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Microbial community production and tolerance in heavy metals polluted sediment

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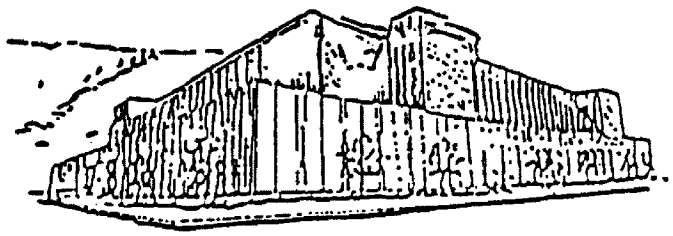

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Microbial Community Production and Tolerance in Heavy Metals Polluted Sediment

By

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Measurement of Heavy Metal Tolerance and Biomass Production in Sediment Microbial Communities

Director: Dr. James E. Gannon

With the aid of four large floods in the late 1800's and early 1900's, mining wastes from the Butte – Anaconda region of Montana established a heavy metal gradient from the headwaters of the Clark Fork River down stream over 200 km. Over time microbial communities have been exposed to different metal concentrations dependant upon their place in the gradient. Microbial secondary productivity and biomass were measured seasonally and were not impaired relative to the metal concentrations in the sediment. Productivity levels were similar to those reported by other researchers in uncontaminated systems. These broad measures of community parameters suggest that microbial communities have adapted to the heavy metal concentrations.

One mechanism for microbial adaptation to heavy metals is increased tolerance. Mine tailings and other mining wastes are routinely washed into the Clark Fork River from the floodplain and interact with the microbial communities. Four sites along the Clark Fork River and one uncontaminated tributary were stressed using increasing volumes of mine tailings and microbial productivity was measured using [^{14}C]leucine incorporation. The sites with the greatest previous metal exposure exhibited the greatest tolerance to the mine tailings. Microbial metal tolerance followed the metals gradient with sites furthest from the source exhibiting the lowest tolerance. These data suggest that the use of mine tailings to stress microbial communities may be a more realistic way to measure microbial tolerance to heavy metals than using conventional metal salt assays. In addition community productivity is still influenced by bank tailings that wash into river sediments.

ACKNOWLEDGMENTS

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CHAPTER 1

GENERAL INTRODUCTION TO HEAVY METAL EFFECTS ON MICROBIAL COMMUNITIES: BACTERIAL PRODUCTIVITY

Over a century of mining activities have left a legacy of hazardous waste throughout many rivers and lakes in the United States. There are an estimated 550,000 abandoned hardrock mines nationwide and 12,000 miles of rivers polluted with heavy metals (38).

Mining in the Butte, Montana region began in the mid-1860's. By the 1890's over 4,500 metric tons of sulfide ores were being mined daily from Butte Hill. Soon after mining reached these levels one of the world's largest smelters was built in nearby Anaconda, MT. Early mining and smelting activities in the Butte - Anaconda region resulted in the accumulation of large volumes of mining wastes. Over 100 million metric tons of tailings and other mining wastes were routinely discarded directly to the land surface or to the headwaters of Silver Bow Creek. A series of large floods in the late 1800's and early 1900's helped to establish a heavy metals gradient extending over 200km downstream. The floodplain of Silver Bow Creek currently holds vast quantities of mine tailings and heavy-metal laden sediment, which still enter the creek during rainstorms and other disturbances. Extensive fish kills have been documented as a result of the sudden influx of heavy metals into the river following thunderstorms, demonstrating that the mining wastes still impact the river today (27). Silver Bow Creek becomes the Clark Fork River several miles downstream. The Clark Fork River Basin is now the largest superfund complex, covering an area approximately $1/5^{th}$ the size of

Rhode Island (37). Metals of concern in this contaminated system include various species of arsenic, cadmium, copper, iron, lead and zinc.

Several studies have explored the effects of heavy metals, including metal tolerance, on sediment microbial communities (13, 23, 40, 41, 23, 34, 47, 48). Research in the heavy metals contaminated New Bedford Harbor has concluded that the biological communities played a significant role in the cycling of metals between sediment and the overlying water, and that the contaminated sediments can serve as a source of heavy metals even after the ultimate source of the heavy metals has been remedied. A study by Wielinga et al. (1994) examined the microbial communities with respect to the geochemistry of a metals-contaminated floodplain and reported a strong correlation between geochemical events and communities (47).

Additional studies have examined the effects of mining wastes in the Clark Fork River on trout, *Daphnia*, and other eukaryotic organisms (9, 19, 30, 39, 49). However, studies on the effects of heavy metals on Clark Fork River microbial communities have been limited (13, 47, 48). Previous studies on microbial communities in the Clark Fork River suggest an increased resistance to arsenic and copper in the headwaters (13). They have also demonstrated an increase in electron transport activity in the areas of greatest metal resistance (13). Other studies have characterized the microbiology and geochemistry of the floodplain of the Clark Fork River headwaters (47, 48). The authors found that the mixing of mining wastes and uncontaminated flood deposits had created a highly heterogeneous environment and that the distribution of specific guilds of bacteria (ex. SRBs) did not readily conform to predictions.

The impact of heavy metals to microbial communities has been well studied. Exposure to elevated concentrations of heavy metals has the potential to negatively impact many microbial community parameters including biomass, respiration, nitrogen fixation, composition and productivity (1, 3, 5, 11, 12, 16, 17, 23, 25, 28, 29, 34, 40, 41). The natural variation in ecosystems often makes determining the particular impacts of heavy metals on microbial communities difficult. In addition to the heavy metals many other factors can influence microbial processes and often studies of this nature examine more than one parameter in order to isolate the heavy metal-induced response relative to natural variation (12).

When elevated concentrations of heavy metals are introduced to an environment where there has been no previous exposure many organisms will be impaired, while others will not (3, 6, 16, 17, 40). Susceptible organisms may not be able to compete effectively and many will die (16). In any given community there will be a population of tolerant organisms. Although this population may be impaired by the presence of heavy metals, it has the mechanisms necessary to survive in such an environment. Evidence suggests that broad measures of microbial communities such as thymidine incorporation can rebound after an acute stress, while a chronic stress is more likely to leave a lasting change (26). Specific functional measures may be necessary to observe any lasting community changes if broad measures indicate community recovery. Greater species diversity may provide the community with increased redundancy, and thus make more diverse communities better equipped to handle such a stress (36).

Numerous authors have shown that microbial communities chronically exposed to elevated concentrations of heavy metals can develop a tolerance to these metals (4, 6, 7,

13, 17, 29, 34). The effects of Cd, Cu, Ni, Pb, and Zn are most commonly studied. Díaz-Raviña and Bååth (1996) demonstrated that exposure to greater concentrations of heavy metals led to greater tolerance to those same metals (16). This trend held true for Cd, Cu, Pb and Zn. The length of time that the microbial communities were exposed to the metals also influenced the development of metal tolerance. Longer exposure times (up to 14 months) led to greater tolerance in subsequent challenges with heavy metals. Although community metal tolerance appears to develop fairly slowly, this tolerance can be lost rapidly (15). In soils artificially contaminated for one year with high concentrations of Cd, Cu and Zn, 70% to 90% of the gained tolerance was lost within one week of being inoculated into uncontaminated soil. Thus, microbial community metal tolerance appears to require consistent metal exposure to be maintained. The exposure to increased levels of one heavy metal can even induce the development of tolerance to multiple metals (3, 6, 16, 17). For example, soils experimentally polluted with only copper induced tolerance to copper as well as tolerance to zinc, cadmium and nickel (17).

The development of metal tolerance in microbial communities is a sensitive indicator of metal stress. Communities that are exposed to greater concentrations of heavy metals respond with a greater degree of tolerance (3, 6, 16, 17, 29). To determine the extent of tolerance development researchers subject microbial communities to elevated concentrations of heavy metals added in the laboratory and measure productivity (3, 6, 8, 15, 16, 17, 29, 34, 40). Productivity is frequently measured using thymidine or leucine incorporation rates, although other measurements have been used (3, 6, 15, 16, 17, 40). Other metal tolerance studies subject microbial communities to elevated concentrations of metals by plating them on agar spiked with heavy metals (8, 29). The

heavy metals added in the laboratory are generally added in a range of concentrations from slightly inhibitory to completely inhibitory. The metals used for these experiments are in the form of metal salts obtained through chemical supply companies. Do salts such as $CdSO_4$, $CuSO_4$, $ZnSO_4$, $Ni(NO_3)_2$ or $Pb(NO_3)_2$ added in the laboratory truly represent what microbial systems experience in the field? Heavy metal contamination can occur due to direct application, atmospheric deposition, river transport, groundwater transport or a host of other primary, secondary, and tertiary mechanisms (37). Heavy metals can be distributed in the soluble form or in the particulate phase on the surface of rocks, sediment or mine tailings, and leach over time. *In situ* microbial communities are subject to multiple heavy metals simultaneously that are often accompanied by a change in pH. Methylation can also play a significant role in determining the toxicity of a particular metal. While metal salt studies are well accepted, reproducible, and can elucidate the role of individuals, they may not accurately reflect conditions *in situ.*

Many studies have looked at the effects of bioavailable metals on higher trophic levels in river systems. These studies generally use some accepted technique to extract available metals and then add them back to a test organism, such as midge larvae or trout (9, 19, 30, 49). Most of these studies report reduced growth and increased bioaccumulation with increased exposure to available metals. Although these studies provide much needed information on the effects of bioavailable metals on the ecosystem, they do not include microbial communities in their study of the effects of metal stress.

Jonas et al. (1984) conducted one of the first studies on the ecotoxicity of heavy metals to microbial communities by subjecting microbial communities to various mercuric salts and organometals. This study used thymidine incorporation and glutamate

metabolism as indicators of metal toxicity (28). Since then, the incorporation of radiolabeled thymidine or leucine into bacterial biomass has become the common methods for the study of heavy metal effects on microbial communities. The point at which saturation of thymidine or leucine uptake and incorporation processes occurs (V_{max}) can be compared across data sets. V_{max} is a function of the microbial community and provides an estimate of *in situ* productivity. When the microbial community is thriving, the rates of both protein synthesis and nucleic acid synthesis will both increase. New DNA is synthesized only when the cell divides, while protein synthesis is a fairly continuous process, but varies with intensity due to the needs of the cell. This difference results in the greater sensitivity of the leucine technique (2, 15, 40). This sensitivity is greatest during periods of unbalanced growth (43). A study by Reichart et al. (1993) compared the effects of different heavy metals on sediment microbial communities using multiple methods of measuring the community response (40). The authors concluded that, in general, the leucine technique had a higher degree of sensitivity than the thymidine technique for metal toxicity studies.

Several studies have looked at the rate of leucine incorporation in sediment microbial communities (22, 35, 40, 46). Leucine incorporation is a reliable and sensitive measure of microbial productivity. Protein comprises a fairly consistent fraction of cell biomass (~60%) and leucine comprises a fairly constant fraction of protein (~7.3%) (31). In addition leucine is used almost exclusively for incorporation into protein. It is seldom transformed into other molecules, even at saturating levels (32). Under saturating conditions de novo synthesis is repressed and the external pool of leucine is used almost exclusively (22). The point at which leucine uptake is saturated varies by ecosystem, but

the highest reported concentration of leucine required for saturation is about 50μM (22, 35). These factors are of important consideration for calculating other rates such as bacterial carbon production.

