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Long Term Effects of Heavy Metal Contamination on Bacterial Communities in the Upper Clark Fork River Canyon

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Long Term Effects of Heavy Metal Contamination on Bacterial Communities in the Upper Clark Fork River Canyon

Submitted in partial fulfillment of the requirements for graduation with honors to the Department of Natural Sciences at Carroll College, Helena, Montana

Marcus Simonich

April 4, 2005
This thesis for honors recognition has been approved for the

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Acknowledgements

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Microorganisms and their activities drive most ecosystems and therefore must be assessed in order to study ecosystem stability. Heavy metal contamination is known to have adverse effects on soil bacteria. In this study, bacterial populations from polluted (PV and PB) and non-polluted (NV) soils were compared in order to better understand soil resiliency, ecosystem stability, and the long-term effects of heavy metals have on bacteria. Biological parameters of soil quality including diversity, structure, activity, and heavy metal tolerance were compared and assessed. The numbers of Zn and Cu tolerant bacteria were determined by spread plating diluted soil samples onto agar plates containing 0.5, 2, and 10 mM concentrations of the metals. Arginine and iodonitrotetrazolium assays were used to measure the extent of ammonification and dehydrogenase activity in the soil. And, 16S rDNA cloning and sequencing was used to identify 60 individual microorganisms from both the NV and PV soils. Neighbor-joining phylogenetic trees, constructed with the 16S rDNA clones, were then used to analyze differences in bacterial diversity and community structure. I hypothesized that the population in the NV soil would: 1) have higher activity levels, 2) be more diverse, 3) contain fewer metal tolerant microorganisms, and 4) show a different community structure than the bacterial populations isolated from the polluted soils. All hypotheses were found to be correct except for the hypothesis addressing the diversity of the two soils (NV and PV). It was found that both soils were very diverse in bacterial species and that 60 clones from each soil was not a big enough sample size to address the differences in diversity between the two bacterial populations isolated from the two soils.
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Introduction

History has repeatedly shown that mismanagement of the soil can lead to poverty, malnutrition, and economic disaster (Doran et al., 1994). The Upper Clark Fork Valley is a prime example. More than 65,000 acres in this region have been affected by heavy-metal and particulate matter discharges into air and stream as a result of the operation of the Anaconda Copper Mining Company (CFRTAC, 2004). The Milltown Dam on the Clark Fork River separates the polluted stretches of the Clark Fork from the significantly less polluted stretches below the man-made barricade. Over the past 100 years the Milltown Dam has acted as a settling pond, reducing the flow rate of the Clark Fork River to a standstill, which resulted in the sedimentation of heavy metal laden sediments from the river water being trapped behind the dam. In this study, long-term effects of these copper and zinc tailings on soil bacterial communities in the upper Clark Fork River Valley were investigated.

While some scientists have studied the effects of heavy metals on microorganisms (Renella et al., 2001; Yin et al., 2000), very little is known about the long-term adverse effects of heavy metals on bacterial communities and their relationship to soil resiliency. Intrinsic soil properties governing soil resilience that are also important to soil quality are biodiversity, soil structure, soil water, cation-exchange capacity, organic matter content, and pH (Doran et al., 1994). Doren et al. (1994) defined soil resiliency as the ability of a soil to: 1) tolerate stress 2) return to a new dynamic equilibrium after disturbance, and 3) resist adverse changes under a given set of ecological and land use conditions. And,
according to Diaz-Ravina and Baath (1999), the capacity of the soil to restore itself after disturbance needs further investigation.

It has been assumed that ecosystem stability and productivity are influenced by the diversity of soil microorganisms (Doran et al., 1994; Yin et al., 2000). Ecosystems are maintained by soil and the processes carried out by the microorganisms in soil. The soil provides nutrients for plants, which are the main staples of animal diets (Doren et al., 1994). In addition, microorganisms detoxify organic materials and recycle many nutrients and global gases (Doren et al., 1994). Previous studies have shown that metal contamination can have adverse effects on soil quality (Dull, 2003; Grantz et al., 2003; Palmer, 2004). Soil quality has been referred to as the fitness of a specific kind of soil, to function within its capacity and within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation (Doren et al., 1994). Unlike water or air quality, soil quality is difficult to measure since different chemical, physical, and biological factors contribute to the overall quality of soil (Doran et al., 1994). Only the biological parameters of soil quality are investigated in this study. These parameters include bacterial productivity, population numbers, structure, diversity, and heavy metal tolerance.

