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Relationship of Heavy Metal Contamination to Soil Respiration

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Introduction

This report explores the hypothesis that heavy metal contamination, specifically on Grant-Kohrs Ranch and on Bureau of Land Management (BLM) sites along the Clark Fork River, has resulted in reduced soil function as measured by soil respiration. Soil respiration is a measure of the decomposition of organic carbon by both plant roots and the soil/root-associated microbiota. This report is a product of a two year study where in the first year 15 plots (3 x 3 meters) were evaluated from mid September to mid November, and in the second year 30 larger plots (4m x 4m) were measured 4 times from June – September.

The choice of using soil respiration as a measure of soil injury is from evidence that soil activity (e.g. nutrient cycling) has an essential influence on plant growth, hence production. In soils with low or significantly altered soil communities, nutrients tend to accumulate in forms unavailable to the indigenous plants (Berg et al., 1995; Vesterdal et al., 1995) and plant growth and productivity are severely compromised. Heavy metal contamination is known to hamper the decomposition of litter (Berg et. al, 1991) and reduce soil respiration and microbial biomass (Bååth, 1989, Nordgren et al., 1988, Nordgren et al., 1986, Lighthard et al., 1984, Nordgren et al., 1983, Dumontet et al., 1992; Fostegard et al., 1993; Vanhala and Ahtiainen 1994, Valsecchi et al., 1995; Saviozzi et al., 1997; Palmborg-Cecilia et al., 1998). Several research groups have shown that the microbial community composition changes in response to mining associated heavy metal contamination (Frostegard et al., 1993; Pennanen et al., 1996) such as that existing in the riparian areas along the Clark Fork River.

There are always potential complications with studies concerning cause and effect. In the case of heavy metal contamination, metals levels may show varying effects. In some studies, heavy metals only showed a negative effect on soil respiration when concentrations of heavy metals reach very elevated levels. For example, in two studies about 1000 ug Cu $g^{-1}$was needed to significantly reduce soil respiration (Nordgren et al., 1988; Bååth, 1989). In another study, however, it was found that reduction in soil microbial activity occurred at 60 ug Cu g^{-1} and 120 ug Zn g^{-1} (Tyler and Westman, 1979). This suggests that metals have varying " availability" and/or other variables (including community adaptation/tolerance) significantly influence soil respiration (e.g. organic carbon, moisture, pH) and that the importance of a given variable may change spatially and/or temporally. Another potential complication is that in areas with low or intermediate contamination, where large spatial variation and known (see Wielinga et al.,1999) heterogeneity of fluvial deposited tailings exist, it may be difficult to differentiate effects of heavy metals from other soil influences. Other parameters that are known to affect microbial respiration are soil carbon quality, nutrient availability, microbial structure, and moisture (Palmborg et al., 1998, Palmborg and Nordgren, 1996).

Goal and Objectives

The goal of the study was to evaluate the relationship of soil heavy metal content to soil respiration. The basic objectives were as follows:

a. Using heavy metal data provided by the Murdock Environmental Biogeochemistry (MEBL) lab at The University of Montana, identify approximately 15 sites (season 1) and in the second year another 30 sites where the sites were chosen (random stratified design) across a heavy metal gradient.
b. At each site, and at each sample period, collect, homogenize and composite samples, and conduct analyses for soil organic matter, soil moisture, microbial biomass, and microbial community structure (using phospholipid fatty acid profiling).
c. At each site install respiration collar(s) and monitor soil respiration (using an *insitu* infrared gas analysis) at 3-5 time periods throughout the season.
d. Analyze the data using a combination of different multivariate statistical methods so that the variation in microbial parameters can be separated into a heavy metal component and other soil components.
e. Present the final data in terms of a final report and when appropriate a peer-reviewed publication.

Experimental

Site Selection

2000 Field Season -In year one, using the 1ft core metals data generated by the MEBL, we regressed Cu with Zn and thereby ordered metal concentrations from all the 1ft core samples. From the points on the resulting curve we randomly selected 5 sites on the low end, 5 sites in the intermediate zone, and 5 sites on the high end. Using GPS we located these sites and staked out one 3m x 3m plot at each. In selecting the precise area for the plot we found the closest $3m^2$ area to the north and west of the original stake that was visually homogenous (no ditches/depressions, bushes, fence posts, etc) in appearance. A short site description table is provided below (Table 1).

Table 1: Site Description (2000 Field Season)

Site No.	Metal Load *	Type	Description
MT9	364.6	Pasture	Always Damp, on fringe of marshy area
MT3	390.0	Pasture	Irrigated horse pasture (Sterwart Field east)
MT4	1487.8	Pasture	Irrigated horse pasture (Sterwart Field west)
MT14	1922.4	Fenced Riparian	Thick grasses
MT12	2350.6	Fenced Riparian	Grasses, near bushes
MT1	3366.4	Pasture	Horse and cow pasture, located in clover patch
MT7	3405.5	Fenced Riparian	Grasses
MT2	3750.5	Fenced Riparian	Fringe of slickens, mossy ground cover
MT8	3871.4	Fenced Riparian	Grassy path with alder/willows nearby
MT5	3963.3	Fenced Riparian	Thistle
MT15	4148.9	Fenced Riparian	Sparse grasses
MT13	4441.5	Fenced Riparian	Grasses
MT11	5309.9	Fenced Riparian	Sparse, dead grass
MT6	6775.6	Slicken	No vegetation, large slicken
MT10	7469.1	Slicken	No vegetation, small slicken

*The sum of As, Cu, Cd, Pb and Zn (ppm)

2001 field season (MP sites) - These sites (30) were selected in a manner very similar to that used in the 2000 season (i.e. using a random stratified site selection procedure). Briefly, from the 12" core metal data of 2000, we plotted Cu vs. As (As data had a strong negative correlation with soil respiration in Year 1), and then fractioned out the sites based on As concentration as shown in Table 2 below. From the Cu vs. As coordinates were randomly picked sites (unlabeled dots on the graph) beginning with the > 600 first. Essentially all sites in the > 600 and all the 401-600 were used so that we would could obtain the minimum number of sites desired. Nine of the 13, and 10 of the 15, sites in the 201-400 and 0-200 ranges, respectively, were also selected.

Table 2- 2001 Site Selection Data

Arsenic Range	Sites Selected (SS sites from 12" cores)
0-200	21, 65, 33, 69, 24, 72, 79, 36, 34, 62
201-400	19, 68, 78, 100, 77, 71, 18, 22, 42
401-600	51, 60, 59, 56, 66, 67
>600	58, 35, 57, 53, 70

Underlined sites are former MT sites from year one (see below)
Italicized sites have well nests already installed

Special Site selection procedures for previous MT sites: When previous MT sites were selected, the following uniform procedure was followed. After finding the MT plot, a site 5 m to the north was used and given an MP designation. This was done in a effort to include 30 new sites.

Plot layout

2000 Season - For the 2000 season (MT 3 x 3m plots), each plot was sampled as diagrammed in Fig. 1. The gray circles represent respiration measures (6/site/time period) while the white and gray circles combined represent 15 cm soil cores collected for the other measures shown in Table 3. The nine cores (Fig 1: all circles) were pooled in groups of three for analysis of all soil parameters including soil metals (see report from MEBL). The shaded circles represent soil respiration collars where respiration measures were made. For each sampling period new collar locations were used. The boxes indicate block soil samples taken for concurrent phytotoxicity studies.

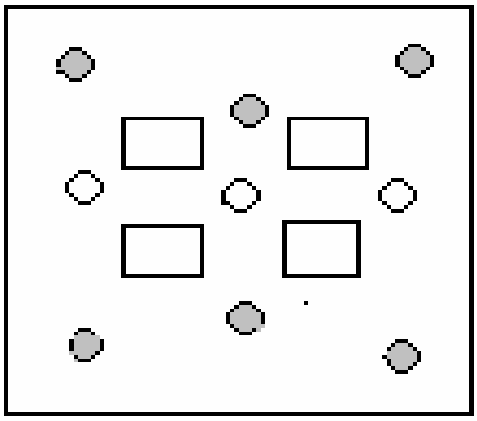


Figure 1: Plot layout 2000 season

2001 Season - The plot layout for the 2001 season was slightly different from that used in the 2000 season (Figure 2). The main differences were a) the plot size was expanded to 4 x 4m, 2) a uniform vegetation pattern was not a requirement and 3) the metal and soil analysis was conducted on the pooled blocks of soil sample collected for phytotoxicity work rather than separate cores adjacent to the respiration measurements (as in year 1). These changes were made in an attempt to increase the robustness of the data relative to making broader interpretations of microbial impact.

