using the method outlined by Dull (2003), which was a modification of Griffiths (1989). One gram of soil from each sample was combined with 1.5 mL of deionized water and 2.0 mL of 0.3% iodonitrotetrazolium violet (INT) in a test tube. Controls were established by autoclaving sample soils and were treated in the same manner as the experimental samples. The soil and solution were mixed thoroughly and incubated overnight in the dark at room temperature. Following incubation, 5 mL of extracting solution (1:1 dimethylformamide: methanol) were added to each sample and control. The mixture was then vortexed for 1 minute every 10 minutes for a 30-minute period. Finally, 1.5 mL of solution were transferred to a microcentrifuge tube and centrifuged at top speed for 5 minutes. Absorbance was read in a quartz cuvette at 460 nm on a spectrophotometer (Spectronic® Genesys) and compared to a standard curve
that had been constructed using successive 1:10 dilutions of a 500 μg/mL of iodonitrotetrazolium formazin in extracting solution. Deionized water, INT, and extracting solution were combined in the above proportions to make a blank for use in the spectrophotometer.

Arginine-Ammonification Assay. The method used was similar to that of Bonde et al. (2001). The following reagents were prepared prior to sampling: Phenol-alcohol reagent (10 g phenol dissolved in 95% ethyl alcohol and brought to final volume of 100 mL), sodium nitroprusside (1 g of nitroferricyanide dissolved in deionized water to final volume of 200 mL, and stored in a dark bottle for not more than one month), alkaline complexing reagent (100 g trisodium citrate and 5 g of sodium hydroxide dissolved in deionized water and brought to final volume of 500 mL), sodium hypochlorite (commercial bleach), and oxidizing solution (25 mL of alkaline complexing reagent plus 25 mL sodium hypochlorite, made fresh daily).

The following calibrants were also prepared prior to sampling: 1000 ppm NH₄-N (4.7168 g of dry (NH₄)₂SO₄ were dissolved in approximately 900 mL of deionized water in 1 L volumetric flask and labeled Stock Solution A), and 100 ppm NH₄-N (10 mL of Stock Solution A were diluted to 100 mL in a volumetric flask and labeled Stock Solution B). Using these calibrants, a standard curve was constructed based on concentrations of NH₄-N of 1000 μg/L, 750 μg/L, 500 μg/L, 200 μg/L, 100 μg/L, 50 μg/L, and 0 μg/L.

One gram of soil was weighed into a sterile screw-cap plastic vial, and 1 mL of 1 mM arginine was added. Arginine-free samples were also made.
Otherwise, treatment of controls was identical to treatment of samples. Vials were incubated at room temperature in the dark for 24 hours. Following the addition of 1 mL of 2 M KCl, the vials were shaken for one hour on a rotary shaker. In a microcentrifuge tube, 1.5 mL of this solution was centrifuged at high speed for 5 minutes.

In labeled test tubes, 1 mL of sample or control was combined with 40 μL of phenol solution, 40 μL of nitroferricyanide, and 100 μL of oxidizing reagent. This solution was mixed well and allowed to develop for 1-3 hours in darkness. Periodic mixing occurred during incubation. Absorbance was read on a spectrophotometer (Spectronic® Genesys) at 630 nm and absorbance values were compared to a standard curve. As a blank, a mixture of water, phenol, nitroferricyanide, and oxidizing reagent was used.

**Bacterial Population Density.** Serial 10:1 dilutions of soil samples were performed ranging from $10^{-1}$ to $10^{-6}$ in 0.01% NaCl. Spread plating of 100 μL of solution, in duplicate, was performed on nutrient agar media to which heavy metals had been added: 0 mM metals, 0.5 mM Cu, 0.5 mM Zn, 2 mM Cu, 2 mM Zn, 10 mM Cu, and 10 mM Zn. For all media, pH was adjusted to 7.5-8.0. For media containing 0 mM and 0.5 mM metal concentrations, $10^{-4}$, $10^{-5}$, and $10^{-6}$ dilutions were used for plating; on media with 2 mM metal concentration, $10^{-3}$, $10^{-4}$, and $10^{-5}$ dilutions were used for plating; and on media with 10 mM metal concentration, $10^{-2}$, $10^{-3}$, and $10^{-4}$ dilutions were used for plating. Plates were stored inverted in the dark and soil population density was determined based
upon bacteria colonies that were counted after 48 hours and 6 days.