Diaz-Ravina and Baath (1996) studied the time scale for the development of tolerance in soil bacterial communities exposed to known concentrations of metals. This was similar to the studies carried out by Sykes (2005) and Davis (2005), which were performed under laboratory conditions with known heavy metal concentrations and exposure lengths. Unlike these studies, the present study was performed in a natural
setting were the concentrations of heavy metals (copper and zinc) and the exposure lengths of soil microorganisms to these metals could have varied significantly. In soil, the ratio of metal-tolerant to metal-sensitive bacteria (determined by plate counts) has been suggested as a relevant measure of community tolerance to metals (Diaz-Ravina and Baath, 1996). The types of the microorganisms present after heavy metal contamination is important to determining the role bacterial communities play in maintaining soil system structure and resiliency. For this reason, bacterial community tolerance to heavy metals was measured in three populations of bacteria, and the overall proportion of Zn and Cu tolerant microorganisms from each soil type was determined.

Metal tolerant microorganisms have been found at higher frequencies in polluted habitats (Diaz-Ravina and Baath, 1996), and they suggested three means by which bacterial communities might become tolerant to heavy metals: 1) an immediate, toxic effect killing sensitive species; 2) a selection of metal tolerance due to the different competitive abilities of surviving bacteria; and 3) adaptation of bacteria developing in these polluted soils due to physiological and/or genetic changes. It has also been discovered that ATPases regulate heavy metal tolerance in prokaryotes (Rensing et al., 1999; Beard et al., 1997).

Heavy metals deposited directly into soil negatively impact nutrient cycling, oxygen fixation, and nitrogen fixation carried out by soil microorganisms (Grantz, 2003). It has been shown that heavy metals decrease ammonification and oxygen fixation rates in bacterial communities (Dull, 2003). In the past, activity levels for various metabolic processes have been used to assess the health and viability of bacterial communities in soil (Renella et al., 2001). In this study, arginine-ammonification (Bonde et al., 2001)
and dehydrogenase (Griffiths, 1989) assays were used to measure and assess microbial activity. These assays were performed on three different soils: non-polluted vegetated (NV), polluted vegetated (PV), and barren polluted (PB).

Culture independent techniques including 16s rRNA sequencing, denaturing gel gradient electrophoresis (DGGE), and RIKEN integrated sequence analysis (RISA) have been shown to be suitable methods for studying bacterial diversity and community structure in different microbial environments (Borneman and Triplett, 1997; Ovreas et al., 1997). Since a majority of bacteria cannot be grown under laboratory conditions, culture independent analysis of the 16S ribosomal gene has provided a means to study many culturable and unculturable microorganisms without the need to first grow them on laboratory media (Torsvik et al., 1998; Borneman and Triplett, 1997). Using 16s rRNA gene sequence analysis, Borneman and Triplett (1997) found the microbial community structure of an Amazonian forest’s soil to be significantly different than the same soil that had been deforested and used as pasture. In the present study, 16S rDNA sequence analysis was used to examine the differences in diversity and community structure between bacterial communities from polluted and non-polluted soils taken from the Clark Fork River Valley. Phylogenetic analysis of 60 clones from the NV and PV soils provided a detailed look at the community structures of both communities. With this information, ways by which microorganism communities adapt or shift in structure in order to accommodate to heavy metal contamination were explored.