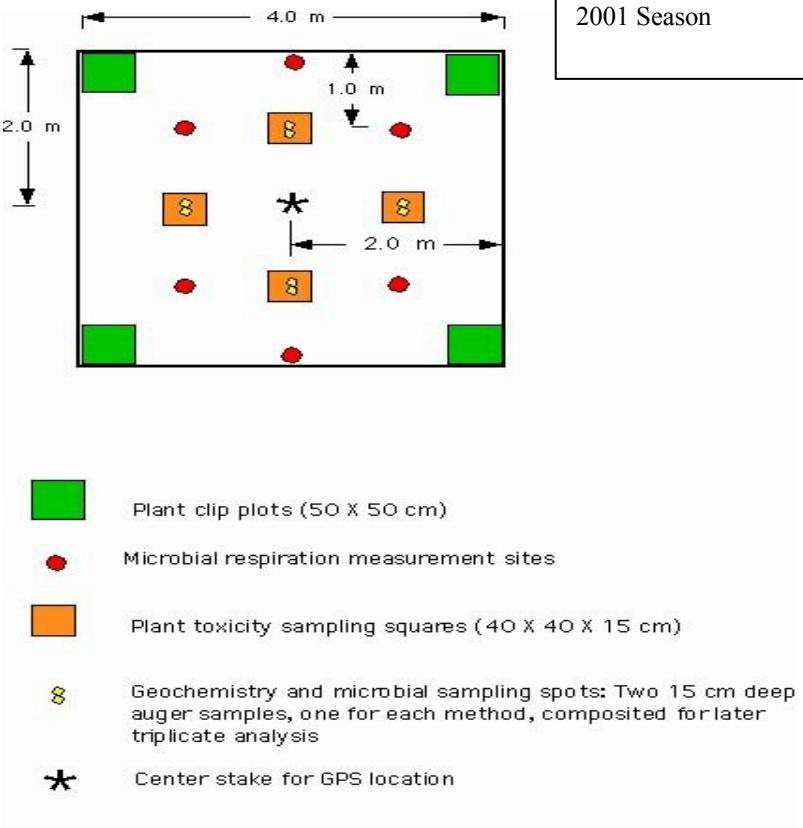


Figure 2: Plot Layout for the 2001 Season

Sample Frequency

At the indicated sample periods, measurements shown in Table 3 and Table 4 were made. Samples for soil metals (2000 season) and pH were collected during the Sept. 14th period only. We assume that the total metal concentration would not change from the first time point to the subsequent time points.

Table 5 provides additional information relative to the methods used.

Table 3: Sampling time and measures made for 2000

Measurement	Sep 14-18	Sep 26,30	Oct 10,11,16	Nov 2-3
Respiration	✔	✔	✔	✔
Loss on Ignition (350°C)	✔		✔	
Microbial Biomass	✔		✔	
Soil Temperature	✔	✔	✔	✔
Soil Moisture	✔		✔	
PH	✔			
Metals	✔			
PLFA	✔		✔	

Table 4: Sampling time and measures made for 2001

Measurement	June 6-7	June 21-23	July 1-3	Sept 24-26
Respiration	✔	✔	✔	✔
Loss on Ignition (350°C)	✔			
Microbial Biomass	✔			
Soil Temperature	✔	✔	✔	✔
Soil Moisture	✔			✔
PH	✔			
Metals	✔			
PLFA	✔			

Table 5 – Sample measure methods

Type of Measurement	Method(s)[a]
Respiration	GM-08
Loss on Ignition (350°C)	GM-04
Soil Biomass	GM-02
Soil Temperature	GM-08
Soil Moisture	GM-03
pH	MEBL[b]
Metals	MEBL
PLFA	GM-06, 07

a. The method designation refers to a specific SOP.
b. MEBL– refers to the Murdock Environmental Biogeochemistry Lab, Department of Geology, at the University of Montana. Refer to their material for procedures etc.

Soil Sampling and Sample Preparation

We anticipated a great deal of spatial variation at each site and attempted to reduce this somewhat by pooling several samples across each plot into a composite sample.

In the 2000 season 9 cores from each plot were composited into 3 sets of 3. The samples were collected via auguring to a depth of 6 inches. Each sample was placed in a labeled sterile whirl-pak bag and transported to the laboratory on ice (usually within 8 hours). At the laboratory, the three samples (3 cores each) were sieved (4mm) to remove roots and then homogenized. An aliquot from each was removed for analysis; triplicates were removed for every 20th sample. For example for Site MT1 there was three measurements made labeled in the data section as MT1-A, MT1-B, and MT1-C. Storage and further preparation of the sample is described in each specific procedure.

In the 2001 season, four blocks of soil were removed from each plot (see MEBL sample collection protocol). The blocks were pooled and we received a subsample. Our subsample was sieved (4mm), roots removed, and an aliquot subsampled for each analysis. Every fifth sample was analyzed in triplicate to evaluate laboratory error.

Sample analysis

In situ -Soil Respiration (GM-08)– The primary processes that produce carbon dioxide in soil are respiration by roots and soil organisms from the decomposition of organic matter. The production of CO_2 therefore normally correlates with soil organic matter as well as with soil temperature and moisture. At each test plot 6 four-inch soil collars were

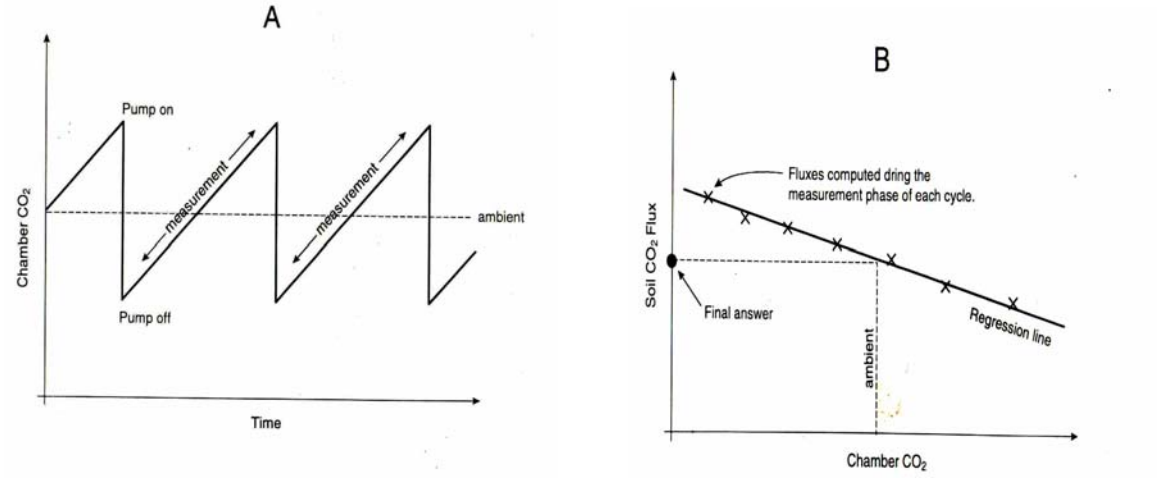


Figure 3: Diagram explaining how soil CO_2 efflux (Respiration) is determined using the Licor 6400 fitter with a soil chamber. Panel A shows a cycle draw down

is logged as the CO_2 in the chamber rises above ambient. Flux values are computed across repetitive measurement cycles and a regression is drawn (Panel B). Final CO_2 flux is determined at ambient CO_2.

installed (3-4cm deep) in an area that had been cleared of surface vegetation (grass was cut to surface level with scissors). At each sample time period, a portable Li-6400 (Licor Instruments, Lincoln, Nebraska) infrared gas analyzer with a 6400-09 soil chamber was

fitted on to the collar. The measurement protocol was fully automated, in brief, the instrument circulates the air in the chamber through a soda lime CO_2 scrubber effectively lowering the chamber CO_2 concentration to a prescribed level (delta value) below ambient CO_2. Ambient CO_2 is measured and recorded initial as part of the calibration protocol (this data can be found in Appendix A). As soil respiration occurs, the CO_2 in the chamber rises. Every two-three seconds the CO_2 flux is computed based on a running average of change of CO_2 concentration with time. This process is repeated through a number of cycles and the intermediate flux data are fit with a regression, which is then used to calculate soil respiration (umol CO_2 m^{-2} s^{-1}) at ambient CO_2 (see Figure 3). The average of the 6 measures at each site are used to report soil respiration at each time point. The raw data is given in Appendix A.