Subsequently, plates were stored at 4°C for further use.

**Metal Extraction and ICP Analysis of Soil Metal Content.** To test for total metal concentration, approximately one gram of soil was weighed into a test tube and 5 mL of concentrated nitric acid were added. Tubes were placed in a beaker of sand on a hot plate and heated to near boiling in a fume hood for 2-4 hours, until most of the liquid had evaporated. Samples were then filtered on #41 filter paper and diluted to 100 mL in a volumetric flask. Some additional dilutions were necessary upon analysis.

To test for available metal concentration, approximately one gram of soil was weighed into a test tube, and 5 mL of 0.01 mM CaCl₂ were added. Tubes were then covered with parafilm and placed on a rotary shaker for 24 hours. Samples were filtered on #41 filter paper and diluted to 100 mL in a volumetric flask.

Using an Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) (Perkin Elmer Optima 2000 DV), samples were analyzed for both total and available metal concentration. A pre-set method, pre-made standards, and a water blank were used.

**Percent Moisture.** Soil was weighed into small glass beakers, and the mass of soil in each beaker recorded. Soil samples were then heated in a 60°C oven for 24 hours. Upon removal, samples were allowed to cool and reweighed in order to determine moisture content lost during heating.
pH. Two-gram samples of soil were weighed into small beakers. Two parts
deionized water were added to one part soil and stirred to make a paste. After
the paste settled for one hour, samples were analyzed using a pH meter
(Accumet AB15).

DNA Extraction. DNA was extracted according to directions found in the Bio
101® FastDNA SPIN Kit for Soil (Catalog #6560-200) made by Q-BIOgene.
Instructions in revision #6560-999-1D06 of the Application Manual (p. 7-8) were
followed exactly, except 785 µL of sodium phosphate Buffer, 98 µL of MT Buffer,
and 750 µL of binding matrix were used.

Polymerase Chain Reaction (PCR). The methodology followed for PCR
analysis closely resembled that outlined by Øvreås (1997) with minor
modifications. The reagents were combined to make PCR Master Mix as seen in
Table 1. After the reagents were combined, 24 µL of Master Mix and 1 µL of
DNA were added to each PCR tube. A control tube containing no DNA was also
made. The PCR temperature protocol was as follows: 92°C for 2 min, 92°C for
1 min, 55°C for 30 sec, 72°C for 1 min, and 72°C for 6 min. Cycles 2 through 4
were repeated 30 times each. Samples were then loaded with 5 µL of loading
dye and run on 1% agarose gel for 15-20 minutes at 120 V to determine success
of the PCR.

RISA Gel Analysis (Community Fingerprinting). Methodology was as
suggested by Ranjard et al. (2000), though modifications were made for the use
of a DGGE tank. PCR amplified intergenic spacer region fragments were loaded
onto a 0% denaturing polyacrylamide gel in a DGGE tank of 1X TAE buffer at
60°C. After 3.5 hours, the gel was removed, stained in dilute ethydium bromide solution for 15 minutes, and examined under a UV light.

**Statistical Analysis.** Data was tested for correlations between independent and dependent variables using Microsoft Excel® and Statistica®.