The primary focus of this study was to analyze the differences between biological parameters (heavy metal tolerance, diversity, structure, and activity) of polluted and non-polluted soils in order to gain insight as to how heavy metal contamination effects soil
microorganisms, soil quality, and ultimately, the ecosystem. The Clark Fork Valley provided ideal conditions for such a study due to the presence of both polluted and nonpolluted soils, which were both similar in composition and climatic condition. In light of the fact that the soil along the Upper Clark Fork has been polluted with heavy metals since 1884 and the lower site has received less contamination by metals because of the Milltown Dam (Carlson, 2004; CFRTAC, 2004), I hypothesized: 1) the bacterial populations isolated from the polluted soils would have more Zn and Cu tolerant microorganisms than the bacterial population isolated from the non-polluted soil, 2) the non-polluted soil samples would have greater ammonification and dehydrogenase activity levels, 3) the bacterial population obtained from the non-polluted vegetated soil would be more diverse in microorganism species than the bacterial population obtained from the barren polluted soil, and 4) the structure of the bacterial community in the polluted soil would be different than the structure of the bacterial community observed in the non-polluted soil.
Materials and Methods

**Soil Sampling.** Three different soils were collected: nonpolluted vegetated (NV), polluted vegetated (PV), and barren polluted (PB). The polluted samples were taken from a site contaminated with heavy metals near Galen, Montana, while the nonpolluted samples were collected below Milltown Dam near East Missoula. A soil sample consisted of three soil cores mixed together in a ziplock bag, and for each soil type four soil samples were taken. Soil cores were 2 cm in diameter by 10 cm deep. The soil corer was washed before new core samples, from a different soil type, were collected. The collected samples were then placed in a styrofoam cooler with ice and transported back to the laboratory in Helena, Montana, for testing.

**Bacterial Population Density.** In the lab, the soil samples were serially diluted (10:1) from $10^{-1}$ to $10^{-6}$ in 0.01% NaCl. Copper or zinc was added to 10% nutrient agar media at four different concentrations: 0 mM, 0.5 mM, 2 mM, and 10 mM. The pH of each medium was adjusted to 7 prior to autoclaving. The $10^{-4}$, $10^{-5}$, and $10^{-6}$ soil dilutions were used for plating on agar media containing 0 mM and 0.5 mM concentrations of Cu or Zn, and the $10^{-2}$, $10^{-3}$, and $10^{-4}$ dilutions were plated on the media amended with 2 mM and 10 mM concentrations of Cu or Zn. Plating was executed in duplicate using 100 μL of the corresponding dilution and a sterile glass spreader. To prevent condensation from gathering on the growing bacterial colonies and to imitate an underground environment, the inoculated plates were stored up-side down in a dark room at room temperature. Plates were counted at 2-days and 7-days after inoculation. After counting, the plates were stored in a cold room at 4°C.
**Dehydrogenase Activity Assay.** Soil dehydrogenase activity was measured using a modified version of Griffiths' (1989) procedure, the same method used by Dull (2003). Absorbance of iodonitrotetrazolium formazan (formazan) was measured at 460 nm in a spectrophotometer. The obtained absorbance readings were then used to calculate the rate of dehydrogenase activity using a fitted-line equation from a standard curve, which was constructed with successive dilutions of 500 µg/mL of formazan in extracting solution. The blank used for the dehydrogenase assay was a mixture of deionized water, iodonitrotetrazolium chloride (INT), and extracting solution.

**Arginine-Ammonification Assay.** The procedure used for the arginine-ammonification assay was a modified version of the procedure carried out by Bonde et al. (2001). The modified version is exactly the same as the procedure used by Palmer (2004) in his study concerning the adverse effects of zinc on bacterial communities. Modifications included the use of one gram of soil from each microcosm and the incorporation of 1 mM arginine into the final reaction mixture. Absorbance readings from serially diluted NH₄-N concentrations provided the information needed to construct a standard curve for the arginine-ammonification assay. Stock solutions of 1000 ppm NH₄-N and 100 ppm NH₄-N were combined to make seven different concentrations of NH₄-N: 1000 µg/L, 750 µg/L, 500 µg/L, 200 µg/L, 100 µg/L, 50 µg/L, and 0 µg/L. Absorbance readings of the solutions were read at 630 nm on a spectrophotometer.