Soil Microbial Community Structure: Ecological theory predicts that stress on a community will change the metabolism and assemblages of populations within the community (see Odem, 1985). Alteration of the microbial community to substantiate the effect of heavy metals on soil function is based on the premise that metal toxicity exerts a selection pressure, which induces a change in the composition of the community. Since microorganisms have discernible lipid patterns, PLFA composition gives an integrated picture of all living organisms in the soil, and changes in the PLFA pattern of the soil indicate an altered species composition due to, e.g. metal toxicity (Baath et al., 1998). A number of other workers have also demonstrated the value of PLFA in understanding soil damage due to heavy metal contamination; hence, this provides relevant cause and effect information (Frostegard et al., 1993; Palmborg et al., 1998, Palmborg and Nordgren, 1996). We have conducted these analyses on Clark Fork River sediments and they have proved valuable in determining changes in microbial community structure across a heavy metal gradient. From these data, the goal was to determine a) if different sites have altered community structure due to a given variable (e.g. heavy metals), and b) the ratio of relevant groups of organisms present at each site (e.g. fungal to bacterial ratio, gram negative to gram positive ratio). The specific methods used to preprocess the soil, extract, purify, and analyze the fatty acids are given in GM 07-08 .

Soil microbial biomass - Soil microbial biomass is an important component of the soil organic matter, regulating the transformations of soil nutrients and carbon. It is a labile component of the soil organic fraction containing 1 to 3% of the total soil carbon (Smith and Paul 1990).

The chloroform fumigation extraction method (Horwath and Paul 1994) was used to measure soil microbial biomass carbon. This is a standard method that has the advantage over related methods (e.g. chloroform fumigation incubation) of short analysis time, and low interference from non-microbial labile C. The soil samples were sieved (4mm) as this size mesh has been determined not to affect the soil microbial biomass size (Ross et al. 1995). Briefly, fumigated (chloroform, for 5 d at 25˚C) and un-fumigated replicates were extracted on a shaker for 1 hr with 0.5 M K_2SO_4. This soil suspension was filtered, and the filtrate collected and frozen until analyzed. A blank filtrate, extractant alone, was run for each batch of samples and analyzed to determine background levels of C in both the filter paper and extractant. The soluble C in the extract was analyzed on a C analyzer. Biomass carbon is calculated as (Cf – Cuf)/ Kec, where Cf = C in fumigated extract, Cuf

= C in the un-fumigated extract. Kec is the proportion of the microbial C that is extracted from the soil, a general value used for soils being 0.35 (Voroney et al. 1991). The protocol is described in detail in GM-02.

Soil organic matter - Soil organic matter is defined as the organic fraction of soil, including plant, animal and microbial residues at all stages of decomposition, and the relatively resistant soil humus. Soil organic matter generally scales with soil organic carbon content (Horwath and Paul 1994). Soil organic matter was analyzed by the loss on ignition (LOI-350) method at 350˚C (Nelson and Sommers 1982). The protocol is described in detail in GM-04.

Soil Moisture: The sieved soil was weighed in triplicate, dried at 105^{o} C for 72 hours and reweighed. (see GM-03).

Results

Respiration and the relationship to soil metals – The data presentation is organized as follows:

Part 1: *Presentation of 2000 data (MT sites)*
Part 2: *Presentation of 2001 data (MP sites)*
Part 3: *BLM Respiration Data*
Part 4: *Presentation of pooled 2000, 2001, and BLM data.*

Part 1, 2000 Data:

Respiration vs. Metals: The respiration averages for the 4 time points in the 2000 season are summarized in Table 6. This data can also be found in Appendix A (Summary Respiration Data and Licor Respiration Output Files). The data show that sites MT1, 3, 4, 9, and 14 have the highest average respiration across the 4 sample periods (average = >5 uM/m^2/s). In general, these sites have the lower total metals and the higher organic matter concentrations. It can also be seen that soil respiration declined from the 14th of September through the 2nd of November. This was attributed to a significant decrease in soil temperature. Soil temperature data can be found on the Licor output files (Appendix A).

Table 6: 2000 Respiration Data Set

Site	14-Sep	SD	26-Sep	SD	10-Oct	SD	2-Nov	SD
MT1	12.272	1.636	4.702	0.626	4.088	0.464	2.542	0.556
MT2	2.618	1.013	1.145	0.285	0.850	0.336	0.386	0.068
MT3	11.574	2.386	4.678	0.244	4.088	0.729	1.442	0.230
MT4	10.870	1.467	5.941	2.305	2.309	0.326	1.756	0.147
MT5	6.658	0.847	3.665	0.545	2.052	0.255	0.658	0.330
MT6	0.296	0.082	0.197	0.064	0.146	0.068	0.245	0.117
MT7	5.666	0.371	2.639	0.257	2.216	0.426	0.671	0.147
MT8	1.504	0.610	0.746	0.092	0.421	0.173	0.363	0.071
MT9	7.891	2.343	2.980	0.747	2.180	0.343	0.591	0.922
MT10	0.597	0.367	0.415	0.131	0.541	0.206	0.195	0.185
MT11	1.040	0.187	0.435	0.036	1.080	0.139	0.247	0.053
MT12	4.861	0.882	2.000	0.179	2.419	0.602	0.912	0.362
MT13	4.078	0.690	1.499	0.389	2.877	0.885	0.690	0.202
MT14	7.392	1.907	3.162	0.756	3.729	0.723	1.084	0.581
MT15	1.061	0.176	0.608	0.093	0.609	0.118	0.332	0.091

Table 6 contains the respiration data collected during the Fall of 2000. SD = the standard deviation of all 6 measures on each plot and represents the variation across the plot.

Multicollinearity – Before analyzing metal data relative to respiration, we examined for multicollinearity. Table 7 shows a correlation matrix (numbers are Pearson's product-moment correlation coefficients) of four metal concentrations. Only these metal concentrations were included in our analysis, since others were not significantly correlated with respiration. A high Condition Number (> 100), calculated for this set of variables, indicated multicollinearity. As stated above this means that modeling the data in a multiple regression is not valid, since multicollinearity of predictor variables violates one of the assumptions of that procedure. In order to extract a pattern from the data we performed a Principal component analysis (PCA).

Table 7: Correlation Matrix of four important metals

	Cu	As	Pb	Zn
Cu	1.00	0.82	0.78	0.49
As	0.82	1.00	0.98	0.39
Pb	0.78	0.98	1.00	0.28
Zn	0.49	0.39	0.28	1.00

Principal component analysis (PCA)–An explanation of Principal Component Analysis is given in Appendix B. Principal component scores are shown in Table 8. The PCA was based on the correlation matrix (Table 7), and was carried out on the pooled data per site along the metal transect.

Table 8: Principal component (PC) scores

Site (MT)	PC1	PC2	PC3	PC4
1	1.2443	-1.7038	-0.7670	-1.2171
2	-2.1556	0.3442	-0.9333	-0.5540
3	0.9460	1.5887	-0.2842	0.8975
4	0.4218	1.0462	-0.3030	-0.8442
5	-0.5209	-0.1240	-0.1333	0.2018
6	-0.1991	0.4284	2.5356	-0.7221
7	-0.2070	0.4226	0.1290	-1.4415
8	0.1400	-1.4650	-0.9070	1.1320
9	1.0281	1.6489	-0.1987	1.3214
10	0.5747	-1.3188	2.0518	0.9129
11	-1.3046	-0.3506	0.1330	0.6625
12	0.6902	-0.4479	-0.7780	0.8311
13	-1.0042	0.0804	0.0713	0.0565
14	1.1019	0.0611	-0.3931	-1.6896
15	-0.7556	-0.2106	-0.2230	0.4529

Associated with the PC's are the factor loadings (Table 9) of the four PC axes. Factor loadings can aid in the interpretation of the PC axes, which are linear combinations of the original variables. The higher the (absolute) factor loading of a variable on an axis, the more influential that variable is for the axis. In other words, factor loadings represent the correlation between an original variable and its factor.