The thymidine incorporation technique can produce results with greater variability because thymidine incorporation does not always follow Michaelis-Menton kinetics (2, 44). It has been hypothesized that this is due to diffusive transport mechanisms that are unable to be saturated (33). Because of this, conversion factors, which allow for the calculation of carbon production from rates of uptake, are generally less variable with the leucine technique than with the thymidine technique. Another disadvantage of the thymidine technique is that thymidine binds readily to sediments, and thus becomes unavailable to microbial communities (14). Isotope dilution can be a concern with the leucine incorporation technique; however it is possible to determine it experimentally. Isotope dilution is generally a greater concern with low concentrations of leucine because at higher leucine concentrations de novo synthesis is inhibited.

The first objective of this study was to compare microbial community biomass and productivity in sites along the Clark Fork River metals gradient with each other and with uncontaminated reference sites over the course of one year. It was expected that these broad measures of microbial communities would reveal that communities with the greatest chronic exposure would be inhibited in comparison to both uncontaminated reference sites and sites along the river that have been exposed to lower concentrations of heavy metals.

The second objective was to examine microbial community tolerance to heavy metals along the Clark Fork River metals gradient. This study examined metal tolerance

to native metal species through the use of mine tailings or secondarily deposited tailings, which for simplicity, will both be referred to as tailings herein. The use of tailings stressed microbial communities in a manner consistent with what regularly occurs *in situ* when mining wastes from the floodplain slough off into the streambed. It was anticipated that microbial communities that have been exposed to the greatest concentrations of heavy metals would exhibit the greatest tolerance to the addition of metals. These same communities are routinely exposed to tailings from stream bank erosion, which would contribute to their metal tolerance.

CHAPTER 2

SEASONAL MICROBIAL PRODUCTIVITY AND BIOMASS ALONG A GRADIENT OF MINING CONTAMINATION IN THE CLARK FORK RIVER OF WESTERN MONTANA

Abstract

Over a century of mining in the headwaters of the Clark Fork River has left a legacy of contamination that has formed the largest Superfund site. Numerous studies have examined the geochemistry of this system and have monitored the effects of heavy metals on aquatic organisms (5, 6, 8, 9, 24, 27, 28, 29, 35, 36, 37). The study of microbial communities in this system has been limited to this point (9, 35, 36). The study presented here examined microbial biomass and the rate of [^{14}C]leucine incorporation into protein along the Clark Fork River over the course of one year. Biomass ranged from 10^6 to 10^8 cells per gram of sediment over the course of the study, while the rate of leucine incorporation ranged from no incorporation to 8375pmol g^{-1} hr^{-1}. Comparison of Clark Fork River study sites with uncontaminated reference sites suggests that the microbial communities have adapted to the chronic metal stress and that biomass and productivity have rebounded to pre-exposure levels. The observed biomass and productivity values in this study were often similar to those values reported in the literature from uncontaminated systems. A seasonal pattern in biomass direct counts was observed, although no measures were made to correlate this pattern with stream conditions.

Introduction

Early mining and smelting activities in the Butte - Anaconda region have resulted in the large-scale contamination of the Clark Fork River basin by heavy metals. Between 1878 and 1925 over 10 million metric tons of mining and smelting wastes were discarded into the headwaters (28). During this same period four large floods occurred, transporting much of this waste downstream. A gradient of heavy metal contamination now extends from the River's headwaters in Butte downstream over 200km.

Ecotoxicology studies in the Clark Fork River thus far have primarily focused on midge larvae, benthic invertebrates and various species of trout (5, 9, 24, 29, 37). Few studies have examined the toxicity of heavy metals to lower trophic levels in the Clark Fork River system (9, 35, 36). The goal of this study is to characterize the microbial communities along the metal gradient with respect to their biomass, as measured by direct counts, and their productivity, as measured by [^{14}C]leucine incorporation, over the course of one year.

Studies have shown that biomass at sites chronically contaminated with heavy metals are often lower than biomass at uncontaminated sites (1, 3, 7, 20, 23, 30). Elevated heavy metals can also result in reduced microbial productivity (2, 4, 12, 13, 22, 30, 31). However, most microbial community productivity studies involving heavy metals examine the tolerance of these communities to a subsequent heavy metal challenge, which is the subject of Chapter 3. Comparatively few microbial productivity studies have looked at the effects of *in situ* microbial activity at heavy metal contaminated sites (9, 30). Burton et al. (1987) and Roane and Kellog (1996) offer conflicting evidence as to the effects of heavy metals on microbial activity. Burton et al.

conducted a study in the Clark Fork River and found increases in electron transport activity, proteolytic activity, galactosidase activity and glucosidase activity in areas of higher metals, while Roane and Kellogg observed a decrease in microbial activity, as measured by fluorescein diacetate hydrolysis, with increasing metals (9, 30).

We expect differences in productivity and biomass between test and reference sites to be greatest between the site with the greatest chronic heavy metal contamination and it's reference site. Biomass and productivity along the gradient should reflect the heavy metal content of the sediment; sites with the greatest sediment metal content should be the most impaired.

Materials and Methods

Study Sites. Three sites along the Clark Fork River metals gradient were chosen for this study so that high, intermediate and low metals sites were represented. Each site consisted of three subsites. Silver Bow Creek at Miles Crossing is the most contaminated of the sites and is located in the headwaters of the Clark Fork River in Butte, MT (SBMC). The second site along the Clark Fork River is above the confluence of Gold Creek, west of the town of Garrison (CFGC). The final site on the Clark Fork River is above the confluence of Rock Creek, east of the town of Clinton (CFRC). This site is the least contaminated of the test sites. Each of these test sites is paired with an uncontaminated reference site. The test and reference sites are identified in Figure 1. Site pairs were selected based on the similarity of watershed effects ecoregions, geology, geomorphology and hydrology. SBMC is paired with a site on the Little Blackfoot River

east of Garrison Junction (LBGJ). CFGC is paired with the Big Hole River near Notch Bottom fishing access (BHNB). Finally, CFRC is paired with Rock Creak near Ekstrom Station (RCES). This information is summarized in Table 1.

Table 1: Test and Reference Sites for this Study

Corresponding Number on Fig 1	Test Site (Abbreviation)	Paired Reference Site (Abbreviation)
1	Clark Fork River @ Rock Creek (CFRC)	Rock Creek @ Ekstrom Station (RCES)
2	Clark Fork River @ Gold Creek (CFGC)	Big Hole River @ Notch Bottom (BHNB)
3	Silver Bow Creek @ Miles Crossing (SBMC)	Little Blackfoot River @ Garrison Junction (LBGJ)

Sample Collection. Baseline productivity was studied by collecting sediment cores from each test and reference site. During the summer of 2000, sediment was collected, sieved (1.7-2.36mm) and packed into acid-washed PVC cores. The cores were returned to their respective sites and allowed to equilibrate for one month. Over the course of thirteen months these cores were collected and analyzed. Column sediment was washed thoroughly in the field. Sediments for productivity measurements were transported back to the lab on wet ice and were processed within four hours. Sediment samples for biomass analysis were transported back to the lab on dry ice and lyophilized before use.

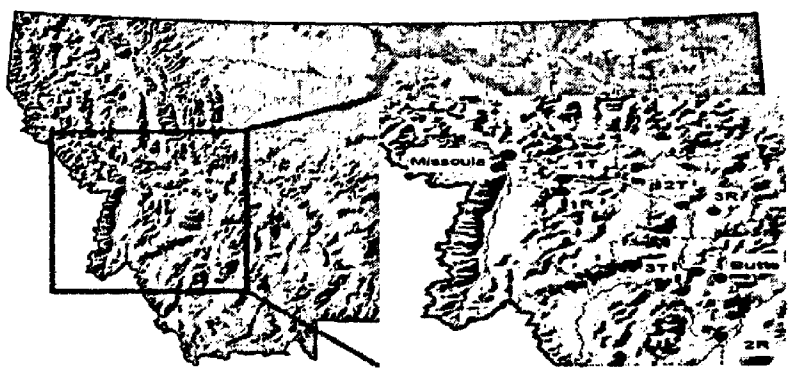

Figure 1: Map of study sites in western Montana. 'T' indicates a test site. 'R' indicates a reference site. Numbers correspond to the sites listed in Table 1.

Biomass Measurements. One gram of lyophilized sediment was sonicated (117V, 100W) for 10 minutes in a 0.1% Tween-80 solution. The samples were allowed to settle for 20min before 1mL was withdrawn and diluted into 9mL of 0.1% Tween-80. One milliliter of each sample was incubated in the dark for 15 minutes with the Live/Dead Baclite® Stain in accordance to the manufacturer's instructions (Molecular Probes, Eugene, OR). The stained bacteria were collected by vacuum filtration onto a 0.2 μm-pore-size black polycarbonate filters (Millipore, Bedford, MA). The stained bacteria were counted using epifluorescence microscopy on a Zeiss Axioskop microscope (Carl Zeiss, Germany) and ImagePro Plus software (Media Cybernetics, Silver Spring, MD). Cells were counted until 30 fields-of-view or 400 cells were reached. One slide was made for each subsite.

Productivity Measurements. Sediment was diluted 9x into conical centrifuge tube with 50μM leucine suspension (spec. activity 125Bq/nmol). Leucine was suspended in filter-sterilized water from the same site as the samples and [^{14}C]leucine was diluted with the appropriate volume of cold leucine. All samples were then incubated at 14°C for two hours before the addition of formaldehyde to stop the reaction (final concentration 5%). An additional set of samples was assayed and formaldehyde was added to the sediment prior to the addition of leucine in order to correct non-specific uptake and binding of leucine. The incorporation rates measured in this set were subtracted from the test samples.

Fixed samples were sonicated for 10 minutes followed by the extraction of the proteins by hot (95°C) trichloroacetic acid (final concentration 5%) for thirty minutes. The samples were then placed on ice for 30min in order to precipitate the proteins. An

aliquot was filtered through a 0.2μm-pore-size polycarbonate filter. Each filter was rinsed with filter-sterilized water in order to remove any unincorporated leucine. The filters were then placed into 6mL scintillation vials with 0.5mL of solvent and 4.5mL scintillation fluid (Hionic Fluor, Packard Bioscience, Downers Grove, IL). The filtrate was periodically collected in order to account for all radioactivity used in these assays. Radioactive decay was measured in a Beckman LS6500 scintillation counter (Beckman Coulter, Inc., Fullerton, CA). The method described here has been modified from the one previously described by Fischer and Pusch (17).

Isotope Dilution. Isotope dilution was measured in a manner consistent with other studies (26). Concentrations of cold leucine were increased, while maintaining a constant concentration of radioactive leucine. Initial concentrations of [^{14}C]leucine were 20μM and 50μM (specific activity 125Bq nmol^{-1}).

Sediment Metals Analysis. The metal content of river sediments from each site was measured in April, July and September of 2000. Sediments and tailings were digested overnight in a nitric and hydrochloric acid solution, filtered and analyzed using a Thermo Jarrell-Ash Atom-Comp 800 ICP. The procedure was adapted from procedure EPA 350 B.

Statistics. Relationships between seasons and test and reference site communities were analyzed using JMP version 3.1.6.2 (SAS Institute, Inc, Cary, NC).

Results

Sample Collection. Samples were not collected from BHNB in November due to thick ice on the river. Samples were not collected CFRC in April due to high, turbid flows, which obscured the columns. Locating columns proved difficult in October and samples from BHNB, SBMC and LBGJ were kept on wet ice too long before productivity measurements were made. This sampling problem most likely resulted in underestimated productivity. Biomass measurements from the same October samples were most likely unaffected.