Table 1. Reagents and Volumes for PCR Master Mix

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume per Sample (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile Deionized Water</td>
<td>14.25</td>
</tr>
<tr>
<td>0.5M Tris Buffer (pH 8.3)</td>
<td>2.5</td>
</tr>
<tr>
<td>25 mM MgCl</td>
<td>2.5</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA), 10 mg/mL</td>
<td>1.25</td>
</tr>
<tr>
<td>5 mM dNTPs</td>
<td>1.25</td>
</tr>
<tr>
<td>Forward Primer (1522, 5 pmol/mL)</td>
<td>1</td>
</tr>
<tr>
<td>Reverse Primer (132, 5 pmol/mL)</td>
<td>1</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>0.25</td>
</tr>
</tbody>
</table>
RESULTS

Soil Properties. Determination of available and total heavy metal concentrations in study soils revealed higher concentrations of both copper and zinc in soils containing mine tailings (polluted soils, Galen, MT) than in soils lacking mine tailings (non-polluted soils, Missoula, MT). The total concentration of copper in polluted soils was more than 20-fold higher than in non-polluted soils, averaging 2976 ppm compared to 133 ppm in non-polluted vegetated soil, and the total zinc concentration in polluted soils was 3-6-fold higher than in non-polluted soils, averaging 135 ppm compared to 27 ppm in non-polluted vegetated soil. The available copper concentration in polluted barren soil was 50-100-fold higher than in polluted and non-polluted vegetated soils, averaging 153 ppm in polluted barren soil and only 1.25 ppm in polluted and non-polluted vegetated soils. Available zinc concentration in polluted barren soil was 3-5-fold higher than in polluted and non-polluted vegetated soils, here averaging 11.5 ppm in polluted barren soil and 0.75 ppm in polluted and non-polluted vegetated soils (Table 2).

pH was found to be higher in non-polluted vegetated soils than in either of the polluted soils. In fact, pH in the non-polluted vegetated soils was over 7 at both sampling times. Similarly, the pH of polluted vegetated soils was 6.2 and 6.5 respectively, while the pH of polluted barren soils was near 4.8 in June, 2003 and 5.0 in July, 2003 (Table 2).
Table 2. Table of Soil Properties

<table>
<thead>
<tr>
<th></th>
<th>Polluted Barren Soil</th>
<th>Polluted Vegetated Soil</th>
<th>Non-polluted Vegetated Soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Available Cu (ppm)</td>
<td>166</td>
<td>140</td>
<td>3</td>
</tr>
<tr>
<td>Available Zn (ppm)</td>
<td>9</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>Total Cu (ppm)</td>
<td>2598</td>
<td>3020</td>
<td>3253</td>
</tr>
<tr>
<td>Total Zn (ppm)</td>
<td>105</td>
<td>93</td>
<td>154</td>
</tr>
<tr>
<td>pH</td>
<td>4.8</td>
<td>5.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Percent Moisture</td>
<td>26.7</td>
<td>18.9</td>
<td>36.4</td>
</tr>
</tbody>
</table>

Dehydrogenase Activity Assay. Soil dehydrogenase activity was 3-5-fold higher in non-polluted vegetated soil than in polluted vegetated soil over the course of both sampling dates. Polluted barren soils taken from Galen, MT, had the lowest soil dehydrogenase activity of all study soils and were 2-8-fold lower than vegetated polluted soils at the same site (Figure 1). Soil dehydrogenase activity was negatively correlated with available copper and available zinc concentrations in soil (p=0.004 and p=0.016 respectively) (Table 3). Multiple regression analysis was also performed using dehydrogenase activity as the dependent variable and available copper concentration and available zinc concentration as the independent variables, yielding an overall p value of 0.01 (Table 4). Both available copper concentration and available zinc concentration
showed negative correlation patterns with dehydrogenase activity (Figures 2 and 3).

![Graph showing soil microbial activity in soils from the Clark Fork River Valley with different levels of available and total metals.](image)

**Figure 1.** Soil Microbial Activity in Soils from the Clark Fork River Valley with Different Levels of Available and Total Metals.