Table 9 : Factor Loading Table

Variables	PC1	PC2	PC3	PC4
Cu	-0.374882	-0.411857	0.830561	-0.002146
As	-0.924118	-0.166059	0.324223	0.115365
Pb	-0.912453	-0.316429	0.225357	-0.128516
Zn	-0.250158	-0.914304	0.318492	-0.005664

Factor loadings show that PC1 is very strongly influenced by both As and Pb, while PC2 is most strongly influenced by Zn, and PC3 by Cu. The following table (Table 10) of eigenvalues and cumulative percentages explained suggests that PC4 can be eliminated, as PC1, 2, and 3 together explain already over 99% of the variability of the data set. The Scree plot criterion for choosing the number of axes to include is also shown.

Table 10: Eigen values and cumulative percentages

No.	Eigenvalue	Percent	Percent	Scree Plot
1	1.889681	47.24	47.24	|||||||||
2	1.133281	28.33	75.57	||||||
3	0.947176	23.68	99.25	|||||
4	0.029862	0.75	100.00	|

We hence choose PC axes 1 to 3 for inclusion in the multiple regression procedure. These three axes explain together 99.25% of the metal data. We have also carried out

subsequent multiple regression analyses with only PC1 and 2 included; these yielded similar results leading to the same conclusions.

Multiple Regression: Respiration vs. metals - We used the following multiple regression model:

$$(1) \qquad y = b_o + b_1 x_1 + b_2 x_2 + b_3 x_3$$

For illustration, we show a representative normal probability plot (Fig. 4) of residuals (for the September 14, 2000 respiration data).

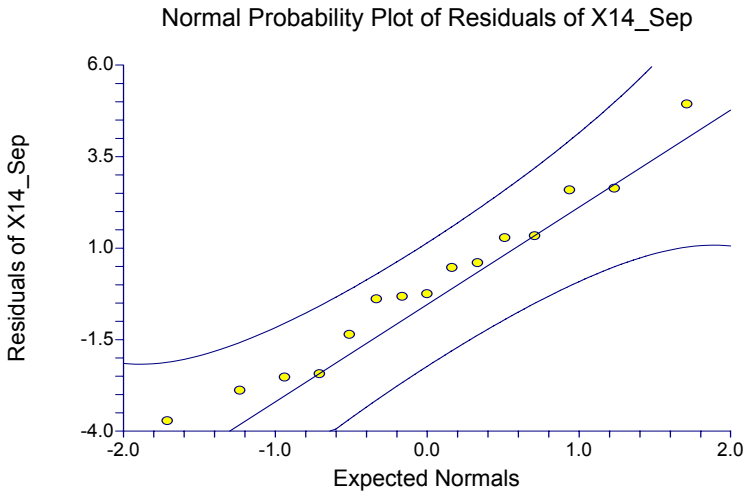


Figure 4: Probability Plot of Residuals

The following table (Table 11) summarizes the results of multiple regressions carried out for the 4 respiration measurement data sets. Given are r^2 and adjusted r^2 (in brackets; the latter seeks to remove distortions due to small sample size), and model fit (F and P value and Power given alpha = 0.05).

Table 11: Multiple Regression Summary (Respiration vs. metals)

Parameter	14 Sept 2000	26 Sept 2000	10 Oct 2000	2 Nov 2000
r^2 (adjusted r^2)	0.67 (0.58)	0.58 (0.47)	0.54 (0.41)	0.49 (0.35)
F	7.46	5.14	4.28	3.52
P	0.0053	0.018	0.0312	0.052
Power	0.92	0.79	0.71	0.62

These data indicate that a significant portion of the variability of respiration rates across the 15 sites examined can be explained using a linear combination (principal components) of 4 soil metal concentrations. Regression coefficients were positive for PC1 and 2, and negative for PC3. Comparing this to the sign of the factor loadings (Table 9) this translates to decreased respiration with increasing metal concentrations.

In addition, it can be seen that the strength of the relationship between metals and soil respiration declines from the September 14th sample period to the November 2nd sample period. This suggests that other variables (i.e. soil temperature) become more important at limiting respiration across the seasonal changes.

Loss on Ignition: The LOI measure is an estimate of organic material in the soil. Organic matter is a substrate for respiration and, therefore, should correlate positively with respiration. Organic matter was measured at two time points. These data are presented in Table 12. In general the data agree well. We expect that there would be some difference in organic values between sample time points since each time point had a unique core collected from different locations in the plot. The differences, although minor, reflect heterogeneity. In Fig. 4, column one, the degree of correlation of organic matter with respiration (r^2= 0.66, September 14th data set) is plotted. The correlation of LOI with metals is also discussed below (Table 13). As expected, sites with higher organic matter had higher respiration. We found organic matter to also be positively correlated with pH (Fig 10) : as pH went down LOI decreased.

Table 12: Loss on Ignition (Soil Organic Matter)

Date	14-Sep		10-Oct	
Site	LOI (%)	STDEV	LOI (%)	STDEV
MT1	11.349	0.68	11.456	0.979
MT2	4.002	0.821	4.767	1.514
MT3	15.516	3.129	17.727	8.224
MT4	16.126	1.401	12.451	1.373
MT5	15.034	0.895	11.327	0.459
MT6	3.073	0.823	3.927	1.272
MT7	13.256	0.444	12.17	0.429
MT8	3.539	0.361	3.329	0.36
MT9	15.04	1.837	12.38	0.352
MT10	3.217	0.393	3.914	0.854
MT11	5.861	0.669	6.858	1.003
MT12	4.394	0.578	4.815	1.335
MT13	6.949	0.788	8.099	2.01
MT14	7.72	0.039	8.118	0.908
MT15	7.446	1.715	8.895	0.979

Multiple Regression: LOI (soil organic matter) vs. metals. – We used the same regression model (1) to fit the LOI data. Results are shown in Table 13.

Table 13: Multiple Regression Summary (LOI vs. metals)

Parameter	14 Sept 2000	26 Sept 2000	10 Oct 2000	2 Nov 2000
r^2 (adjusted r^2)	0.53 (0.40)	n.d.	0.54 (0.42)	n.d.
F	4.10	n.d.	4.34	n.d.
P	0.035	n.d.	0.03	n.d.
Power	0.69	n.d.	0.72	n.d.

n.d. = not done

These data indicate that a significant portion of the variability in LOI across the 15 metal transect sites is explained by the 3 principal component axes representing metal data.

Regression coefficients were positive for PC1 and 2, and negative for PC3. Comparing this to the sign of the factor loadings (Table 7) this translates to decreased LOI with increasing metal concentrations. This is explained by the fact that sites with higher metals have more tailing deposition and less organic matter.

Soil pH -The soil pH data is given in Table 14. Soil pH ranged from 4.89 (slickens site) to 8.28 at site MT 9. Sites with lower soil pH had higher metal concentrations and lower respiration values. There was a positive correlation ($r^2 = 47$, September 14th sample period) between soil pH and respiration (Figure 5), meaning that as soil pH decreased, soil respiration decreased.

Table 14: Soil pH

Site	pH	Site	pH
MT1	7.42	MT9	8.28
MT2	7.15	MT10	5.30
MT3	7.72	MT11	6.84
MT4	6.96	MT12	7.07
MT5	7.85	MT13	6.80
MT6	4.89	MT14	7.82
MT7	7.84	MT15	5.82
MT8	6.65		