Biomass. Biomass, as measured by direct counts, was measured between September 2000 and October of 2001. Cell numbers varied between 10^6 and 10^8 cells per gram of sediment (dry weight) over the course of the study (Fig 6). On average biomass was lowest in April and greatest in November (Figure 6). The greatest variability occurred in October samples.

A Student's t-test was performed for each test and reference site pair at each time point. CFRC and RCES, the low metals difference pair, were significantly different during the months of July and October ($p \leq 0.05$), but not at any other time point (Fig 2). Although biomass direct counts were higher at the intermediate metals site at each sampling point than at it's reference site, this relationship was only significant during the month of September ($p \leq 0.05$). This data is presented in Figure 3. Biomass direct counts at the high metals site were significantly greater than it's paired reference site in September ($p \leq 0.05$). They were not significantly different at any other time point (Fig 4).

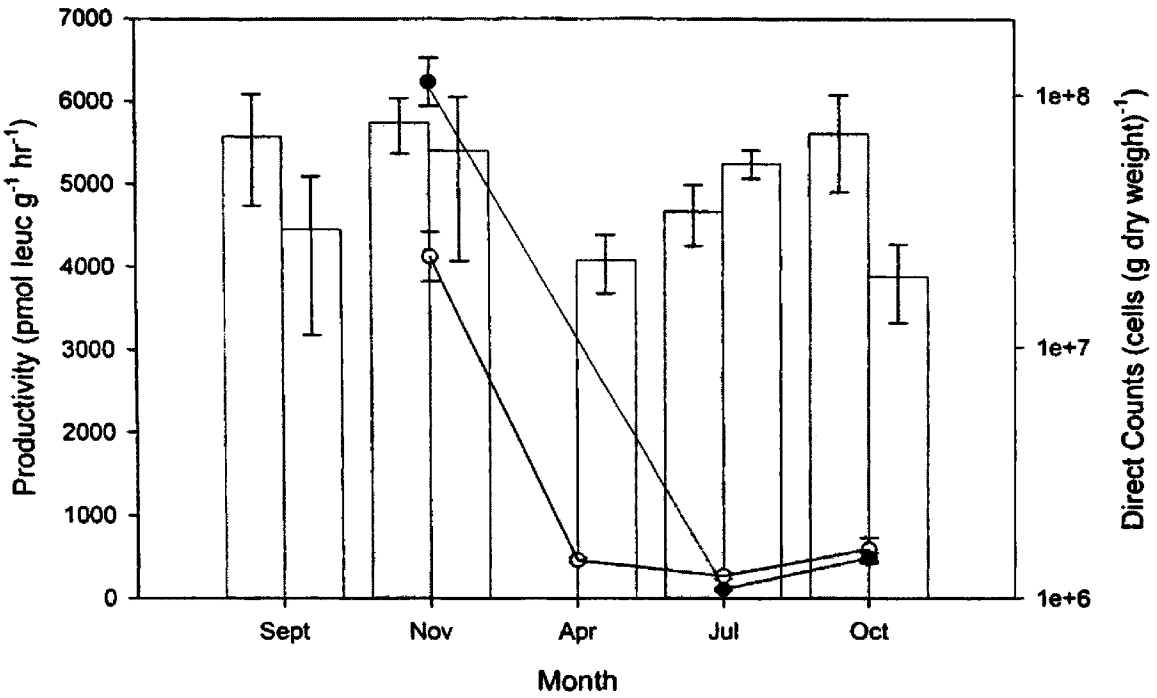

Figure 2: Low Metals Difference Pair. Comparison of biomass direct counts and productivity at CFRC and it's reference site, RCES. Columns represent biomass and circles (○) represent productivity. Shaded columns and filled circles (●) are CFRC, open columns and open circles represent RCES. All values are means with standard deviations. For biomass measurements n = 3. For productivity measurements n = 12, except in October when n = 6.

Productivity. Productivity was measured from November of 2000 through October of 2001. Leucine incorporation over the course of the study varied from no incorporation to 8375 pmol leucine hr^{-1} (gram dry weight of sediment)$^{-1}$.

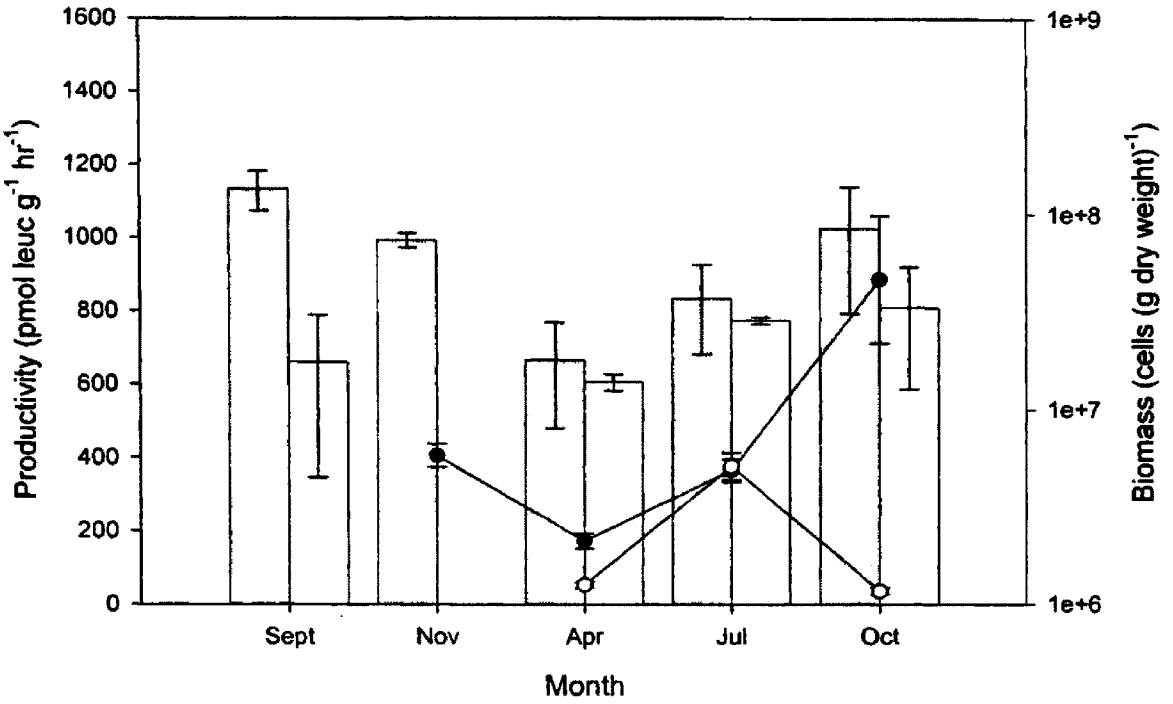

Figure 3: Intermediate Metals Difference Pair. Comparison of biomass direct counts and productivity at CFGC and it's reference site, BHNB. Columns represent biomass and circles (○) represent productivity. Shaded columns and filled circles (●) are CFGC, open columns and open circles represent BHNB. All values are means with standard deviations. For biomass measurements n = 3. For productivity measurements n = 12, except in October where n = 6.

Productivity between test and reference sites showed very few differences. In November and July productivity was significantly different between CFRC, the low metals site, and its reference site, RCES ($p \leq 0.05$). In both April and October productivity was significantly greater at the intermediate metals site than at it's reference pair ($p \leq 0.05$). There was a significant difference in productivity between the high metals site and it's reference pair during the months of November, April and October ($p \leq 0.05$).

There was no evident correlation between direct counts and productivity at any of the sites with the exception of CFGC, the intermediate metals site, where they were weakly correlated ($R^2 = 0.72$).

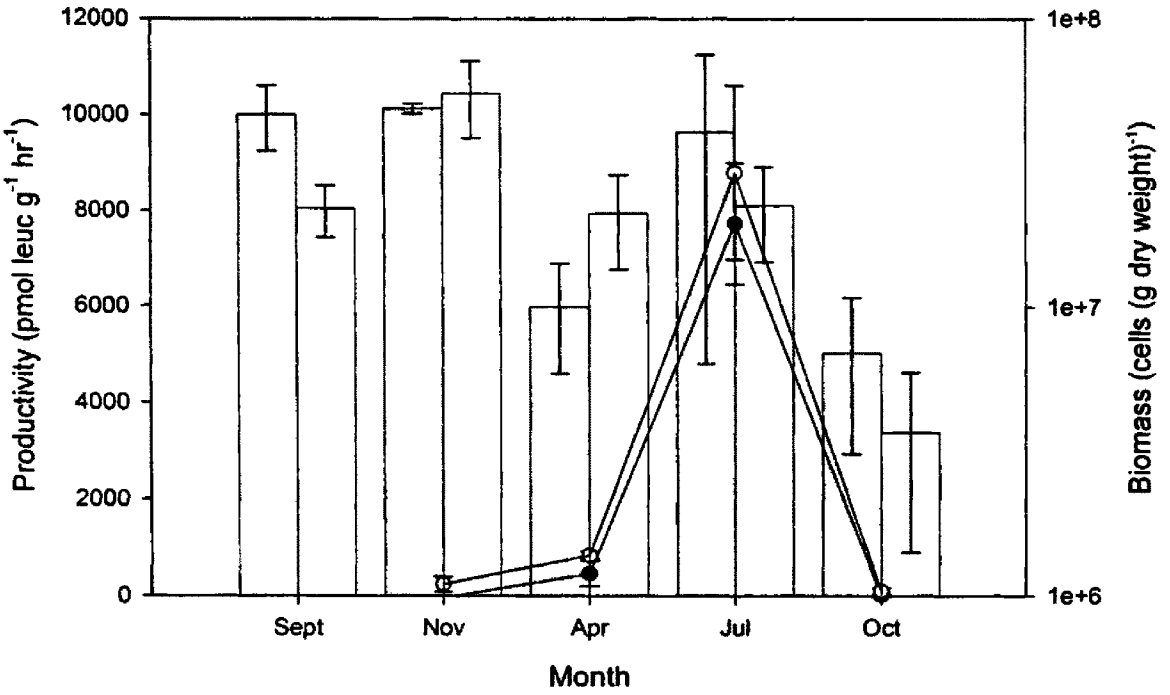

Figure 4: High Metals Difference Pair. Comparison of biomass direct counts and productivity at SBMC and it's reference site, LBGJ. Columns represent biomass and circles (○) represent productivity. Shaded columns and filled circles (●) are SBMC, open columns and open circles represent LBGJ. All values are means with standard deviations. For biomass measurements n = 3. For productivity measurements n = 12, except in October where n = 6.

Metal Analysis. Background metal concentrations were measured across the gradient and at reference sites and are given in Table 2. Metals were highest at SBMC and declined along the Clark Fork River metals gradient. Metal concentrations in each pair were always lower at the reference site.

Table 2: Concentrations of key metals in sample sediments. Values are mean ppm (μg/g) with standard deviation in parentheses.