**Table 3.** Table of Statistical Values Using Activity as the Dependent Variable

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variable</th>
<th>R² Value</th>
<th>β Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity*</td>
<td>pH</td>
<td>0.154</td>
<td>0.058</td>
<td>0.071</td>
</tr>
<tr>
<td>Activity*</td>
<td>Total Cu</td>
<td>0.089</td>
<td>-0.030</td>
<td>0.178</td>
</tr>
<tr>
<td>Activity*</td>
<td>Total Zn</td>
<td>0.055</td>
<td>-0.044</td>
<td>0.291</td>
</tr>
<tr>
<td>Activity*</td>
<td>Available Cu</td>
<td>0.372</td>
<td>-0.041</td>
<td>0.004**</td>
</tr>
<tr>
<td>Activity*</td>
<td>Available Zn</td>
<td>0.280</td>
<td>-0.069</td>
<td>0.016**</td>
</tr>
<tr>
<td>Activity*</td>
<td>Available Total Metals</td>
<td>0.095</td>
<td>-0.023</td>
<td>0.162</td>
</tr>
</tbody>
</table>

* Activity was expressed in μg/g*h for analysis.
** Statistically significant p values.
Table 4. Table of Values from Multivariable Statistical Analysis

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variable</th>
<th>R² Value</th>
<th>B Value</th>
<th>F Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity*</td>
<td>Available Copper</td>
<td>0.40</td>
<td>-0.990</td>
<td>5.40</td>
<td>.01</td>
</tr>
<tr>
<td></td>
<td>Available Zinc</td>
<td></td>
<td>-0.410</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Activity was expressed in μg/g*h and available metal concentrations in ppm for analysis.

Figure 2. Correlation Analysis of the Effect of Available Copper Concentration on Bacterial Activity

Figure 3. Correlation Analysis of the Effect of Available Zinc Concentration on Bacterial Activity
Arginine-Ammonification Assay. No consistent results were obtained from this assay.

Zinc and Copper Tolerance. Higher proportions of the total soil bacterial population were tolerant to both copper and zinc in polluted vegetated soil than in non-polluted vegetated soil. Eighty percent of the soil bacterial community from polluted barren soil sampled in June, 2003 was tolerant to 10 mM copper and 60% was tolerant to 2 mM zinc. In contrast, for the later sampling date, less than 7% of the community was tolerant to 10 mM copper and less than 1% was tolerant to 2 mM zinc (Figures 4 and 5). There was a significant correlation (p=0.053) between concentration of available copper (ppm) and the proportion of the microbial community tolerant to 10 mM copper as measured by viable plate counts on copper containing agar plates (Table 5). When the data from both sampling dates, June 2003 and July 2003, were combined, a negative correlation ($y = -0.0159x + 0.108$) was observed (Figure 6). However when the two time points were analyzed separately, a positive correlation ($y = 0.0097x - 0.0056$) between available copper and the proportion of tolerant bacteria was observed in June (Figure 7a) while a negative correlation ($y = -0.0297 + 0.173$) was observed in July (Figure 7b).
Figure 4. Comparison of Bacterial Populations of Three Soils from the Clark Fork River Valley. (a) June, 2003, Copper Media  (b) July, 2003, Copper Media
Figure 5. Comparison of Bacterial Populations of Three Soils from the Clark Fork River Valley. (a) June, 2003, Zinc Media (b) July, 2003, Zinc Media
Table 5. Table of Statistical Values Using Metal Tolerance as the Dependent Variable