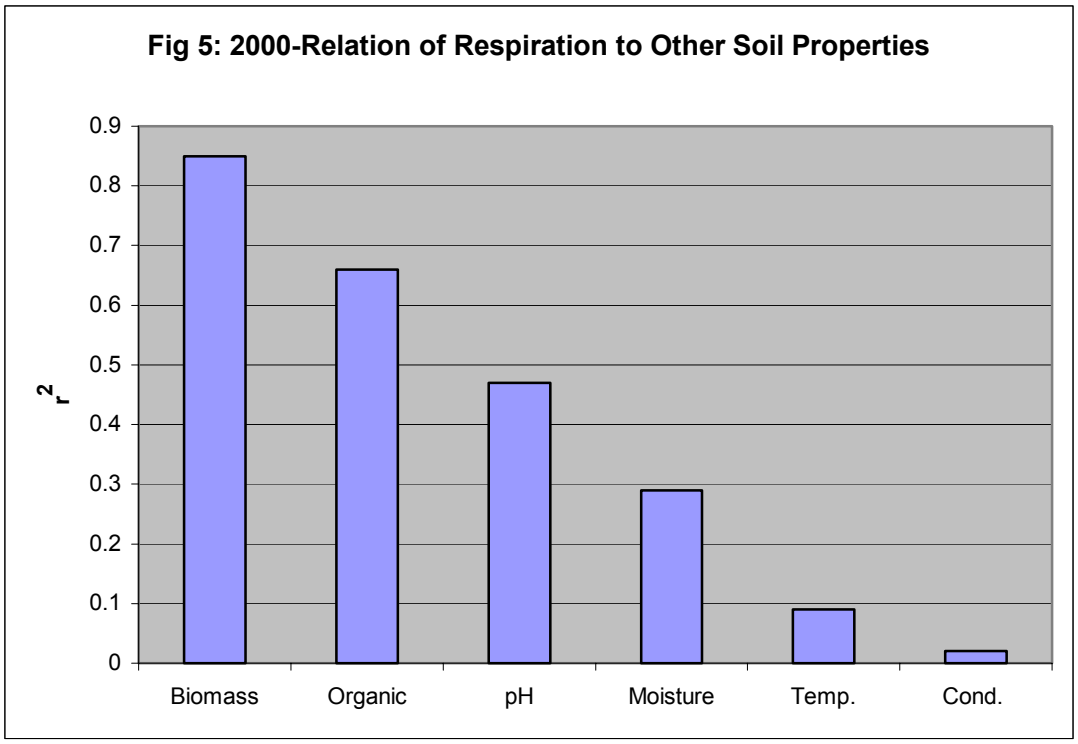


Figure 5: Linear Regressions of Soil Respiration to other soil factors for the September 14th 2000 sample period. The regression coefficients of respiration to the indicated parameter are plotted.

Soil Moisture: Within some range, microbial activity (e.g. respiration) can depend on soil moisture. The soil moisture data is given in Table 15. We found considerable variability across the plots. There was a weak but positive relationship between soil moisture and respiration (Fig. 5). With the exception of sites MT 11 and 12, soil moisture readings agree well between sampling periods. Moisture at these sites increased significantly between sample periods.

Table 15: Soil Moisture - 2000

Site	Sep-14	Stdev	10-Oct	Stdev
MT1	22.04	1.01	27.88	0.93
MT2	8.25	0.95	9.76	0.81
MT3	28.00	6.00	31.23	0.16
MT4	17.40	1.54	20.70	1.59
MT5	32.70	0.87	34.16	1.32
MT6	15.58	2.48	16.70	1.63
MT7	31.90	1.04	31.91	1.22
MT8	11.60	2.05	10.42	0.21
MT9	50.10	1.11	51.10	0.25
MT10	12.90	1.33	14.98	1.20
MT11	11.10	0.70	23.12	0.81
MT12	12.10	1.68	23.30	5.37
MT13	12.90	2.22	14.85	8.54
MT14	27.60	1.16	27.68	0.65
MT15	13.10	5.42	17.28	4.69

Soil Microbial Biomass: The soil microbial biomass data is show in Table 16. Biomass had a strong positive correlation (r^2=0.85) with soil respiration. This was expected, as the soil biomass is partially (along with roots) responsible for CO_2 evolution. Biomass had a negative correlation (r^2= 0.47) with soil metals.

Table 16: Soil Biomass

Site	Biomass
MT 1	107.7
MT 2	12.0
MT 3	171.6
MT 4	98.4
MT 5	56.8
MT 6	3.0
MT 7	29.4
MT 8	16.0
MT 9	66.2
MT 10	3.0
MT 11	11.0
MT 12	34.0
MT 13	18.6
MT 14	61.1
MT 15	12.0

Microbial Community Structure (PLFA): Phospholipid fatty acid data was collected at two sampling periods. These data can be found in Appendix C. The PLFA data indicates that factors related to heavy metal contamination are among the most important determinants of differences in microbial community structure between study sites at Grant Kohrs Ranch. Principle component analysis was used to extract variables from phospholipid fatty acid profile data. The variables, called principle components, are linear combinations of response variables. The first principle component (PLFA PC1), is the variable that accounts for the greatest amount of variation between sites. Regressions of PLFA PC1 reveal it to be correlated to metal concentration in the study sites.

Fig. 6: PLFA PC 1 v.Total (As, Cu, Cd, Zn, Pb)

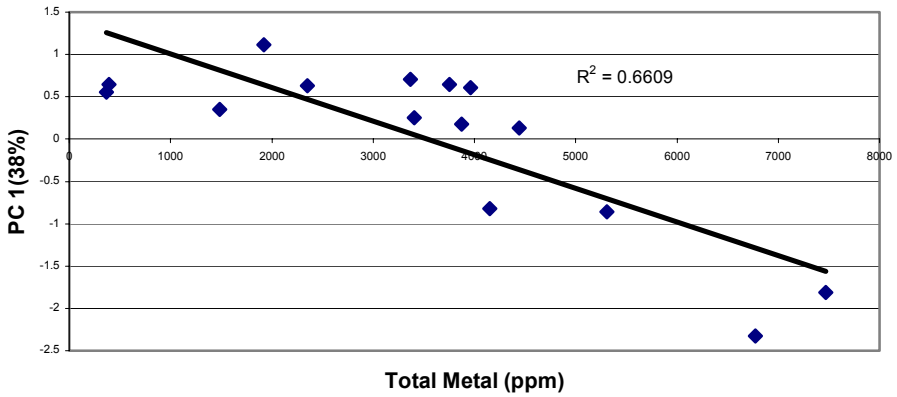


Fig. 6: Linear Regression of PLFA principal component data (PC 1) with the sum of the metal data described in the text as described in the text.

This analysis shows that the microbial community structure based on PLFA patterns is strongly negatively correlated with soil metals. In other words, the community composition is significantly altered and this alteration is explained in part by the heavy metal gradient. In order to view this relationship we have plotted PFLA PC1 against a sum of total biologically important soil metals (As, Cu, Cd, Zn, and Pb) (Fig. 6).

Part 2: 2001 Season data

As described above we identified 30 additional sites to study in the 2001 season. Of the 30, three (MP18, MP33, and MP62) sites were excluded since they were flooded for part or all of the analysis period and respiration measurements could not be made across all time points.

Respiration vs. Metals: Respiration data for the 2001 season is shown in Table 17. There did not appear to be a trend across the season. At some sites, the average respiration increased where at others it was constant or decreased.

Table 17- 2001 Respiration Data Set

Site	7-Jun-01	SD	22-Jun-01	SD	2-Jul-01	SD	24-Sep-01	SD
MP18	Flooded	-	Flooded	-	Nd	-	nd	-
MP19	4.382	0.70	6.258	0.87	7.104	1.52	4.664	1.04
MP21	2.256	0.69	3.439	1.07	1.494	0.69	2.061	0.82
MP22	5.072	1.18	6.906	1.09	7.289	1.62	5.276	1.77
MP24	8.408	1.33	7.466	1.53	8.091	1.39	7.891	1.78
MP33	Flooded	-	Flooded	-	Flooded	-	5.778	0.56
MP34	2.867	0.76	4.211	1.73	4.557	1.38	3.377	0.70
MP35	2.078	0.50	2.741	1.23	2.150	0.89	1.721	0.94
MP36	4.228	0.95	4.527	1.57	2.471	0.27	3.520	0.53
MP42	3.492	1.01	9.208	2.53	10.276	2.28	7.300	1.08
MP51	2.051	0.99	2.717	2.05	3.179	2.25	1.598	0.99
MP53	2.499	1.16	1.797	0.80	2.819	0.93	2.167	0.53
MP56	3.664	1.37	3.421	0.68	5.057	1.02	2.415	0.58
MP57	5.015	0.65	4.966	0.90	6.556	1.61	2.865	0.54
MP58	2.396	0.33	4.288	0.78	4.079	0.85	2.443	0.48
MP59	3.833	0.66	4.116	0.61	4.341	0.65	3.577	0.51
MP60	2.213	0.45	2.628	0.81	3.372	1.28	3.571	0.53
MP62	Flooded	-	Flooded	-	Nd	-	Nd	-
MP65	3.261	0.67	6.826	0.90	5.485	1.44	6.625	2.25
MP66	0.573	0.34	1.955	0.81	1.727	1.02	0.356	0.13
MP67	2.841	1.08	3.168	0.77	3.668	1.72	1.557	0.46
MP68	5.916	1.58	8.454	0.61	8.139	2.54	3.149	0.74
MP69	4.939	0.33	5.722	0.37	5.915	0.32	2.627	0.25
MP70	3.186	1.25	4.077	1.14	3.889	1.59	2.467	0.67
MP71	0.324	0.09	1.595	1.76	0.999	0.37	0.667	0.21

MP72	Flooded	-	6.644	1.22	6.311	0.90	3.637	0.39
MP77	5.931	0.67	5.653	1.00	4.692	0.98	3.016	0.91
MP78	4.338	0.46	5.752	0.27	4.750	0.81	2.761	0.72
MP79	3.071	0.73	2.324	0.45	3.098	0.87	3.241	0.87
MP100	4.188	0.95	5.936	0.69	4.782	0.61	3.516	0.40

Respiration = CO_2 efflux (uM CO_2/m^2/sec): SD= standard deviation for all measures across the plot.