Site	As	Cd	Cu	Pb	Zn
CFRC	5.1 (1.1)	0.52 (0.08)	33 (4.00)	8.9 (1.3)	120 (17)
RCES	2.4 (0.50)	0.11 (0.00)	2.3 (1.03)	2.6 (0.29)	2.3 (1.8)
CFGC	7.5 (1.9)	0.58 (0.06)	75 (16.39)	16 (5.0)	160 (21)
BHNB	4.5 (1.6)	0.31 (0.38)	1.9 (1.39)	5.0 (2.3)	12 (7.9)
SBMC	55 (22)	1.7 (0.39)	400 (210)	93 (26)	440 (120)
LBGJ	5.5 (1.0)	0.26 (0.03)	4.2 (0.89)	8.0 (0.93)	22 (2.2)

Isotope Dilution. A linear relationship between the added leucine concentration and the reciprocal of radioactive decay indicates that leucine incorporation is following

Michaelis-Menton kinetics. Figure 5 suggests that leucine incorporation into protein is following Michaelis-Menton kinetics at a concentration of 50μM, but may not be at a concentration of 20μM. The negative intercept with the y-axis indicates the dilution of the added leucine. At a concentration of 20μM the dilution is 7.5μM. For the plot of 50μM leucine the line passes through the y-axis with a positive intercept, therefore we can assume that no isotope dilution is occurring. Thus a concentration of 50μM leucine was chosen for these studies and no isotope dilution was assumed.

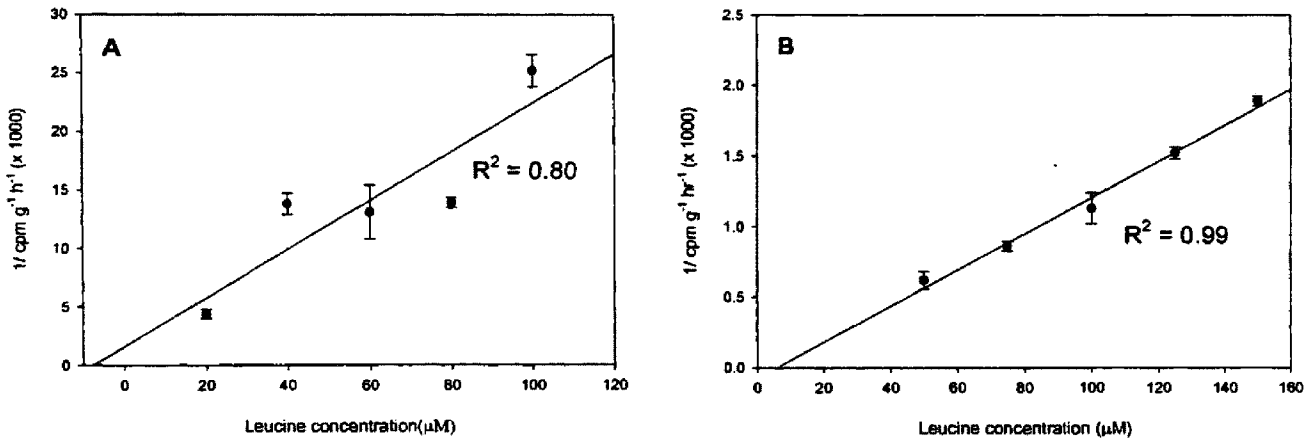

Figure 5. Isotope dilution plot indicating that incorporation processes are saturated at 50μM (B), whereas they may not be at 20μM (A).

Discussion

Biomass. With the exception of July at the low metals pair, the only significant relationships that exist between pairs in the biomass data indicate greater biomass at the contaminated sites. These significant relationships were few in number, but are contrary to our hypothesis. All direct counts within one log value of each other with the exception of CFGC, which reached $1x10^8$ cells g^{-1} in September and LBGJ and SBMC, which were on the order of 10^6 cells g^{-1} in October. Given the inherent variability associated with direct counts we concluded that there is no real difference in the biomass values.

Seasonal trends in biomass measurements are shown in Figure 6. Even though an ANOVA indicates that these relationships are not significant, they do suggest that something other than heavy meals are driving direct counts. Biomass in September of 2000 after a hot, dry summer that saw record wildfires, was variable. In general biomass was higher in the Clark Fork River sites in September than in the reference sites. Biomass generally increased and became less variable in November as precipitation and flow returned. Organic matter from deciduous trees and shrubs enter the rivers during this time and introduce nutrients. Organic matter is then diluted by the high flows of spring runoff and water temperatures are only slightly above freezing in April. Flows have decreased and temperatures have increased by July. Biomass was varied widely between sites in October 2001 with half of the sites increasing in biomass from the previous measurement in July and the other half declining in biomass. Mean biomass did not change from July 2001 to October 2001.

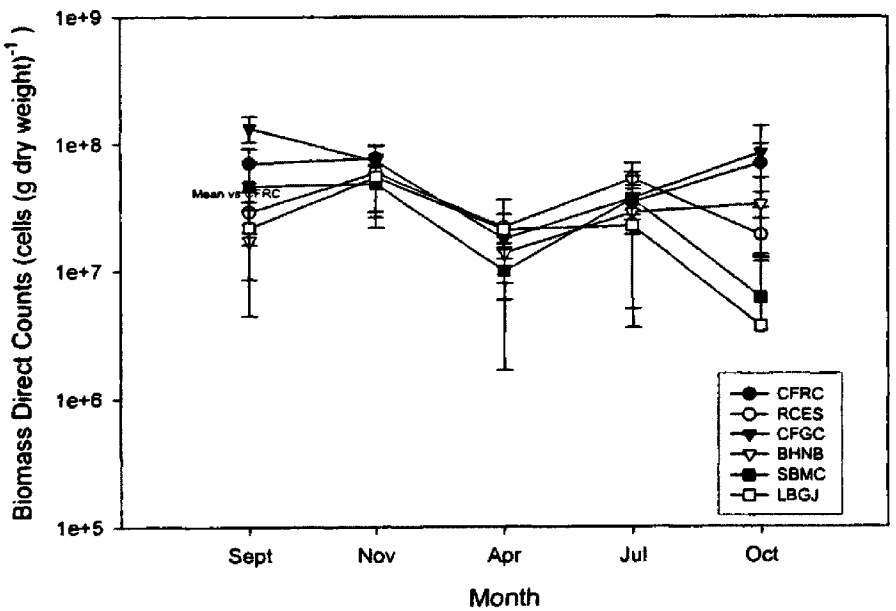

Figure 6: Biomass, as measured by direct counts, followed a seasonal trend.

This study was conducted as part of a larger study, which also studied microbial diversity using phospholipid fatty acid (PLFA) signatures and denaturing gradient gel electrophoresis (DGGE) patterns in Clark Fork River microbial communities. This study was not designed as a true seasonal study. If this study were to be repeated with the goal of capturing seasonal variations, other measures, such as dissolved oxygen, dissolved organic carbon, pH, flow and water temperature would need to be recorded.

Ellis et al. (1998) found that direct cell counts, water table elevation and DOC all exhibited the same seasonal pattern in the Flathead River, which is a major tributary of the Clark Fork River below the study sites (14). The seasonal pattern in the Flathead River, studied in 1988 and 1989, exhibited a similar drop in biomass in April and a corresponding increase in mid-summer. However, too few sample points overlapped between our study and the study by Ellis et al. to draw any further conclusions. Schallenberg and Kalff (1993) suggest that microbial abundance in sediments may be inversely related to sediment organic matter and positively correlated with sediment temperature (32). The strongest correlation observed was a negative relationship with sediment water content. A seasonal study by Findlay et al. (1986) related both bacterial biomass and bacterial carbon production with sediment organic content (16).

Although the watershed effects, geomorphology and hydrology of the test and reference sites in this study were paired closely, land use patterns did vary and may explain some of the variability between test and reference sites. Direct counts presented here support the hypothesis that microbial biomass levels have adapted to the elevated heavy metal concentrations and that other factors may be more important in controlling biomass.

Productivity. Although some significant relationships were observed, as a whole there was no clear trend as to the relationship between rates of leucine incorporation at Clark Fork River sites and reference sites. Rates of leucine incorporation into protein in the Clark Fork River was generally lower than productivity values determined by Fischer and Pusch (1999) in an unpolluted German river (17). Values observed by Fischer and Pusch ranged from approximately 500 to 1300 pmol leuc g^{-1} hr^{-1}. The average grain size of sediment used was 0.5mm, where the average grain size in this study was 2mm. This would result in greater surface area in Fischer and Pusch's sediments, which may result in greater microbial abundance per gram of sediment. They observed the lowest productivity in February, highest productivity in March and intermediary productivity in May. The study by Fischer and Pusch was not designed as a seasonal survey and no explanation was offered as to these observations.

Productivity in the Clark Fork River system was often higher than productivity in an unpolluted stream in Germany, where leucine incorporation averaged 75pmol g^{-1} hr^{-1} (26). The average grain size of the sediment studied was 0.5mm, which presents the same issues as with the study by Fischer and Pusch. Although this stream was not observed seasonally, productivity and sediment organic matter content were observed at several of the same sample sites as productivity and the two were closely related. Tuominen (1995) observed rates of protein synthesis in the range of 950 to 1820 μmol hr^{-1} L^{-1} in lake sediments (34). These values were higher than those observed here, however, the sediments were of a heterogeneous grain size and milliliters of sediment were used to correct for surface area to volume differences.

A study by Díaz-Raviña and Bååth (2001) re-inoculated soil microbial communities that had been exposed to elevated concentrations of heavy metals into unpolluted soil (11). The highest observed rate of productivity using [^{14}C]leucine incorporation was approximately 50 pmol leuc mL^{-1} hr^{-1}.

The rate of leucine incorporation into protein was converted to bacterial carbon production (BCP) as outlined by Kirchman (1993) and is shown in Table 3 (25). No isotope dilution was assumed as suggested in Figure 5. BCP is readily compared between data sets and published values, even when different methods are used. The BCP values observed in the Clark Fork River study were similar and often higher than those reported by Marxsen (1996) where BCP in stream-bed sediments were reported 0.1 and 1 μg C mL^{-1} hr^{-1} (26). BCP values reported by Tuominen were on average slightly higher than those reported here and ranged from 1.5 to 5.7 μg C mL^{-1} hr^{-1} (34). Cole et al. (1988) observed BCP covering a wide range of values from <1 to >20 mg C m^{-2} hr^{-1} in planktonic bacteria (10). Tömblom and Petterson (1998) examined BCP over the course of one and a half years. BCP values ranged from 0.2 to 2mg C m^{-2} hr^{-1} (33). Variations in BCP generally followed the seasonal variations in temperature with the exception of during a period of sedimentation by diatoms when BCP was elevated. None of these systems were exposed to elevated concentrations of heavy metals, suggesting that BCP in the Clark Fork River is not hindered by elevated metals.

Table 3: Bacterial carbon production at study sites (μg C g^{-1} hr^{-1}). Values are means with standard deviation. Sites with asterisks had no observations.

	Nov	Apr	Jul	Oct
CFRC	9.6 (2.7)	*	0.2 (0.1)	0.8 (0.2)
RCES	6.4 (2.8)	0.7 (0.3)	0.4 (0.4)	0.9 (0.4)
CFGC	0.6 (0.3)	0.3 (0.1)	0.6 (0.3)	1.4 (0.8)
BHNB	*	0.1 (0.1)	0.5 (0.3)	0.1 (0.0)
SBMC	0.0 (0.0)	0.7 (0.4)	12 (2.0)	0.0 (0.0)
LBGJ	0.4 (0.2)	1.3 (0.1)	14 (2.8)	0.2 (0.1)

The results presented here suggest that heavy metals have not resulted in significant long-term change to microbial communities detectable by broad parameters such as direct counts or protein synthesis measurements. Numerous other studies have reported changes in either microbial community biomass and activity as a result of heavy metal stress (1, 2, 3, 7, 12, 13, 20, 22, 23, 31, 30). Why were observations in the Clark Fork River different? Observations presented here are contrary to research by Griffiths et al. (2000) that suggests that microbial productivity can return to its original level after a transient metal stress, but a chronic stress is likely to impair the return to pre-stress levels (21). From this study it appears that direct counts and the rate of leucine incorporation into protein were not sensitive enough measures to detect the impacts of heavy metals on Clark Fork River microbial communities. The use of other microbial community based measurements could likely elucidate the effects of over a century of mining wastes on microbial communities. Seasonal variations and land use patterns may have obscured any effects of heavy metals on biomass and productivity.