**DNA Extraction.** Total genomic DNA from soil was extracted using a Bio101® Fast DNA SPIN Kit (Catalog #6560-200), manufactured by Q-BIOgene. The application manual was followed exactly except 785 µL of sodium phosphate buffer, 98 µL of MT buffer, and 750 µL of binding matrix were used instead of the protocol volumes.
**PCR primers and protocol.** The method used to amplify raw soil DNA from each microcosm was similar to the method used by Øverås (1997). For the nested PCR, the 16f to 1400r region of the 16S ribosomal DNA was first amplified using primers 16f (AGAGTTTTGATCCTGGCTCAG) and 1492r (AAGGAGGTGATCCAGCCGCA) (Lane, 1991). The presence of this PCR product was confirmed by analyzing 20 μl of PCR product with 5 μl of loading dye on a 1% agarose gel for 20 minutes at 120 V. The 16f-1492r fragment of the 16S rDNA was then cut from the gel using a clean razor blade and placed into a microcentrifuge tube with 20 μl of dH2O. The tubes were then stored in the refrigerator at 4°C overnight, which allowed the DNA to diffuse out of the agar and into the water. PCR was again performed in which the 16f to 1492r fragments were used as the DNA template for the amplification of the 16f to 518 region of the 16S rDNA. One micro-liter of the 16f-1492r fragment/water mixture was used along with primers 16f (AGAGTTTTGATCCTGGCTCAG) and 518r (ATTACCGCGGCTGCTGG) for the second half of the nested PCR (Lane, 1991). Success of the PCR replication process was confirmed via gel electrophoresis.

In each PCR tube, 24 μl of master mix was combined with 1 μl of template DNA. The 25 μL PCR mixture contained the following ingredients at the indicated amounts: 1 μl template DNA, 14.25 μl 0.5 M Tris buffer, 2.5 μl 25 mM MgCl, 1.25 μl Bovine Serum Albumin (BSA), 1.25 μl 5.0 mM deoxynucleoside triphosphates (dNTPs), 1 μl of each primer, and 0.25 μl of *Taq polymerase*. PCR was performed in a Perkin-Elmer-Cetus thermal cycler with 30 cycles of 92°C for 1 min, 55°C for 30 sec, 72°C for 1 min, and a final extension of 72°C for 6 min.
**Cloning.** Following PCR amplification of the 16r-518f fragment of the 16S ribosomal gene, the amplified 500-bp fragments were isolated via gel electrophoresis and excised from the gel with a razor blade. The DNA fragments were purified from the gel using a Qiagen QIAEX® II gel extraction kit (Catalog #20021). The fragments were then inserted into the pGEM®-T Easy plasmid and transformed into *E. coli* JM109 (Promega). Fifty micro-liters of transformed cells were then inoculated into 950 µl Luria-Bertani broth (LB) and 50 µl ampicillin, in a microcentrifuge tube. The inoculated microcentrifuge tubes were incubated at 37°C on a shaker for 90 minutes. After incubation, 100 µL of cells in the LB broth were spread plated onto LB agar plates amended with X-gal, IPTG, and ampicillin (50mg/L). Bacterial colonies that grew on the amended plates were individually picked and used to inoculate additional microcentrifuge tubes containing 950 µl of LB broth and 50 µl of ampicillin. The tubes containing the cultured isolates were incubated at 37°C overnight.

**Plasmid Extraction and Sequencing.** Plasmids containing the amplified 16S rDNA fragments within the *E. coli* clones were extracted using the Novagen® Spinprep™ plasmid kit (Catalog #70851-3AT) or Qiagen QIAprep spin miniprep kit (Catalog #27104). The extracted plasmids were then sent to Macrogen, South Korea, to be sequenced. Sequencing reactions were performed in a *MJ Research PTC-225 Peltier Thermal Cycler* using ABI PRISM BigDye™ Terminator Cycle Sequencing Kits with *AmpliTaq DNA polymerase* (Applied Biosystems).

**Sequence Analysis.** Sequence analysis was done using the computer program ChromasPro 1.22 (http://www.technelysium.com.au/ChromasPro.html). In this program, blast searches (www.ncbi.nlm.nih.gov/blast/Blast.cgi) were performed on the V3 region
of the sequenced 16S rDNA, and species identification was executed by matching known sequences with unknown sequences. Identified sequences were then aligned using Clustal (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) and then used to construct neighbor joining phylogenetic trees using Paup*4.0b10 (Swofford, 2002).