Multicollinearity – Before analyzing metal data relative to respiration, we examined the metals data for multicollinearity. Table 18 shows a correlation matrix (numbers are Pearson's product-moment correlation coefficients) of the measured metal concentrations. Low condition numbers (< 100), calculated for this set of variables, indicated that for this data set (as opposed to the year 2000 data) multicollinearity was not a problem. In addition to the multiple regressions we also carried out a variant, ridge regression (which is less sensitive to multicollinearity violations), and obtained the same results.

Table 18: Correlation matrix of measured metal concentrations

Variable	As (ppm)	Cd (ppm)	Cu (ppm)	Pb (ppm)	Zn (ppm)
As	1.000000	0.178412	0.858509	0.850837	0.493285
Cd	0.178412	1.000000	0.338380	0.257473	0.811437
Cu	0.858509	0.338380	1.000000	0.862236	0.623496
Pb	0.850837	0.257473	0.862236	1.000000	0.409850
Zn	0.493285	0.811437	0.623496	0.409850	1.000000

Table 19: Eigen values of correlations

No.	Eigenvalue	Incremental Percent	Cumulative Percent	Condition Number
1	3.233198	64.66	64.66	1.00
2	1.334309	26.69	91.35	2.42
3	0.250076	5.00	96.35	12.93
4	0.130844	2.62	98.97	24.71
5	0.051573	1.03	100.00	62.69

We carried out multiple regressions including all 5 measured metal concentrations as predictor variables. Results for respiration are shown in Table 20.

Table 20: Multiple regression results for respiration

Parameter	2 June 2001	21 June 2001	1 July 2001
r^2	0.29	0.26	0.21
F	1.63	1.51	1.13
P	0.19 (n.s.)	0.22 (n.s.)	0.37 (n.s.)
Power (5%)	0.45	0.43	0.32

n.s. = not significant at alpha 0.05

These data indicate that only 20-30% of the variability of respiration rates across the sites examined can be assigned to soil metal concentrations. The results of a linear regression

of respiration with total soil metals is shown in Figure 7 ($r^2 = 0.25$). These coefficients were much lower than that observed in the 2000 season and are likely due to site heterogeneity factors including different vegetation types on a given site.

Soil Organic Matter- Soil organic matter measurements were conducted by the MEBL. The data is shown in Table 21. Organic matter was positively correlated with soil respiration ($r^2 = 0.41$). These data can be found in Appendix D.

Table 21 - Summary of Soil Factors - 2001

Site	Organic Matter %	Respiration [a]	Biomass	pH	%Soil Moisture
MP19	3.2	5.602	91.7	7.53	34.54
MP21	0.9	2.313	23.1	7.24	10.99
MP22	2.5	6.136	130.1	7.77	24.99
MP24	14.6	7.964	223.1	7.27	60.98
MP34	1.0	3.753	26.0	7.59	15.27
MP35	2.9	2.172	134.3	5.09	27.09
MP36	4.9	3.687	28.0	6.95	21.01
MP42	6.2	7.569	134.3	6.62	46.96
MP51	2.8	2.386	51.4	5.85	25.53
MP53	2.7	2.321	66.3	5.20	27.67
MP56	8.8	3.639	106.0	6.25	43.73
MP57	4.5	4.850	65.9	7.32	31.38
MP58	4.4	3.301	102.6	6.35	33.96
MP59	5.0	3.966	102.0	6.11	31.96
MP60	2.2	2.946	60.0	5.30	21.42
MP65	8.1	5.549	296.0	6.98	46.64
MP66	1.7	1.153	142.0	4.50	24.71
MP67	4.3	2.808	108.0	7.29	33.59
MP68	5.9	6.415	120.9	7.32	34.24
MP69	4.6	4.801	123.7	7.05	31.92
MP70	4.0	3.405	107.1	5.76	31.70
MP71	1.3	0.896	112.0	4.23	18.60
MP77	2.6	4.823	100.0	7.50	24.79
MP78	3.0	4.400	122.3	6.54	27.79
MP79	2.1	2.933	95.4	8.25	20.14
MP100	3.1	4.605	123.4	7.49	22.80

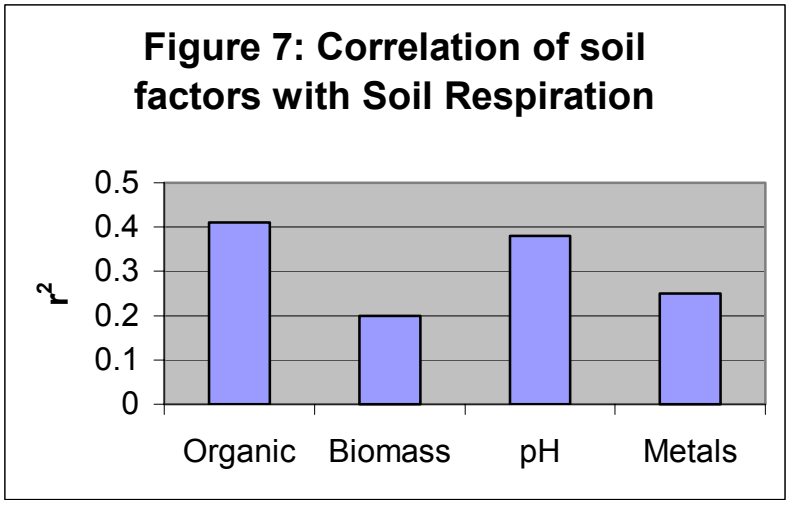


Figure 7: Regressions were applied for each factor vs Respiration.

Soil Microbial Biomass: Soil microbial biomass data is also shown in Table 21 and the linear regression with respiration is plotted in Figure 7 ($r^2 = 0.20$). This is in contrast with the 2000 season where a very strong positive correlation with biomass was observed.

We carried out a multiple regression on soil microbial biomass data with soil metals; results are shown in Table 22. Again, the model fit was not significant.

Table 22: Multiple regression results for soil microbial biomass

Parameter	
r^2	0.10
F	0.50
P	0.77
Power (5%)	0.15

Soil Moisture: We carried out a multiple regression on soil moisture data; results are shown in Table 23. Again, while soil metal concentrations accounted for 32% of the variability in soil moisture, the model fit was not significant. Soil moisture (Table 21) did not correlate with soil respiration.

Table 23: Multiple regression results for soil moisture

Parameter	
r^2	0.32
F	2.00
P	0.12
Power (5%)	0.55

2001 Microbial Community Structure (PLFA) -Phospholipid fatty acids were extracted from the soil samples collected at each site and quantified by gas chromatography (GM-07, GM08). The data can be found in Appendix C. Principle component analysis, a multivariate data reduction technique, was used to simplify the data by creating new variables representative of variation between samples. These variables are referred to as principal component axes. Each axis represents a portion of the total variation between samples. The new variables are amenable to further statistical analysis. Principal component analysis and multiple regression modeling were conducted using SPSS v.10 software. ANOVA's and post-hoc comparisons were made using JMP v. 3.1.6.2 software.

A multiple regression model of the metal index against principal components 1 (41%) and 2 (16%) of the PLFA data indicates a weak but significant correlation between metal contamination and microbial community structure (r^2=0.274, F=5.091, p=0.013). At low concentrations of metals the effects of contamination can be expected to be subtle because the effects of metals become obscured by variation between communities due to differences in vegetative cover, soil moisture, and organic material. The obscuring effects of natural heterogeneity in community structure can cause fairly low r^2 values. Additional analysis can help illustrate the information contained in the PLFA data.