Even though there was no direct correlation between biomass and heavy metals or productivity and heavy metals, it would not be correct to state that heavy metals are not interacting with microbial communities. Over time free metals from the sediment enter

the soluble phase and interact with the microbial communities. It is possible that after several decades of oxidation in the river, the available metals in the sediment may be very limited, thus microbial exposure is limited. However, Ford et al. (1998) concluded that even after the source of heavy metals contamination had been remediated that heavy metals in sediments could present a long term source of additional metals (18). Additionally, SBMC mining wastes routinely enter the streambed presenting microbial communities with available heavy metals. Although these metals enter the stream and initially present an acute toxicity, not all of the sediments are washed downstream. The mining wastes that remain behind result in additional chronic heavy metal exposure. Also, the sampling strategy for this study may have limited the heavy metals induced variation in the system. Diel variations are known to cause significant elevations in the soluble metal concentrations at night (6). This could potentially result in a decrease in microbial productivity at night, although a significant decrease biomass would not be expected in such a short exposure.

The observed results may be explained by the measurement of other community variables, such as metal tolerance and composition. There is plenty of evidence to suggest that this is possible (2, 3, 4, 11, 12, 13, 23). Development of microbial tolerance is a slow process that has been shown to continue for up to 2 decades (12). The development of metal tolerance has been demonstrated on the Clark Fork River and this is the topic of Chapter 3. Metal tolerance development has been linked to changes in community composition (11, 19). Other research on the Clark Fork River has demonstrated that differences in microbial community composition exist both between test and reference sites and between test sites along the metals gradient (15). These

differences are greatest between SBMC and LBGJ, where heavy metal differences are also the greatest.

Conclusions

The results presented here suggest that either microbial communities in the Clark Fork River were able to maintain or recover to an original level of biomass and productivity from metal stress or that too many other variables existed for these broad measurements to show the impacts of over a century's exposure to heavy metals. Seasonal fluctuations in biomass were observed and such factors as temperature and particulate organic matter may play a more important role in determining microbial community biomass than the concentration of heavy metals. Rates of bacterial carbon production were similar or slightly lower than those reported in studies of unstressed systems. It is likely that microbial community structure and tolerance have been altered in response to elevated concentrations of heavy metals and that broad community measures such as biomass and productivity don't reflect this change.

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CHAPTER 3

HEAVY METAL TOLERANCE OF SEDIMENT MICROBIAL COMMUNITIES TO MINE TAILINGS

Abstract

Over a century of mining and smelting activities in the headwaters of the Clark Fork River in Western Montana has resulted in the largest Superfund complex in the United States. Around the turn of the century a series of large floods helped establish a gradient of metals extending over 200km down stream. Earlier we analyzed sediment community productivity across the gradient and found essentially no loss in productivity relative to metal concentration. We hypothesized that one possible explanation might be an increase in metal tolerant communities. Native sediment samples were collected from four sites along the metal gradient, and microbial community productivity was measured by [^{14}C]leucine uptake in the presence of increasing mine tailings. The use of mine tailings to stress microbial communities is a change from conventtional techniques and was undertaken in order to stress microbial communities in a manner representative of how they are stressed *in situ*. Metal tolerance was related to the microbial community's place in the gradient. Communities from areas of highest sediment metal concentration demonstrated the greatest tolerance, while communities from further down the gradient, where sediment metal concentrations were lower, demonstrated lower tolerance. The results also show that the bank tailings, which still enter the creek today, interact with the microbial communities, select for a metal tolerant community, and reduce sediment productivity. To the best of our knowledge this is the first work demonstrating that

alluvially deposited mining wastes entering the streambed not only impact sediment microbial communities but also continue to select for tolerant communities.

Introduction

Large-scale mining and smelting operations in the headwaters of the Clark Fork River in Western Montana resulted in large volumes of mining wastes. A series of floods in the late 1800's and early 1900's established a heavy metals gradient with the highest metal contamination in the headwaters and declining concentrations over 200km downstream. The floodplain of Silver Bow Creek, which forms the headwaters of the Clark Fork River, contains vast quantities of heavy metal-laden mining wastes (tailings), which enter the streambed through erosion events. For over a century, microbial communities have been exposed to different concentrations of heavy metals dependent upon their place along the gradient.

Exposure to heavy metals exerts a selective pressure on microbial communities resulting in changes in community structure (2, 3, 4, 5, 12, 13, 16). This can lead to the selection of a more metal tolerant community (5, 16). Many studies have shown that the degree to which tolerance is developed is a function of the level of the community's exposure to heavy metals (2, 4, 12, 20). Common methods for studying microbial heavy metal tolerance include labeled thymidine incorporation into DNA, labeled leucine incorporation into protein, or viable counts in the presence of elevated metals. These studies are generally conducted by subjecting microbial communities to increasing concentrations of heavy metal salts, such as $CuSO_4$, $ZnSO_4$ and $Pb(NO_3)_2$. This method

uses artificial contamination but is well accepted. However, in contaminated soil and sediment a mixture of metals and metal complexes are often present and these metals vary considerably in their availability. Heavy metals reach the environment by a variety of means including tailings and waste rock deposition, atmospheric deposition and groundwater contamination amid a host of other means (22). *In situ*, the bulk of the metals are therefore rarely free salts in soil pore spaces or dissolved in the water column, but sorbed to the soil or sediment surface. Mobilization of these metals from surfaces over time results in soluble metals capable of affecting microbial communities. Biogeochemical processes results in the release of free metal species and organometals. Protons are often sorbed along with metal ions to the surface of sediments associated with mining wastes and release of the metals into solution is associated with the release of these hydrogen ions and a decrease in pH. Thus, pH also plays a role in many systems where microbial communities are presented with heavy metal stress (9).

The authors of this study have previously measured microbial community biomass and productivity along the heavy metals gradient and found no correlation between metals exposure and community biomass or productivity. We hypothesized that this may be a result of development of tolerance to mine tailings or secondary deposits of tailings. Herein we refer to both forms as tailings. In a change from conventional tolerance assays, we developed a tolerance assay using native metal complexes in place of metal salts. Our perspective being that sediment microbial communities in contact with tailings are exposed to the intrinsic (shape, surface properties) and extrinsic factors (metal complexes, pH) associated with tailings and that these factors influence sediment productivity in unique ways. Thus, our tolerance assay using mine tailings represents a

means of stressing microbial communities using a natural source of contamination as opposed to the commonly used artificial contamination. Previous studies in the Clark Fork River have shown an increase in the number of metal tolerant communities in the headwaters using viable counts in the presence of elevated metals (8). The use of a more sensitive method (leucine incorporation) combined with a natural source of contamination (tailings) should give us a better understanding how the Clark Fork microbial communities are responding to metal stress.

Sediment samples were collected from four sites along the gradient and one site from an unimpaired tributary of the Clark Fork River. The sediment samples were mixed with increasing volumes of mine tailings and microbial community productivity was measured by [^{14}C]leucine uptake to study tolerance. Microbial communities that have been routinely exposed to elevated metal concentrations should be more tolerant of a subsequent challenge by heavy metals.

Materials and Methods

Field Sites. Four sites along the Clark Fork River metals gradient were chosen for study (Figure 1). The first site was closest to the source of contamination on Silver Bow Creek at Miles Crossing (SBMC). The unsaturated zone (approximately 2m thick) is composed of a mix of fine and coarse grain mine tailings with elevated concentrations of As, Cd, Cu, Fe, Pb, and Zn (26). Mixed with these tailings are uncontaminated flood deposits. The second site along the gradient is on Silver Bow Creek slightly upstream from Opportunity Ponds (SBOP). As water flows out of these ponds it is treated with

lime and becomes the Clark Fork River. The floodplain at this site is also composed of a mix of fine and coarse grain mine tailings and uncontaminated flood deposits. The third site is situated above the confluence of Gold Creek with the Clark Fork River (CFGC). The river is partially channelized at this point in order to protect roads and the floodplain does not contain mining wastes. The final site along the gradient is the least contaminated and is situated on the Clark Fork River slightly upstream of its confluence with Rock Creek (CFRC). The unimpacted tributary, Rock Creek, flows mainly through wilderness and national forest before reaching the study site (RCES).

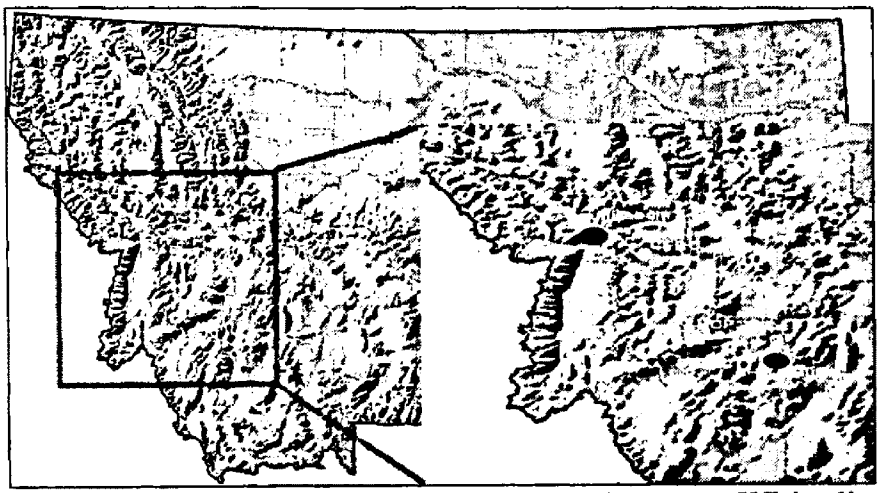

Figure 1: Map of sample sites in Western Montana. White line indicates the Clark Fork River, which is Silver Bow Creek above SBOP.

Sample Collection. Sediment was sieved (1.7-2.36mm) and rinsed in the field. Sediments were transported back to the lab on wet ice and were processed immediately upon returning.

Mine tailings were collected from the Silver Bow Creek floodplain and sieved to the same size fraction as the fresh sediment. The "tailings" were not true fine grain mine tailings. They may be what are referred to as coarse jig tailings or they may be the result

of secondary deposition of soluble metals on sediment surface. Further discussion is presented later. The tailings were stored at -20°C until used.

Metal Tolerance Measurements. Samples from SBOP and SBMC were mixed with tailings and acid washed sediment (AWS) and were assembled as outline in Table 1A. Fresh sediment, tailings, and AWS assemblages for RCES, CFGC and CFRC samples are given in Table 1B. The AWS was of the same size fraction as the fresh sediment and were used to bring the total volume of all samples up to 5g. Tailings and AWS were autoclaved for 20min at 15psi and 121°C prior to mixing with fresh sediment. AWS was washed overnight in a 0.1% HCl solution and brought back to neutral pH by repeated rinsing with filter-sterilized water (FSW).

Each assemblage was mixed with a total of 20mL of filter-sterilized river water. The river water was from the same site as the sediment samples. All samples were then incubated at 18°C for twenty-four hours. At this point 5mL of leucine suspension was added to each sample so that the final concentration of leucine was 50μM (specific activity 125Bq $nmol^{-1}$). The [^{14}C]leucine was diluted with the appropriate volume of cold leucine before addition to the samples. The samples were incubated for two hours before the addition of formaldehyde to stop the reaction (final concentration 5%). An additional set of samples was assayed and formaldehyde was added prior to the twenty-four hour incubation in order to correct for non-specific uptake and binding of leucine. The incorporation rates measured in this set were subtracted from the test samples. Sample pH was monitored at 0hrs and 24hrs using a Corning pH meter (Corning, NY).