**Statistical Analysis.** Statistical tests were performed using the data-analysis tool ANOVA from the computer based program Excel.
Results

Zinc and Copper Tolerance. Higher proportions of the total bacterial population were tolerant to 0.5 mM and 2 mM copper in the barren polluted and polluted vegetated soils than the non-polluted vegetated soil (Figure 1). About thirty percent of the soil bacterial population from the PB soil was tolerant to 0.5 mM copper, while only 15% was tolerant to 2 mM copper. An opposite trend in tolerance was observed for the bacteria plated on 0.5 mM zinc (Figure 2). Interestingly, close to 17% of the bacterial population from the NV and PV soils were tolerant to 0.5 mM zinc, and all three of the bacterial populations studied seemed to exhibit similar tolerance proportions to 2 mM zinc. Very few bacteria from all three populations were tolerant to 10 mM zinc or copper (data not shown). There was a significant correlation \( (P-value = 1.56E-04) \) between the soil copper concentration (ppm) and the number of bacteria tolerant to 0.5 and 2 mM copper, as measured by plate counts on copper amended agar plates (Table 1). Alternatively, no significant correlation was found \( (P-value = 0.62) \) between soil zinc concentrations and the number of zinc tolerant bacteria in a population (Table 1).

<table>
<thead>
<tr>
<th>Tests for Significance</th>
<th>Independent Variable</th>
<th>P-value</th>
<th>F</th>
<th>F_{crit}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydrogenase Activity</td>
<td>Total Metals in Soil</td>
<td>8.91E-06</td>
<td>55.12</td>
<td>4.26</td>
</tr>
<tr>
<td>Arginine-ammonification Activity</td>
<td>Total Metals in Soil</td>
<td>0.013</td>
<td>7.23</td>
<td>4.27</td>
</tr>
<tr>
<td>Copper Tolerance</td>
<td>*Total Cu in Soil</td>
<td>1.56E-04</td>
<td>12.34</td>
<td>3.35</td>
</tr>
<tr>
<td>Zinc Tolerance</td>
<td>*Total Zn in Soil</td>
<td>0.62</td>
<td>0.49</td>
<td>3.26</td>
</tr>
</tbody>
</table>

*Total soil concentrations for Cu and Zn were obtained from Carlson (2004)
FIGURE 1. The proportion of bacteria tolerant to 0.5 and 2 mM copper in the non-polluted, polluted vegetated, and polluted barren populations.

FIGURE 2. Proportion of bacteria tolerant to 0.5 mM and 2 mM zinc in the non-polluted, polluted vegetated, and polluted barren soils.
**Activity Assays.** Soil copper and zinc concentrations were found to significantly affect soil dehydrogenase ($P-value = 8.91\text{E}-06$) and arginine-ammonification ($P-value = 0.013$) activity (Table 1). The bacterial populations taken from the NV, PV, and PB soils had average dehydrogenase activity levels of 1.8, 0.7, and 0.0 $\mu$g/(g*h), respectively (Figure 3). Additionally, average arginine-ammonification activity levels followed a similar decreasing trend at 633.3, 338.8, and 61.12 $\mu$g/(g*h) (Figure 4). Overall, polluted soils showed lower levels of dehydrogenase and arginine-ammonification activity than the non-polluted soil.

![Image of bar chart showing dehydrogenase activity levels in NV, PV, and PB soils.](image_url)

**FIGURE 3.** Dehydrogenase activity levels in the nonpolluted, polluted vegetated, and polluted barren soil samples.
Diversity and Structure. The microbial diversity found in the NV and PV soils is illustrated by phylogenetic tree analyses (Figures 5 & 6; Table 2). The bacterial communities in the NV and PV soils contained twelve taxa. Sixty-two percent of the clones isolated from the NV soil samples were Bacteriodetes and Proteobacteria, with 37% from the α subdivision of Proteobacteria alone. Alternatively, Bacteriodetes, Green non-sulfur bacteria, Proteobacteria, and Planctomycetes encompassed sixty-two percent of the clones isolated from the PV soil samples. Interestingly, about twice as many clones from the polluted soil were determined to be unclassified microorganisms.