Sites were also categorized according to degree of contamination in order to show that sites containing low levels of contamination differ from those containing moderate or high amounts of metals. Creation of site categories allows for the averaging out of variation in communities that is not related to metal contamination. Factors that are not related to metal contamination should not affect one category more than another. In order to investigate whether a metal effect could be illustrated graphically, the sites were categorized into sites with low, moderate, and high levels of contamination. The analysis investigates whether sites with low levels of contamination differ significantly from sites with moderate and high levels of contamination. Sites were ranked according to a natural breakpoint that occurs in the metal index (same metal index as used elsewhere in this report). The 7 non-slickens sites that fall below the index number of 150 are considered to have low levels of contamination. The 7 sites with a metal index greater than 300 are considered to be highly contaminated. Sites with intermediate index numbers are considered to be moderately contaminated. Sites containing un-vegetated ground that appeared to be slickens were considered separately because microbial communities in un-vegetated soils can be expected to differ greatly from those in vegetated soils regardless of contamination.

The chart indicates that microbial communities differ between sites with different levels of metal contamination. Low metal and slickens sites are significantly different from each other and all other groups along PC 1 (Oneway ANOVA- F = 15.17, $p<0.0001$, post hoc comparisons by Tukey-Kramer HSD used alpha = 0.05) . Sites with moderate and high levels of contamination are not significantly different from each other along PC 1 but do differ significantly along PC 2 (Oneway ANOVA- F = 3.41, p= 0.0322, post hoc comparisons by Tukey-Kramer HSD used alpha = 0.05). Low and slicken sites do not differ significantly from the other groups or each other with respect to PC 2.

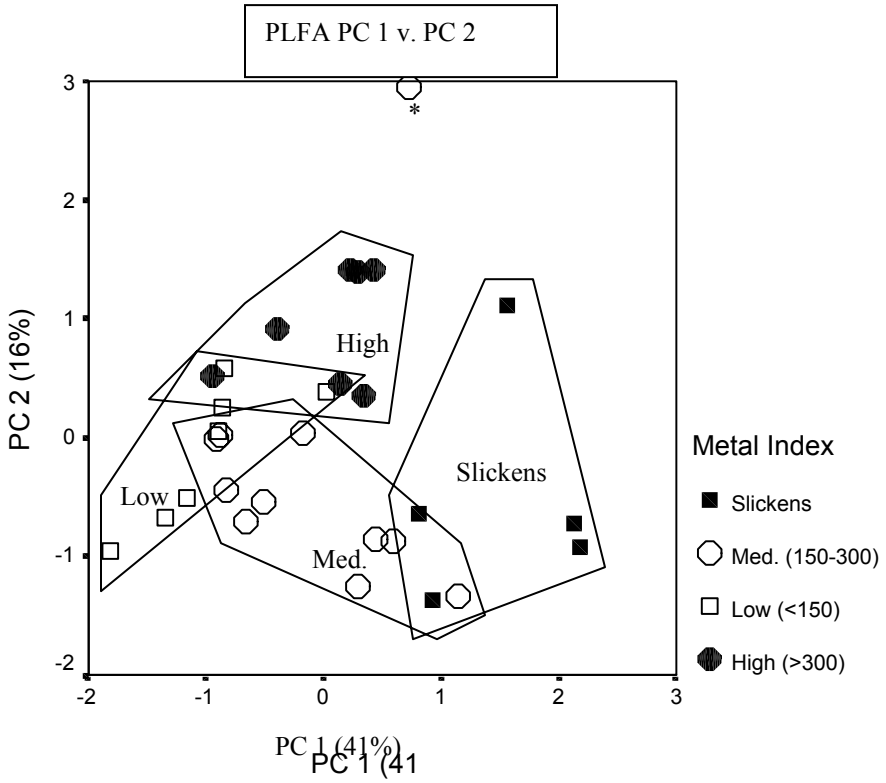


Figure 8. Results of principal component analysis of phospholipid fatty acid data. Circled groups indicate categories that are significantly different from each other along one of the two principle component axes. Samples from sites containing low levels of metal contamination (Low-metal index below 150, hollow boxes) differed significantly from all other groups along PC 1. Samples from sites containing bare ground presumed to be slickens (slickens, filled boxes) also differed from all other categories along PC 1. Sites containing medium (Med.- metal index between 150 and 300, hollow circles) and high (High- metal index above 300, filled circles) differed along PC 2 only. *Although not included in the circled Med. sites this apparent outlier was included in all statistical analysis to establish significant differences between sites.

Part 3: Bureau of Land Management Data

Selection and location of BLM sites can be found in the MEBL report. At each of the indicated sites, we conducted respiration measurements. The complete respiration data set can be found in Appendix A. A summary of the data is given in Table 24 along with the corresponding metal index for the site. Metal index is calculated by the sum of As, Cd, Zn, Pb, and Cu) divided by the sum of the baseline metal concentrations as determined by the MEBL. We performed a regression analysis for respiration and metal index and found no significant correlation.

Table 24: Respiration Values of Bureau of Land Management Sites

Site	Respiration	Metal Index*	Site	Respiration	Metal Index*
T1-1	3.921	47	T1-20	8.136	121
T1-2	10.361	43	T1-21	3.142	76
T1-3	4.570	138	T7-1	2.524	52
T1-4	3.072	116	T7-2	5.132	73
T1-5	5.616	161	T8-3	4.081	30
T1-6	2.066	65	T12-1	7.434	28
T1-7	4.483	161	T12-2	8.444	46
T1-8	0.885	48	T12-3	2.068	28
T1-9	4.169	14	T12-4	4.937	55
T1-10	8.071	118	T12-5	2.887	93
T1-11	4.876	134	T12-6	10.390	47
T1-12	5.023	183	T12-7	5.698	50
T1-13	10.597	26	T12-8	2.066	21
T1-14	3.536	18	T12-9	2.589	67
T1-15	5.402	19	T13-2	4.802	37
T1-16	5.236	63	T13-3	6.348	29
T1-17	8.826	63	T13-4	5.888	52
T1-18	3.519	29	T15-1	1.789	41
T1-19	5.442	55	T15-2	4.489	58

*Metal Index = Σ (metal concentration (As, Cd, Zn, Cu, Pb)/ metal baseline concentration)

Part 4: Presentation of pooled 2000, 2001, and BLM data.

In the following, we present an analysis of the pooled 2000 and 2001 GKR samples (closest time points of respiration measurements), and the BLM tract respiration measurements.

Data presentation: Fig. 9 is a scatterplot of soil respiration vs. metal index, where metal index is calculated as MI = Σ (metal concentration/ metal baseline concentration) for the individual metals.

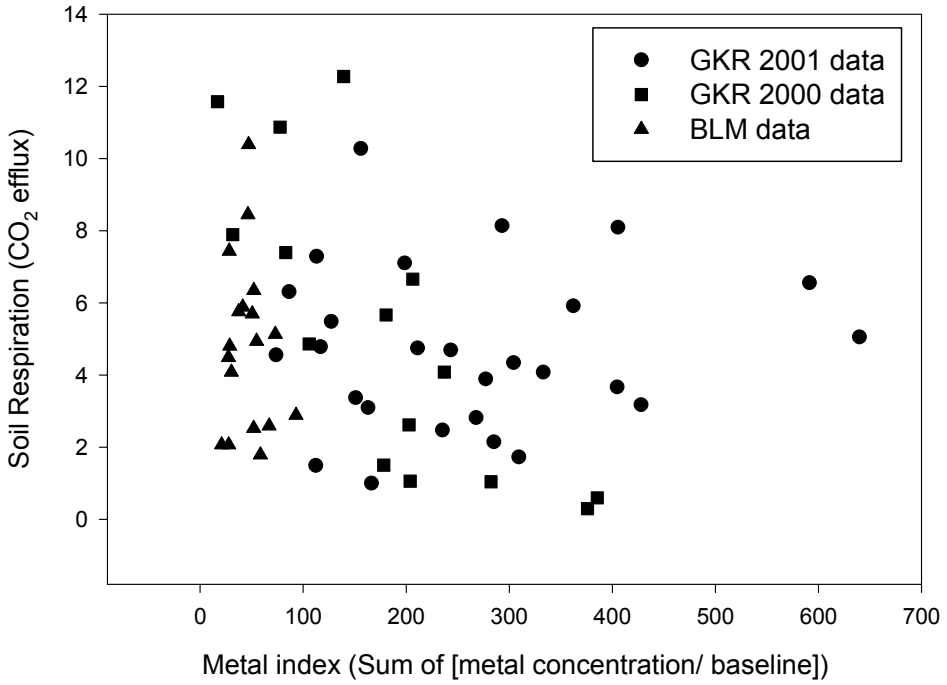


Fig. 9: Scatterplot of soil respiration vs. metal index for pooled GKR (2000, 2001) and BLM data.