Table 1: **(A)** Sample compositions for sediment samples from SBOP and SBMC. **(B)** Sample compositions for sediment samples from RCES, CFRC and CFGC.

A

Tailings (g)	AWS (g)	Fresh Sediment (g)
0	2.5	2.5
0.5	2	2.5
1	1.5	2.5
1.3	1.2	2.5
1.6	0.9	2.5
2	0.5	2.5
2.5	0	2.5

B

Tailings (g)	AWS (g)	Fresh Sediment (g)
0	2.5	2.5
0.2	2.3	2.5
0.4	2.1	2.5
0.6	1.9	2.5
0.8	1.7	2.5
1	1.5	2.5
1.5	1	2.5

Inhibition of productivity by pH. The rate of [^{14}C]Leucine incorporation into protein was measured in a set of samples where the pH was held constant at 6.3 using a 0.2M phosphate buffer. In a second set of samples the pH was held constant at 7.2 and 1g of tailings were added. The final set of samples served as a control and was not amended with tailings and the pH was not adjusted.

Isotope Dilution. Isotope dilution was measured in a manner consistent with other studies (1, 21). Concentrations of cold leucine were increased, while maintaining a constant concentration of radioactive leucine. Initial concentrations of [^{14}C]leucine were 20μM and 50μM (specific activity 125Bq nmol^{-1}).

Measurement of [^{14}C]Leucine Incorporation into Protein. Fixed samples were sonicated for 10 minutes followed by extraction of the proteins by hot (95°C) trichloroacetic acid (final concentration 5%) for thirty minutes. The samples were then placed on ice for 30min in order to precipitate the proteins. From each sample 10mL was filtered through a 0.2μm-pore-size polycarbonate filter (Millipore, Bedford, MA). The filters were then rinsed with filter–sterilized water. The filters were placed into scintillation vials with solvent and scintillation fluid. The filtrate was periodically

collected in order to account for all radioactivity used in the assays. Radioactive decay was measured in a Beckman LS6500 scintillation counter (Beckman Coulter, Inc, Fullerton, CA). The method described here has been modified from the one previously described by Fischer and Pusch (15).

Metals Analysis. A metal digest was carried out on sediments from each of the study sites in order to estimate heavy metal content of sediment surface crusts. The same procedure was conducted to determine the surface metal, or available, content of the tailings. Sediments and tailings were digested overnight in a nitric and hydrochloric acid solution, filtered and analyzed using a Thermo Jarrell-Ash Atom-Comp 800 ICP. The procedure was adapted from procedure EPA 350 B.

Statistical Analysis. Statistical analysis was carried out using JMP version 3.1.6.2 (SAS Institute, Inc, Cary, NC). Regression lines were compared as outlined by Peterson et al (23).

Results

Productivity. Samples from SBMC, the site of highest previous metals exposure, appeared to have a threshold tolerance to the mine tailings and were not affected by concentrations up to 1g (Figure 2). A stimulatory effect was noticed in SBOP samples after the addition of mine tailings. Two grams of tailings were required before productivity at SBOP was approximately equal to the productivity of the no tailings sample.

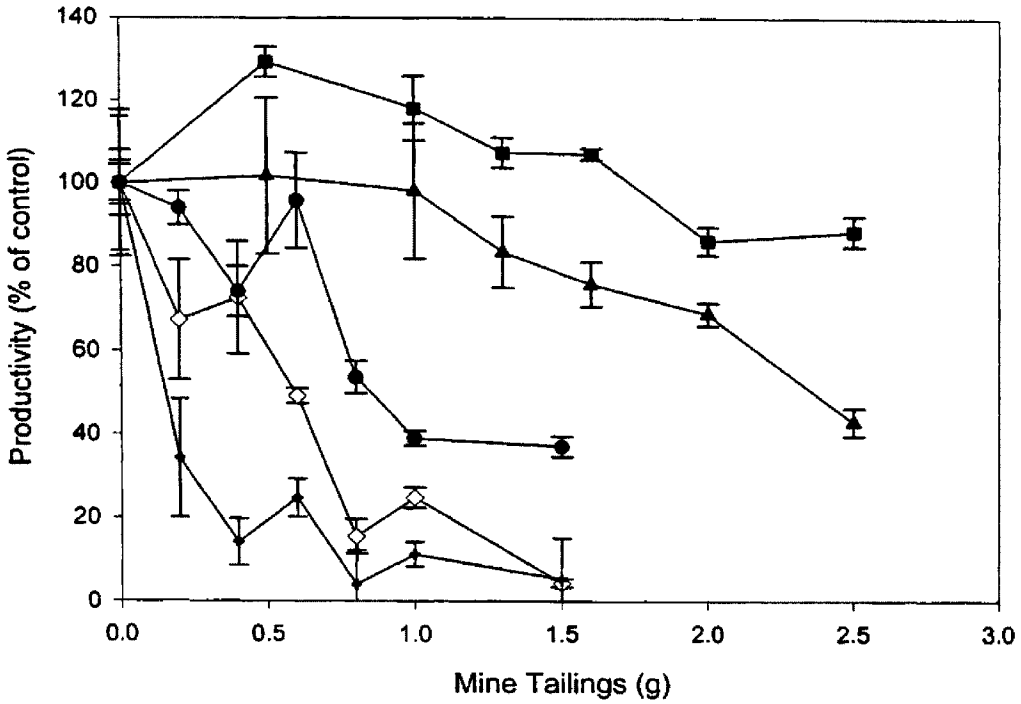

Figure 2: Mine tailings inhibit microbial productivity. Cross-hairs (+) represent RCES samples, circles (●) indicate CFGC samples, diamonds (◊) indicate CFRC samples, squares (■) indicate SBOP samples and triangles (▲) indicate SBMC samples. Values are means and error bars indicate standard error (n=4).

The decline in productivity in RCES, CFRC and CFGC samples was steeper than in the samples from SBOP and SBMC with the steepest decline coming from the microbial communities from the unimpacted tributary. A linear regression was conducted on the means of each point and the results are given in Figure 3. The slope of the line was related to the previous metals exposure. The site with the greatest previous exposure had the lowest slope, while the uncontaminated tributary had the steepest slope. The regressions resulted in two groups. The regression curves for RCES, CFRC and CFGC were not statistically different from each other. Likewise, the regressions of both SBOP and SBMC were not significantly different from each other. Each of the regressions for RCES, CFRC and CFGC were different from the regressions of SBOP and SBMC ($p<0.05$). The zero tailings point was omitted from linear regression for the

SBOP samples due to stimulatory effects of the mine tailings treatment. The last two data points were omitted from the linear regression of RCES assuming that productivity had already declined to zero. There was no statistical difference between the last three points and this allowed the focus to remain on the linear portion of the curve.

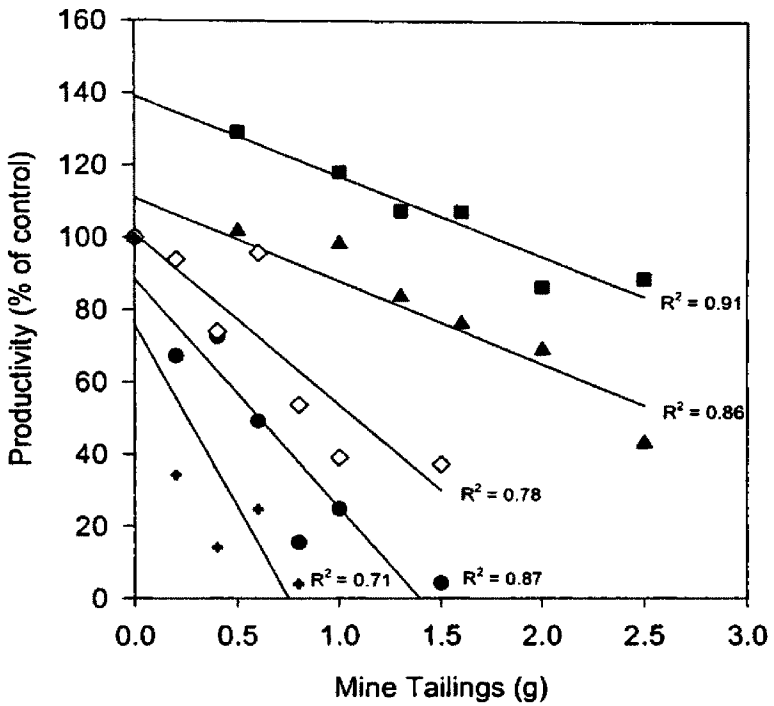

Figure 3: Linear regression plot of mean productivity versus mine tailings. Error bars have been omitted for clarity. Cross-hairs (+) represent RCES samples, circles (●) indicate CFGC samples, diamonds (◊) indicate CFRC samples, squares (■) indicate SBOP samples and triangles (▲) indicate SBMC samples. Values are means (n=4).

Isotope Dilution. A linear relationship between the added leucine concentration and the reciprocal of radioactive decay indicates that leucine incorporation is following Michaelis-Menton kinetics. Figure 4 suggests that leucine incorporation into protein is following Michaelis-Menton kinetics at a concentration of 50μM, but may not be at a concentration of 20μM. The negative intercept with the y-axis indicates the dilution of the added leucine. At a concentration of 20μM the dilution is 7.5μM. For the plot of

50μM leucine the line passes through the y-axis with a positive intercept, therefore we can assume that no isotope dilution is occurring. Thus, a concentration of 50μM leucine was chosen for these studies and no isotope dilution was assumed.

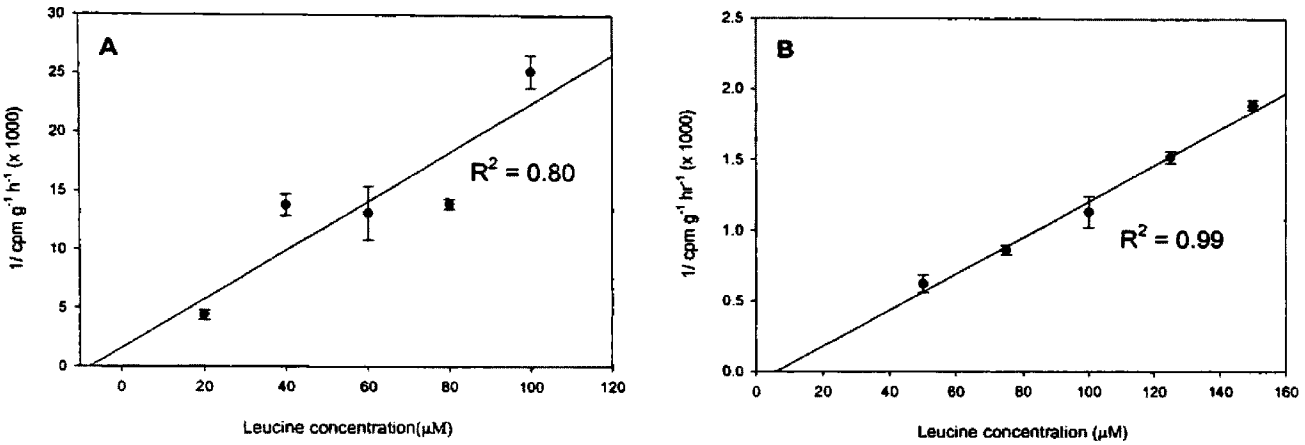

Figure 4. Isotope dilution plot indicating that incorporation processes are saturated at 50μM (B), whereas they may not be at 20μM (A).

pH. Sample pH was recorded at the beginning and at then end of the 24 hour incubation period. As shown in Figure 5, pH dropped initially upon addition of tailings and continued to decline during the 24hr incubation period. The degree to which the pH declined was dependent upon the volume of tailings in the sample. The reduction in approximately 1 pH unit (7.2 to 6.3) resulted in a drop in productivity (Fig 6). This decline in pH is approximately equal to that exhibited in samples after 24hrs when 1g of mine tailings was added. The addition of 1g of mine tailings to sediment microbial communities while holding the pH constant at *in situ* pH resulted in a greater reduction in productivity. These declines in productivity were significant (ANOVA $F = 13.7$, $p < 0.003$).