Significant differences in the number of Acidobacteria, Planctomyces, α Proteobacteria, Gemmatimonadetes, and Green non-sulfur bacteria were found between the two soil types. Acidobacteria, α Proteobacteria, and Gemmatimonadetes were found to be predominantly associated with the non-polluted soil, more Planctomyces and Green non-sulfur were found in the polluted vegetated soil.
TABLE 2. Phylogenetic assignments of 16S rDNA clones for the nonpolluted (NV) and polluted (PV) soils. Sixty clones were sequenced for each soil type.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-polluted</td>
</tr>
<tr>
<td>Acidobacteria / Fibrobacteres</td>
<td>8</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>8</td>
</tr>
<tr>
<td>Bacteriodetes</td>
<td>15</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>0</td>
</tr>
<tr>
<td>Firmacutes</td>
<td>0</td>
</tr>
<tr>
<td>Gemmatimonadetes</td>
<td>8</td>
</tr>
<tr>
<td>Green non-sulfur bacteria</td>
<td>0</td>
</tr>
<tr>
<td>Planctomyces</td>
<td>5</td>
</tr>
<tr>
<td>α Proteobacteria</td>
<td>37</td>
</tr>
<tr>
<td>β Proteobacteria</td>
<td>8</td>
</tr>
<tr>
<td>γ Proteobacteria</td>
<td>2</td>
</tr>
<tr>
<td>Unclassified</td>
<td>9</td>
</tr>
</tbody>
</table>
FIGURE 5. Phylogenetic analysis of 60 16S rDNA sequences from the polluted vegetated soil. Soil clones are represented by the letter m, followed by a reference number. Known sequences used to identify the soil clones were labeled with the letter k, followed by a reference number. Names of the major taxa are shown to the right. The number of substitutions per sequence position, at the median rate, is shown on the scale bar.
FIGURE 6. Phylogenetic analysis of 60 16S rDNA sequences from the non-polluted vegetated soil. Soil clones are represented by the letter m, followed by a reference number. Known sequences used to identify the soil clones are labeled with the letter k, followed by a reference number. Names of the major taxa are shown to the right. The number of substitutions per sequence position, at the median rate, is shown on the scale bar.
Discussion.

It is quite possible that bacterial communities from the Upper Clark Fork River Valley have diverged from a healthy community to a community responding to large doses of heavy metal contamination. In this study, differences in productivity, diversity, structure, and heavy metal tolerance of bacterial communities, from contaminated (above Milltown Dam) and non-contaminated soils (below Milltown Dam), were evaluated in order to investigate the long-term effects heavy-metal contamination has had on soil microorganisms, soil quality, and ecosystem stability.

Polluted and non-polluted soils, varying in both disturbance and contamination intensity, make the Clark Fork River Valley an ideal place for: 1) measuring differences among bacterial community tolerance to Cu and Zn in a natural system, 2) analyzing the effects of heavy-metal contamination on bacterial community diversity over a long period in time, 3) analyzing the long term effects of heavy-metal contamination on the productivity of bacterial communities, 4) evaluating the extent to which bacterial communities shift in structure to accommodate heavy-metal contamination, and 5) studying the role bacterial communities play in maintaining soil resiliency and ecosystem stability.

My first hypothesis was that there would be more Cu and Zn tolerant bacteria in the PV and PB soils than the NV soil. The bacteria isolated from the polluted soils did, in fact, contain a higher proportion of metal tolerant bacteria at 0.5 mM Cu, 2 mM Cu, and 2 mM Zn (Figures 1 & 2). This finding supports the "dosage-response" idea proposed by Diaz-Ravina and Baath (1996). In addition, it agrees with results obtained from Carlson
(2004), who reported that tolerant bacterial populations were more prevalent as metal concentrations increased in the soil. An exception to my first hypothesis was observed at 0.5 mM Zn. At this zinc concentration, the bacterial population isolated from the NV soil contained more Zn tolerant bacteria than the population isolated from the PB soil (Figure 2). However, many studies support the idea that bacteria follow unusual tolerance trends when exposed to low concentrations of zinc (Palmer, 2004; Diaz Ravina and Baath, 1996). Diaz Ravina and Baath (1996) reported that increases in the bacterial community tolerance level were observed only at concentrations above 1 mM of zinc.