Data analysis - The data set exhibits considerable variability, and a simple regression analysis does not suggest a dependence of respiration on metal concentration. However, further examination suggests that metals limit the maximum amount of respiration that can be reached. Hence we chose to analyze the data using a maximum function. The idea of the maximum function is to test whether there is a trend concerning the overall maximum respiration rate with increasing metal index. The following algorithm was used to define this maximum function.

- Pick a start point along the metal index axis
- Pick an interval width along the metal index axis
- Within each interval find the maximum respiration rate. Select this respiration rate, along with the corresponding metal index value to define the x,y pair
- Repeat this process with different interval starting points and interval widths to test if the new line converges reasonably onto a maximum function.

The result of this procedure is presented in Table 25.

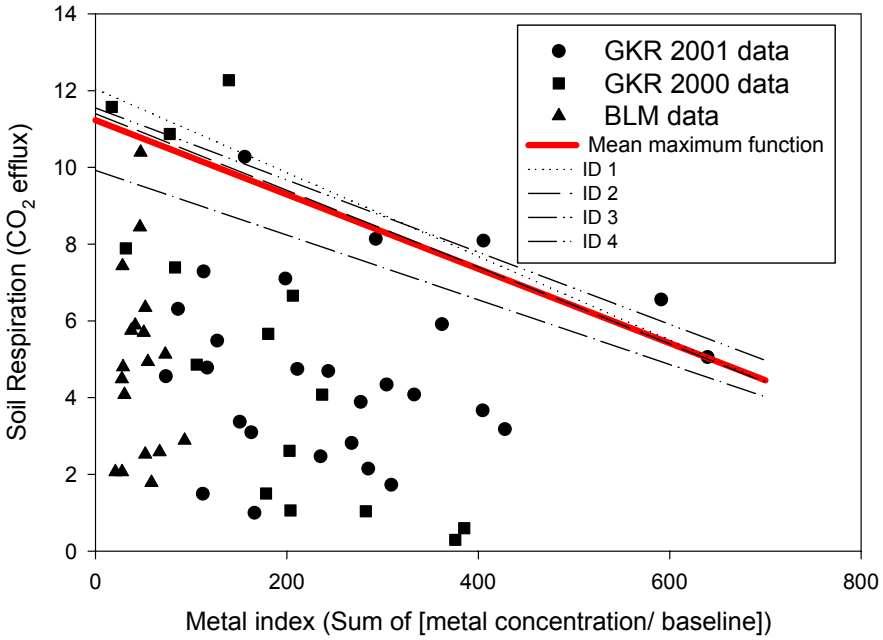
Table 25: Results of using several interval definitions and the regression relationships derived from them, and the average of these (the maximum function). See Appendix G for graphical representation of maximum functions.

Interval definition[a]	**Slope**	**y-intercept**	**r^2**	**F**	**P**
Interval	- 0.0109	12.04	0.79	19.0	**0.007**

definition 1					
Interval	- 0.0100	11.4	0.56	11.4	**0.008**
definition 2					
Interval	- 0.00843	9.92	0.38	5.53	**0.043**
definition 3[b]	(- 0.0079)	(9.70)	(0.35)	(4.77)	**(0.056)**
Interval	- 0.00939	11.55	0.95	80.03	**0.0009**
definition 4					
Mean regression function	**- 0.00968**	**11.23**			

[a]1: start value 0, step 100; 2: start 0, step 50; 3: start 25, step 50; 4: start 50, step 100.
[b] Numbers in brackets refer to regression with BLM data excluded; changes only occurred for this interval definition.

Fig. 10 Scatterplot with average maximum function, and the four different interval definition functions



There are two results:

1. With only 4 iterations of interval definitions, there seems to be reasonable convergence on the regression function listed in the last row of the table (i.e. slope and y-intercept do not vary greatly among the different interval definitions).
2. Each individual regression (and the mean function) has a negative slope, and the P-value testing the null hypothesis that the slope is significantly different from 0 is consistently < 0.05, with high coefficients of determination.

The slope of the maximum function is significantly different from 0, and negative. This is interpreted as a decrease in maximum (potential) respiration rate with increasing metal index.

In order to exclude possible artifacts due to the bin sizes being held constant (equal intervals), while the number of y-values is not (as there are fewer data points as one progresses along the x-axis), we also carried out a similar analysis where the number of

data points selected for an interval are constant, and the x-axis bin sizes are, by necessity, not. The data set was sorted by metal index, and then a set number of x-values were defined as (contiguous) intervals. This process was repeated with starting points of 1st value and 3rd value of the data set, and bin contents of 10 and 5 data points. In each bin, again the maximum respiration value was selected, along with its paired Metal index value. The results are shown in Table 26.

Table 26: Results of using a different algorithm for interval definitions. Here intervals were defined based on equal number of data points per bin, which means that bin sizes were, by necessity unequal. The results are remarkably similar to the first set of permutations.

Interval definition[a]	**Slope**	**y-intercept**
Interval definition A	-0.00937	11.75
Interval definition B	-0.00793	9.86
Interval definition C	-0.00956	11.32
Interval definition D	-0.00409	8.80
Mean regression function	**-0.007**	**10.43**

[a]A: start value 1, bins of 10; B: start 1, bins of 5; C: start 3, bins of 10; D: start 3, bins of 5.

Again, the slopes are all negative, and all but one slope (interval definition D) were very similar again to the previously obtained slopes. The same is also true for the y-intercepts.

The caveat of this analysis is that we did not attempt to explain in a model the variability of the data set. Instead we focused on one aspect of the data, a maximum function. We interpret this maximum function to be biologically meaningful: while at every metal concentration a number of environmental factors are acting upon respiration rates as measured in the field, the potential to achieve a high rate clearly decreases with increased metal index. This means that with increasing metal concentration, metals become a more and more dominant effect with respect to determining respiration.

Discussion

Summary of overall findings

In the first season of field study we found very clear correlations between metal concentrations and respiration. Other correlations (soil biomass, community structure, organic matter, and pH) were also strong. In the second field season, there were similar trends, but model fits were not statistically significant.

A possible explanation for the different results in the 2000 and 2001 season may lie in the spatial scale of sampling and process of averaging. The measurements of respiration and heavy metal levels were co-located in both seasons, and may not necessarily have been distributed over a larger total area in 2001.

- However, in 2000 sampling plots were explicitly selected on the basis of visual homogeneity, versus in 2001 they were selected based on coordinates irrespective of homogeneity.
- Furthermore, in 2000 we had an opportunity to test for the degree of spatial heterogeneity among metal measurements, because pooling occurred at the level of data, not the soil material. In 2000 the three sub-samples always co-located in a principal component ordination (data not shown); i.e. spatial heterogeneity was minimal. In 2001 we did not have an opportunity to test for spatial heterogeneity due to the occurrence of pooling at the soil sample level. This, coupled with the lack of assurance of sample site homogeneity (at least based on visual estimation) could have easily led to the lack of regression fit in 2001 compared to 2000.

Conclusions

From the 2000 field season data, it can be concluded with a high level of confidence that there exists a negative correlation between metals concentration and respiration. When the scale of sampling is broadened where site heterogeneity becomes a major factor, a direct relationship between soil metals and respiration is less clear. However, metals always appear to limit the maximum respiration that can be achieved and this will effect carbon mineralization and other soil processes. A relationship between metal concentration and the degree that respiration is restricted is shown in Table 27.

It may be possible, therefore, to expand the metals data set (using the MEBL SS data set) and to calculate the area of the park that corresponds to a given metal index. From this the overall injury relative to respiration could be determined.

Table 27: Metals Induced Limitation.

Metal Index	Maximum Respiration	% Limitation
50.00	10.75	4.31
100.00	10.26	8.62
200.00	9.29	17.24
300.00	8.33	25.86
400.00	7.36	34.48
500.00	6.39	43.10
600.00	5.42	51.72
700.00	4.45	60.34

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