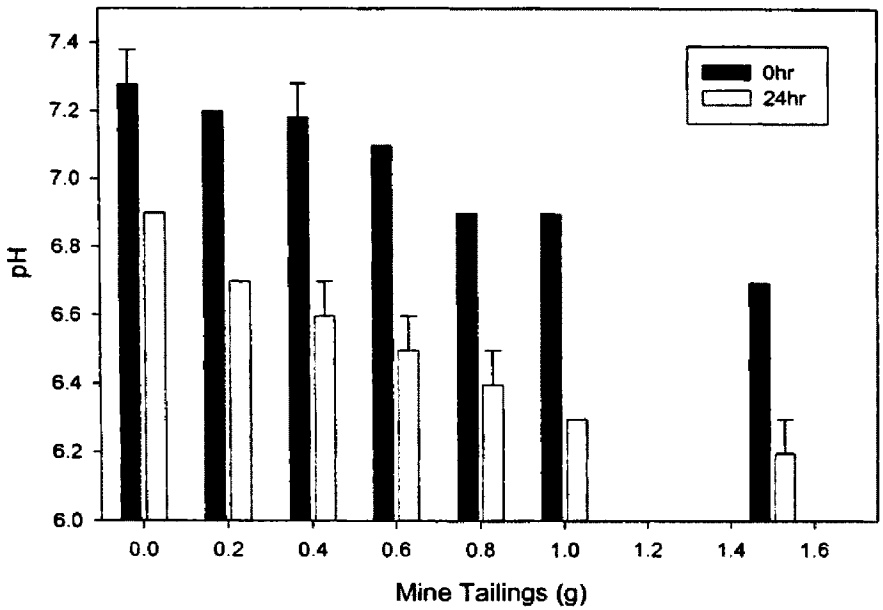

Figure 5: Increased mine tailings results in lowered pH. Error bars represent standard deviation (n=4). Lack of error bars indicate no deviation from the mean.

Metals Analysis. The results of the metal digests of sediment at each of the study sites formed a gradient (Table 2). Metal concentrations in the river sediments declined with increasing distance from Butte, MT. Results of the surface metal digest from the mine tailings used to stress the microbial communities are also shown in Table 2.

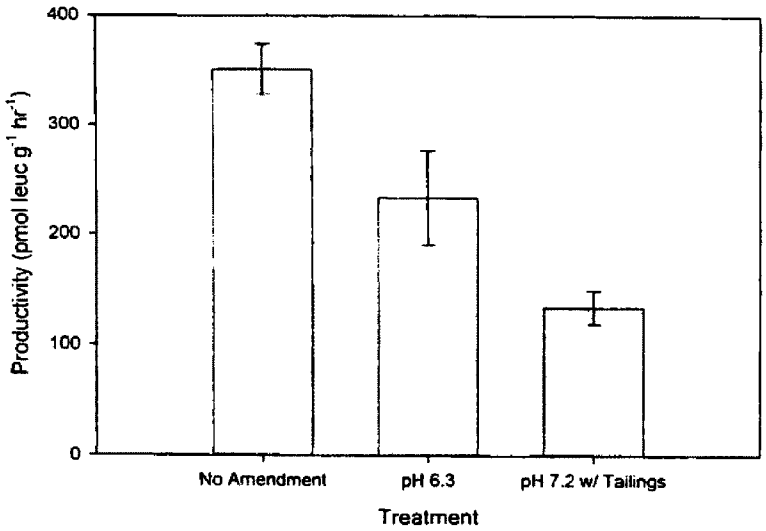

Figure 6: A drop in pH alone resulted in a decrease in productivity. The addition of metals to microbial communities without the influence of pH resulted in a greater drop in productivity relative to the unamended control.

Discussion

Development of Metal Tolerance. The degree of metal tolerance observed at sites along the Clark Fork River was positively correlated with the concentration of metals present in the sediment. Microbial communities have been exposed to different intensities of metal stress based on their place in the metal gradient and this is reflected in the slopes of the regression lines in Figure 3 (Figure 7). It is not possible to determine the exact exposure that these microbial communities have received over the last century. However, the communities in the Clark Fork River have been exposed to large quantities of metals over an extended period of time. A series of floods around the turn of the century were probably very influential in selecting for metal tolerant communities. The use of the current concentration of heavy metals in the sediment does reflect the heavy metal exposure at each site relative to the others.

Table 2: Sediment and tailings metal concentrations at study sites. Values are mean ppm (μg/g) with standard deviation.

Site	As	Cd	Cu	Fe	Mn	Pb	Zn
RCES	2.4 (0.5)	0.1 (0.0)	2.3 (1.0)	4020 (660)	62 (15)	2.6 (0.3)	2.3 (1.7)
CFRC	5.1 (1.2)	0.5 (0.08)	33 (4.0)	4030 (880)	310 (59)	8.9 (1.3)	122 (17)
CFGC	7.5 (1.9)	0.6 (0.1)	75 (16)	4850 (1150)	372 (47)	16 (5)	164 (21)
SBOP	41 (8.1)	0.8 (0.46)	210 (43)	7240 (810)	204 (82)	68 (23)	363 (100)
SBMC	55 (22)	1.7 (0.39)	400 (210)	5310 (920)	470 (200)	93 (26)	440 (120)
Tailings	130 (2.0)	0.85 (0.16)	340 (46)	9670 (39)	260 (87)	220(30)	448 (51)

Sediment microbial communities from the unimpaired tributary, RCES, were less tolerant of the mine tailings than any of the sites along the Clark Fork River. Microbial communities from the low metals site, CFRC, exhibited the greatest drop in productivity of any of the Clark Fork River sites. This site is the furthest downstream and furthest from the source of the heavy metals. CFGC, the intermediate-low metals site, is closer to the source of the heavy metals and communities were more tolerant of the metal additions. Both CFRC and CFGC are below the liming station in Opportunity, MT. Lime is added to the water as it flows from Opportunity Ponds in order to raise the pH of the water and keep the heavy metals in the particulate phase. There is a pronounced increase in the slope of the regression lines below the liming station, indicating less tolerance (Figure 7). Statistically, the sites cluster into two groups. Those above the liming station are not significantly different from each other and those below the liming station are not significantly different from each other. The two groups are significantly different from one another. The greatest metal tolerance, and the shallowest slope, was observed at SBOP and SBMC, where the communities have not only been exposed to the greatest historic selective pressure of any of the sites, but continue to be exposed to

elevated concentrations of heavy metals. The results observed here are supported by numerous other findings where the degree to which microbial communities were previously exposed to heavy metals determined their tolerance to a second challenge by heavy metals (2, 4, 5, 12, 20). However, as indicated by linear regression, the sites below the liming station are not significantly different from each other. There is certainly a trend with decreasing tolerance as sites get further from the source of contamination (Figure 7), but we can only conclude that the communities in the headwaters, Silver Bow Creek, are more tolerant than communities downstream.

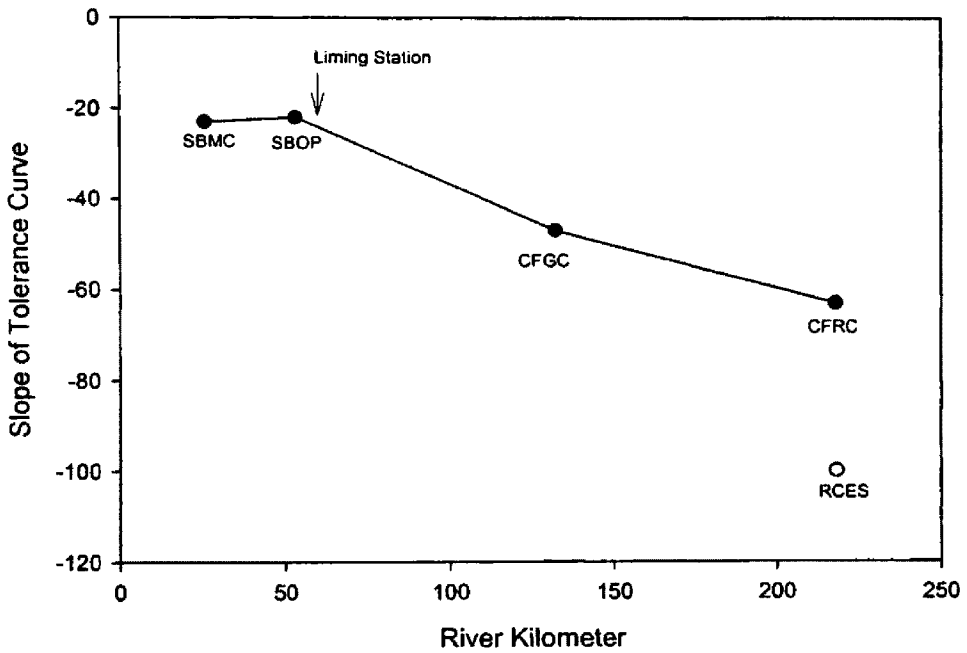

Figure 7: Slope of the curve from Figure 3 plotted against sample position in the heavy metals gradient. RCES is a tributary of the Clark Fork River and is plotted as an open circle to indicate that.

SBMC and SBOP, the high metals site and the high intermediate metals site, exhibited regression lines with the same slope. The two sites are close geographically and similar geologically and hydrologically, however a strong stimulatory effect was observed among SBOP microbial communities. Other studies have observed a stimulatory effect of heavy metals on microbial communities with the thymidine

incorporation technique (24). The stimulatory effect of heavy metals observed in SBOP samples could be the result of the synthesis of metal resistance mechanisms (10, 25).

The observed degree of heavy metal tolerance at the two sites of greatest previous exposure, SBOP and SBMC, indicates that the microbial communities are consistently exposed to heavy metals. Díaz-Raviña and Bååth (2001) observed that when metal tolerant microbial communities were moved to an environment where the metal stress was removed, almost all (70% to 90%) of metal tolerance was lost within one week (10). The sheer volume and availability of highly oxidized mining wastes in the Silver Bow Creek floodplain and the high concentrations of metals in the Silver Bow Creek bed sediment, supports the hypothesis that metals from the eroding stream bank are consistently interacting with the microbial communities at these sites. Further support comes from recent documented fish kills in the Clark Fork River after runoff events (19). These pulses of heavy metals present an acute stress, while the heavy metal contaminated sediment present a chronic stress. Acute exposure to heavy metals would be expected to cause a sharp decrease in productivity. Once the initial pulse of metals has passed, some heavy metal laden sediment would be expected to be left behind, presenting a chronic metal stress. Díaz-Raviña and Bååth also observed that after the metal stress was completely removed, a degree of tolerance was retained for at least 12 months (11). This suggests that even after the stress has been removed some tolerance remains. At other sites, where elevated concentrations of heavy metals are present, but mining wastes do not regularly wash in from the floodplain, other processes would be expected to dominate metal availability. Fuller and Davis (1980) observed that diurnal patterns in pH, due to photosynthetic processes, resulted in similar, but lagging pattern in trace metal

concentrations in the surface water (17). In the Clark Fork River increases of 2 to 3-fold in soluble and acid-soluble fractions of trace metals have been observed at night (7). These processes would be expected to influence productivity, but probably not biomass due to the short exposure time. We hypothesize that these brief metal exposures could also be responsible for selecting and maintaining metal tolerant communities.