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variable</th>
<th>R² Value</th>
<th>β Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu Tolerance*</td>
<td>pH</td>
<td>0.023</td>
<td>0.012</td>
<td>0.503</td>
</tr>
<tr>
<td>Cu Tolerance*</td>
<td>Total Cu</td>
<td>0.003</td>
<td>-0.003</td>
<td>0.816</td>
</tr>
<tr>
<td>Cu Tolerance*</td>
<td>Total Zn</td>
<td>0.000</td>
<td>0.001</td>
<td>0.961</td>
</tr>
<tr>
<td>Cu Tolerance*</td>
<td>Available Cu</td>
<td>0.192</td>
<td>-0.016</td>
<td>0.053**</td>
</tr>
<tr>
<td>Cu Tolerance*</td>
<td>Available Zn</td>
<td>0.123</td>
<td>-0.025</td>
<td>0.129</td>
</tr>
<tr>
<td>Cu Tolerance*</td>
<td>Available Total Metal</td>
<td>0.014</td>
<td>-0.005</td>
<td>0.602</td>
</tr>
<tr>
<td>Zn Tolerance*</td>
<td>pH</td>
<td>0.027</td>
<td>0.030</td>
<td>0.466</td>
</tr>
<tr>
<td>Zn Tolerance*</td>
<td>Total Cu</td>
<td>0.003</td>
<td>-0.007</td>
<td>0.807</td>
</tr>
<tr>
<td>Zn Tolerance*</td>
<td>Total Zn</td>
<td>0.016</td>
<td>0.0301</td>
<td>0.569</td>
</tr>
<tr>
<td>Zn Tolerance*</td>
<td>Available Cu</td>
<td>0.042</td>
<td>-0.017</td>
<td>0.388</td>
</tr>
<tr>
<td>Zn Tolerance*</td>
<td>Available Zn</td>
<td>0.040</td>
<td>-0.032</td>
<td>0.396</td>
</tr>
<tr>
<td>Zn Tolerance*</td>
<td>Available Total Metals</td>
<td>0.050</td>
<td>-0.021</td>
<td>0.318</td>
</tr>
</tbody>
</table>

* Cu tolerance and Zn tolerance are equivalent to the percent of total bacteria tolerant to the appropriate metal. Metal levels were expressed in ppm for analysis.
** Statistically significant p values.
Figure 6. Correlation Analysis of the Effect of Available Copper Concentration on the Presence of Copper Tolerant Bacteria. Data for June and July are combined in this figure.
Figure 7. Correlation Analysis of the Effect of Available Copper Concentration on Presence of Copper Tolerant Bacteria (a) June, 2003 only (b) July, 2003 only

Community Fingerprinting (RISA Gel Analysis). Community fingerprinting showed that bacterial species “richness” decreased with increasing available metal concentration and decreasing pH. Further, soils with high levels of metals had a dominant bacterial community whereas non-polluted soils did not have dominant bacterial communities (Figures 8 and 9), as indicated by smearing in the gel which indicates a broad diversity of bacteria present in a given sample.
Figure 8. Bacterial Community Fingerprints from June 2003. Marker 1 shows non-polluted vegetated soils, marker 2 shows polluted vegetated soils, and marker 3 shows polluted barren soils.
Figure 9. Bacterial Community Fingerprints from July, 2003. Marker 1 shows non-polluted vegetated soils, marker 2 shows polluted vegetated soils, and marker 3 shows polluted barren soils.
DISCUSSION

Concentration of available metals was higher in soils with lower pH (Table 2). Thus, polluted barren soil had the highest available metal concentration and lowest pH, while non-polluted vegetated soil had the lowest available metal concentration and highest pH. Although regression analysis showed no statistical correlation (Table 3) between available metal concentrations and pH, these results concur with the findings of Dull (2003) who found similar trends at nearby sites. In general, for each pH unit decrease there is a subsequent 100-fold increase in the concentration of divalent ions such as Zn$^{2+}$ and Cu$^{2+}$ (Lindsay, 1979). The lack of statistical correlation is likely due to the variability among samples, especially between those taken at two different times. Soil conditions such as temperature, moisture, and nutrient status can change rapidly causing dramatic shifts in soil community structure. More frequent sampling and standardized processing of the samples might lead to more conclusive results.