Several possibilities exist that explain why zinc tolerance was not exhibited by bacteria at low zinc concentrations. Carlson (2004) found that zinc levels in the PV soil samples were not significantly higher than in the NV soil samples, where no zinc was detected. Zinc is a micronutrient that is needed by cells for healthy functioning (Madigan et al., 2003). While high concentrations of zinc (>1mM) may be toxic, a zinc concentration of 0.5 mM in nutrient agar may not have been sufficiently high as to be toxic to many soil bacteria, and may have actually provided sufficient levels of zinc for bacteria suffering from nutrient deficiency. However, the small proportion of bacteria tolerant to 0.5 mM zinc more than likely suggests that this level of zinc is at the borderline of being toxic and that many bacteria in soil can tolerate 0.5 mM concentrations of zinc even in the absence of previous zinc exposure. Another possibility as to why zinc tolerance is not exhibited by bacteria at low zinc concentrations could have to do with the number and type of zinc efflux proteins present inside or on the plasma membrane of the bacterium. Many bacteria have zinc efflux proteins (Madigen et al., 2003; Beard et al., 1997), which pump out excessive zinc ions that find their way into
the cytoplasm of bacteria. Rensing et al. (1999) claim that heavy metal resistance in prokaryotes is maintained by soft-metal-ion-transport ATPases. According to Rensing et al. (1999), zinc uptake is regulated by an ABC transporter consisting of three proteins: ZnuA (binding protein), ZnuB (membrane pump), and ZnuC (ATPase). Likewise, zinc efflux is regulated by ZntA, a zinc-transporting P-type ATPase (Rensing et al., 1999). In my study, 17% of the total bacterial population was tolerant of 0.5 mM Zn in the NV and PV soils. Only about 4% of the total bacterial population was tolerant to 0.5 mM Zn in the PB soil. This may suggest that there are more ZntA efflux ATPases than ZnuABC uptake transporters in the membranes of the bacteria in the NV and PV soils. Quite possibly, bacteria in the BP soil could have more ZnuABC uptake transporters than ZntA efflux ATPases in their membranes, and in environments with low zinc concentrations this ratio of transmembrane proteins could be lethal: the rate of zinc uptake would override zinc efflux and bacteria in the BP soil would overaccumulate zinc ions. Hence, microorganisms able to introduce efflux or uptake mechanisms into their membranes the fastest could have a competitive advantage over other microorganisms. This is in agreement with Diaz-Ravina and Baath (1996), who suggested that long-term increase in metal tolerance of a bacterial community can be attributed to its ability to compete and adapt.

The second hypothesis made was that the NV soil would have greater ammonification and dehydrogenase activity levels than the bacterial population in the PV and the PB soil. Indeed, the NV soil was more active in both dehydrogenase and ammonification activity than the PV and PB soils (Figure 3 & 4). This is in agreement with the regression analysis carried out by Carlson (2004) on the relationship between the
total, measured concentration of metals in the soils and soil activities. These decreased activities could be the result of a number of different mechanisms, all of which involve the adverse effects of heavy-metal contamination on bacterial populations. Heavy metal contamination has been known to affect functional redundancy, species diversity, population size, and cell efficiency (Grantz et al., 2003; Yin et al, 2000; Borneman & Triplett, 1997).