The use of broad measurements of microbial communities, such as biomass and productivity, were unable to detect the impacts of over a century of mining wastes on the Clark Fork River microbial communities. Other studies suggest that these community responses can recover from an acute stress, but may not be able to recover completely from a chronic stress (18). Therefore, we should have observed a decline in both biomass and productivity in the Clark Fork River relative to metals concentrations. It is quite likely that these measures have been impaired by the chronic metal stress, however, seasonal variations and land use patterns may have obscured them. Changes in PLFA and DGGE patterns, coupled with tolerance measurements indicate that the communities have adapted to the metal stress that they are routinely exposed to (14). Community composition and tolerance measurements show that there has been and there continues to be a strong impact from mining wastes on microbial communities.

Metals Analysis. This study did not use metal salts to stress microbial communities as is commonly done. Instead mine tailings were used to test microbial tolerance to metals as they would occur *in situ*. As mentioned earlier the tailings were not true mine tailings. Mine tailings are very fine grain and present a problem, as they are not the same grain size as the study sediment. The "tailings" used in this study may have been coarse jig tailings or may have been the result of secondary deposition of

metals. Secondary deposition occurs when weathering of sulfide minerals in the true tailings release soluble metals that are re-precipitated on the "tailings" as they percolate through the floodplain. When these "tailings" were mixed with sediment in the laboratory, metal cations and protons were released from the surfaces. The protons lowered the pH and furthered the release of soluble metals. Although this study did not use true mine tailings, the "tailings" did serve their purpose as a source of metals for stressing microbial communities in a manner similar to what occurs *in situ*. The tailings that routinely enter Silver Bow Creek are highly oxidized, thus the metals are available and readily interact with sediment microbial communities.

Iron and manganese oxides form a coating on the surface of sediment grains and cations of copper, zinc, and other metals adsorb to this negatively charged coating. Protons are also able to adsorb to these surface coatings. As the pH declines protons compete for binding on the negatively charged oxides, forcing metals and additional protons into solution. Arsenic is the exception since it is actually present as $H_2AsO_4^-$ and the negative charge causes it to act in a manner opposite to the other metal species. Therefore, pH is an important factor in the availability of these metals. As shown in figure 5, sample pH declines from around 7 to around 6.2 as tailings are added up to 1.5g. This pH range follows the sorption edge for iron hydroxides. Thus, as tailings were added to the samples in the laboratory, the pH declined, which affected the surface charge on the tailings, which influenced the concentration of metals in solution. We are studying the effects of natural metal contamination on microbial communities and pH is an inseparable part of this contamination. The inhibition of leucine incorporation rates shown in Figure 2 reflect both inhibition by heavy metals and by a decline in pH. As

demonstrated in Figure 6, both the metals and the pH play a role in inhibiting microbial community productivity with metals probably playing a greater role. Baker et al. (1982) concluded that not only did pH inhibit microbial activity, but it also determined the toxicity of metal ions in solution (6).

Tailings as a means of measuring metal tolerance. Microbial communities from areas receiving mining wastes are not only subjected to multiple different metals simultaneously, but also are often subjected to the effects of a change in pH. The use of metal salts to determine community tolerance allows researchers to determine the contribution of individual metals, but can be misleading because communities often demonstrate a co-tolerance to other metals (4, 5, 13). Although the use of tailings to measure community tolerance does not allow for the elucidation of which metals illicit the greatest tolerance, they are a more natural source of contamination and they represent a very realistic approach to determining how microbial communities respond to metal stress *in situ*. The mining wastes studied here still enter the headwaters of the Clark Fork River and expose microbial communities to highly oxidized metals and to a sharp decline in pH.

Microbial metal tolerance studies have frequently reported a co-tolerance to metals that the microbial communities have not been exposed (4, 5, 13). The development of co-tolerance could possibly be due to similar or shared resistance mechanisms (2, 13). Metal exposure in the Clark Fork River has been to numerous metal species that go beyond those listed in Table 2. Even a well planned study using metal salts would have significant difficulty determining which metals were most important in the development of the observed tolerance.

This work is also one of only a few studies to use the leucine incorporation technique for tolerance studies (11, 24). The leucine incorporation technique has been demonstrated to be a more sensitive measure of community activity than the thymidine incorporation technique (11). Bacterial counts are less sensitive than the thymidine incorporation technique and are limited by our ability to culture communities on agar plates (1).

Conclusions

Many microbial processes are reported to be impaired by the presence of heavy metals. In the presence of a chronic metal stress, however, microbial communities develop tolerant populations due to selective pressure. Upon chronic exposure, broad measures of microbial activity are not likely to indicate stress or injury by the contaminant until some threshold level is reached. Earlier studies in the Clark Fork River indicated that there is essentially no loss of productivity or biomass due to the elevated concentrations of heavy metals. However, microbial communities from sites along the Clark Fork River have demonstrated a tolerance to heavy metals addition relative to communities from an unimpaired tributary. The degree to which tolerance was developed in the Clark Fork River reflected the level of chronic exposure the communities were subjected to.

The metal source used to test for microbial tolerance was not necessarily primary mining wastes (i.e. tailings), but were more likely secondary deposits of metals (metal complexes coating sediment surfaces). This suggests that these metal complexes (even in

circumneutral waters) are not inert and the attached microbial communities interact and are therefore impacted significantly when these deposits slough off of stream banks and are transported downstream. It was also demonstrated that both pH and metals play a role in *in situ* inhibition of community productivity, although metals appear to play a greater role. Finally, this is the first evidence of alluvially deposited mining wastes entering the streambed, interacting with microbial communities, resulting in a prolonged and chronic selective pressure after mining operations have ceased.

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CHAPTER 4

GENERAL DISCUSSION

The study of chronic heavy metal contamination on sediment microbial communities in the Clark Fork River was divided into two parts. The first part examined microbial communities with respect to chronic heavy metal exposure using direct counts to estimate biomass and [^{14}C]leucine incorporation into protein to determine microbial productivity. The second part aimed to determine if, and to what extent microbial communities in the Clark Fork River have developed a tolerance to heavy metals. In order to measure heavy metal tolerance in the most realistic manner possible, natural contamination (mine tailings) were used in place of artificial contamination (metal salts).

Monitoring microbial community biomass along the metals gradient and comparing the observed values with those from the uncontaminated reference sites it was determined that there was no correlation with metal concentrations. This is contrary to numerous studies that have shown that biomass at sites chronically contaminated with heavy metals is often lower than biomass at uncontaminated sites (5, 7, 11, 25, 29, 41). A seasonal biomass pattern did emerge, suggesting other factors may be more important in controlling biomass in this system than heavy metals. Current literature suggests that biomass may be influenced by or correlated with dissolved organic carbon, sediment organic matter, sediment water content or water temperature (18, 21, 42). Future studies may wish to monitor such parameters as pH, DO, sediment organic carbon, flow and temperature in order to better correlate microbial biomass in the Clark Fork River with seasonal changes.

Seasonal measurements of the rate of microbial incorporation of leucine into protein did not appear to be inhibited by the elevated background levels of metals in the sediment either. Rates of bacterial carbon production (BCP) were similar to published values from sediments that were not contaminated by heavy metals, supporting the idea that heavy metals are not the primary drivers of microbial productivity in this system. However, the sampling procedure may have omitted much of the heavy metals induced variation in microbial community productivity. If sampling had occurred immediately after a runoff event, productivity would be expect to decrease due to the influx of heavy metal-laden mine waste washed in from the floodplain at SBMC. Diel cycling of metals can be caused by photoreduction and pH-dependent metal desorption from sediments in river systems (10, 24). These processes have been shown to increase metal concentrations by 2 to 3-fold in the Clark Fork River at night when pH and dissolved oxygen also decrease as a result of the cessation of the light reactions of photosynthesis (10). Thus, the effects of metals on microbial communities may be more pronounced at night.

The observed pattern of microbial productivity in the Clark Fork River is most likely driven by both seasonal changes and land use patterns although no clear pattern emerged. Past studies have linked microbial productivity to seasonal fluctuations such as water temperature and the sedimentation of diatoms (45). However, it is widely accepted that elevated concentrations of heavy metal have an inhibitory effect on both microbial biomass and productivity (3, 5, 6, 7, 11, 16, 17, 25, 28, 29, 40, 41). It is possible that any reduction in biomass or productivity relative to heavy metal concentrations was masked by the seasonal fluctuations and land use patterns. Such broad measures of community

response as these may not be suitable for measuring the effects of chronic metal contamination in the Clark Fork River. It is also likely that microbial communities in the Clark Fork River have adapted to the metal stress that they routinely see. One mechanism of adaptation to metal stress at the community level is a change in community structure and other studies in the Clark Fork River have shown changes in microbial diversity as measured by denaturing gradient gel electrophoresis (DGGE) patterns and phospholipid fatty acid (PLFA) patterns in response to heavy metal contamination. (20).

A change in community composition in response to heavy metal stress can result in a more tolerant community (7). To test this theory of increased metal tolerance we subjected microbial communities to mine tailings at various concentrations. Mine tailings continue to enter the headwaters of the Clark Fork River and thus, their use allows us to test metal tolerance in a manner very similar to how it occurs *in situ*. In this procedure microbial communities were exposed to not only available heavy metals, but also pH and intrinsic properties such as shape and surface properties. The leucine incorporation technique was used to measure productivity in the presence of the tailings.

The degree to which tolerance was developed at each site was positively correlated with the concentration of heavy metals present in the sediment. A pronounced drop in metal tolerance was observed below the liming station, possibly suggesting that efforts to keep metals in the particulate phase have been to a degree successful. All sites in the Clark Fork River exhibited a greater tolerance to the introduced mine tailings than did the microbial communities from the unimpaired tributary. This correlation between increased exposure to heavy metals and increased tolerance is consistent with numerous

studies which have employed artificial contamination (metal salts) as the means of metal stress (3, 6, 7, 13, 16, 29, 34).

When mining wastes enter streambeds they not only subject microbial communities to heavy metals, but also a change in pH. The use of natural contamination (tailings or secondary deposits of metals) to measure community tolerance represents a very realistic approach to determining how microbial communities respond to mining wastes and metal stress *in situ*. The experimentally added tailings resulted in a decrease in sample pH and, in all but the most tolerant samples, a decrease in community productivity. Both pH and the heavy metals played a role in the decrease in productivity with the heavy metals playing the greater role.

A study by Díaz-Raviña and Bååth (2001) showed that microbial communities rapidly lost their metal tolerance when the heavy metal stress was removed (15). These data suggest that the routine influx of heavy metals from the high volume of mining wastes present in the Silver Bow Creek floodplain continually stress the microbial communities and select for a more metal tolerant community. We believe that this is the first evidence of alluvially deposited mining wastes entering a streambed, interacting with microbial communities and applying a prolonged and chronic selective pressure.

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