Microbial activity was lowest in soils with the highest concentrations of available Zn and Cu (Figure 1). Multiple regression analysis showed a significant negative correlation (Table 4) between bacterial activity and available copper concentration and available zinc concentration (Figures 2 and 3). These results support my original hypothesis, that concentration of heavy metals would negatively impact bacterial activity, and they also agree with the findings of Dull (2003). Further, Renella et al. (2002) reported that the main effect of heavy metals is to decrease the overall rate of respiration, especially when the influx of heavy metals occurs over a long period of time. The effect is most likely due to
the toxicity of the heavy metals to many bacterial species. Along these same lines, it has also been suggested that since bacteria are normally acclimated to the pH of the environment in which they live, the decrease in activity may be a result of the increasing metal concentrations (Díaz-Raviña and Bååth, 1996).

With the data for June, 2003 and July, 2003 combined, a higher proportion of the total soil bacterial population was tolerant to copper or zinc in metal-contaminated soils than in non-contaminated soils (Figures 4 and 5). There was not a significant correlation between available zinc concentration and the proportion of the bacterial population tolerant to either Zn or Cu. However, there was a significant negative correlation between available copper concentration and percent of the total bacteria tolerant to copper (Table 5, Figure 6). Previous research at nearby sites found that as the concentration of metals increased, the proportion of tolerant bacteria decreased (Dull, 2003). These results do not agree with Díaz-Raviña and Bååth (1996) who found that the bacterial communities in metal-amended soils were more tolerant of high levels of metals than the communities from non-amended soils.

When the June and July data sets were analyzed independently, the results of regression analysis were quite different. The June time point showed a positive correlation between the available copper concentration and percent of the total bacteria tolerant to copper (Figure 7a) while available copper concentration and percent of the total bacteria tolerant to copper were negatively correlated using only the July data set (Figure 7b). The results of the June data set agree with the work of Díaz-Raviña and Bååth, who (1996) found a similar
effect. They suggest three different mechanisms for the increased tolerance found in contaminated soils: (i) an immediate toxic effect killed all bacterial species sensitive to the high levels of heavy metals; (ii) tolerance to metals was selected based on competition of surviving species; and (iii) the adaptation of the bacteria found in the polluted environment is due to some sort of physiological or genetic change. Díaz-Raviña and Bååth (1996) further noted that the specific mechanism functioning in a soil bacterial community might be related to the level of pollution found there. Small sample size, variability of viable plate counts, changing environmental conditions between June and July, and inconsistent sampling technique may all contribute to the inconsistent results of the two sampling times.

Bacterial species "richness" increased with decreasing available metal concentration and increasing pH. Thus, non-polluted soils contained greater numbers of species than polluted soils. This effect was observed by comparing the banding patterns in the RISA gels (Figures 8 and 9), where one band roughly correlates with one bacterial species. In the most heavily polluted soil, the barren polluted sample, only a few bands are seen. In the other polluted soil, the vegetated polluted soil, a larger number of bands are seen. Finally, in the samples of uncontaminated vegetated soils, smearing is seen. The lack of resolution of individual bands indicates the presence of a greater number of species. Thus, soils with high levels of metals had a dominant bacterial community composed of relatively few bacterial species that were able to withstand the heavy-metal contamination in the soil. Soils with lower levels of
contamination lacked this selective pressure and therefore did not show the presence of a dominant bacterial community. These results support my original hypothesis, and again agree with the work of Díaz-Raviña and Bååth (1996). The three mechanisms mentioned above in explaining the increased tolerance of bacterial populations in contaminated soil again apply. Similar results using the RISA method were also noted by Ranjard et al. (2000), as the enrichment of certain species following contamination by heavy metals was noted.

In summary, five conclusions may be drawn from my research: (i) Availability of the heavy metals (Zn and Cu in particular) was pH-dependent; (ii) bacterial activity was pH- and metal concentration-dependent; (iii) tolerant bacterial populations were more prevalent as pH decreased and available metal concentration increased; (iv) species "richness" decreased as pH decreased and available concentration of metals increased; (v) RISA analysis suggested that soils contaminated with metals had lower species richness as determined by the presence of dominant bacterial communities. Thus, I accept my hypotheses.
LITERATURE CITED