Results from this study suggest that plants are an important aspect of the restoration of soil biological functionality. The bacterial population isolated from the polluted barren soil contained more bacteria tolerant to 0.5 mM Cu, 2 mM Cu, and 2 mM Zn than the population isolated from the polluted vegetated site (Figures 1 & 2). Moreover, the bacterial community in the PV soil showed higher activity levels, in both assays, than the PB soil (Figures 3 & 4). Combined, these observations suggest that plants and their roots may act as biological buffers, reducing, at least to some extent, the harmful effects heavy metals have on soil microorganisms. Interestingly, Yin et al. (2000) found that bacterial functional redundancy increases in relation to the regrowth of plant communities. It is possible that the significant reduction in the number of viable microorganisms present in the (PB) soil, coupled with the idea that plants help to restore functional redundancy (Yin et al, 2000), hints towards the effects of heavy-metals on functional redundancy as a contributing factor to decreased productivity in contaminated soils. But, the actual extent and role of functional redundancy in microbial populations is unknown (Diaz-Ravina and Baath, 1996).

My third hypothesis was that the NV soil would contain more species of bacteria than the PV soil. Taken as a whole, the bacterial diversities in the two soils were large.
This assertion was supported by the fact that very few of the sixty 16S rDNA clones that were sequenced from each soil type were redundant microorganisms. Similar results were found by Borneman and Triplett (1997) in their study on the effects of deforestation on bacterial communities in eastern Amazonian soils. They found that none of the sequenced 100 SSU rDNA clones, taken from pasture soil, were duplicates. Despite the immense diversities of the microorganisms present in both soils, 16S rDNA sequence analysis of about 60 clones from each soil was insufficient to completely describe the differences in microbial species diversity between the two soils. However, twelve different taxa were found to contain the diversity found in both the NV and PV soils (Table 2). By observing the banding patterns in a RISA gel, Carlson (2004) concluded that the NV soil contained greater numbers of species than the polluted soils. This still may hold true for the soils analyzed in this study, but without sequencing additional clones, it is difficult to determine the effect of Cu and Zn on overall species diversity in the NV and PV soils.

In this study, the PB soil was found to be significantly less active than the NV soil in both activity assays (Figures 3 and 4). This observation suggests that decreased activity levels may involve more than the diversity of microorganisms found in each soil type. The number of viable bacteria in the PV and PB soils may have been the direct cause of their low activity levels. The average number of bacteria per gram of soil, as determined by plate counts after 48 hours of incubation on a nutrient agar medium (without metals), was 49 times higher in the NV soil than the PB soil. The difference in the size of the bacterial population in the PB soil compared with the bacterial community in the NV soil associates nicely with the overall productivities of the two populations.
The final hypothesis made in this study was that the structure of the bacterial population in the PV soil would be different than the structure of the bacterial population observed in the NV soil. Phylogenetic analysis of the clones isolated from the NV and PV soil showed that the structure of the two bacterial populations were, in fact, different in many interesting ways (Figures 5 & 6, Table 2). The NV soil contained 3 times as many \( \alpha \) Proteobacteria than the PV soil. In addition, considerably more bacteria were assigned to the taxa Acidobacteria and Gemmatimonadetes from the NV soil than the PV soil. Alternatively, nearly 4 times more bacteria were assigned to the taxon Planctomyces in the PV soil than the NV soil, and Green-non sulfur bacateria not observed in the NV soil were found in abundance in the PV soil (Table 2). These findings suggest that heavy-metal contamination does have an effect on bacterial community structure. These results are also consistent with observations made by Daiz-Ravina and Baath (1996), who reported that gradual changes had occurred in the structure of the bacterial communities in their polluted soil as compared with that of their non-polluted soil. Hence, bacterial populations may shift in structure in order to gain tolerance to heavy metals.

In conclusion, three of the four original hypotheses that I made were supported: the activity levels of the bacteria from the NV soil samples were higher than the activity levels of both the polluted soils; more bacteria were tolerant to heavy metals in the PV and PB soils than the NV soil, with the exception of 0.5 mM zinc; and, the structure of the bacterial populations from the PV and NV soils were different. From these findings, whether the polluted system above Milltown Dam is actually recovering, that is, becoming more stable and productive as the years progress, is difficult to say. There is
no way of actually knowing this unless similar, annual studies are carried out with soils taken from the same sites.
Literature Cited


