Microb Ecol (2009) 58:10–22
DOI 10.1007/s00248-008-9467-7

SOIL MICROBIOLOGY

Molecular and Functional Assessment of Bacterial Community Convergence in Metal-Amended Soils

J. A. H. Anderson · M. J. Hooper · J. C. Zak · S. B. Cox

Received: 24 April 2008 / Accepted: 16 October 2008 / Published online: 22 November 2008

Abstract Species diversity and the structure of microbial communities in soils are thought to be a function of the cumulative selective pressures within the local environment. Shifts in microbial community structure, as a result of metal stress, may have lasting negative effects on soil ecosystem dynamics if critical microbial community functions are compromised. Three soils in the vicinity of a copper smelter, previously contaminated with background, low and high levels of aerially deposited metals, were amended with metal-salts to determine the potential for metal contamination to shape the structural and functional diversity of microbial communities in soils. We hypothesized that the microbial communities native to the three soils would initially be unique to each site, but would converge on a microbial community with similar structure and function, as a result of metal stress. Initially, the three different sites supported microbial communities with unique structural and functional diversity, and the nonimpacted site supported inherently higher levels of microbial activity and biomass, relative to the metal-contaminated sites. Amendment of the soils with metal-salts resulted in a decrease in microbial activity and biomass, as well as shifts in microbial community structure and function at each site. Soil microbial communities from each site were also observed to be sensitive to changes in soil pH as a result of metal-salt amendment; however, the magnitude of these pH-associated effects varied between soils. Microbial communities from each site did not converge on a structurally or functionally similar community following metal-salt amendment, indicating that other factors may be equally important in shaping microbial communities in soils. Among these factors, soil physiochemical parameters like organic matter and soil pH, which can both influence the bioavailability and toxicity of metals in soils, may be critical.

J. A. H. Anderson
Department of Entomology, Iowa State University,
Ames, IA, USA

M. J. Hooper · S. B. Cox (✉)
The Institute of Environmental and Human Health (TIEHH) & the
Department of Environmental Toxicology, Texas Tech University,
Box 41163, Lubbock, TX 79409, USA
e-mail: stephen.cox@ttu.edu

J. C. Zak
Department of Biological Sciences, Texas Tech University,
Lubbock, TX, USA

Introduction

While metals are naturally present in the environment, high concentrations, like those found in areas surrounding mining and smelting operations, have been shown to disrupt many critical biogeochemical processes within soils, due in part to the negative effects they impose on soil microbial communities. Microbial communities are known to be important for a wide range of soil processes, including the cycling of nutrients, breakdown of organic matter [18], interaction with plant roots, and formation of soil structure [25]. The sensitivity of microbial communities to metal contamination has been studied previously in both field-contaminated and laboratory-spiked soils. High levels of soil metal contamination have been correlated with decreased microbial activity [30, 40], and biomass [18, 22], as well as impaired enzyme function and nutrient cycling [7, 19]. Similarly, metal contamination has been shown to impart selective pressure on soil microbial communities, leading to shifts in community structure [8] and decreases in biodiversity [22, 30]. Despite significant attempts to characterize the negative effects of metals on microbial communities, questions remain regarding the variation in soil microbial community responses to metal stress.

In 1934, Bass-Becking theorized that high microbial abundance and dispersal rates foster ubiquitous distribution in the environment [3]. This idea that "everything is everywhere and the environment selects" suggests that differences in soil microbial community composition is a result of the collection of stresses and/or substrates available in the immediate soil environment [35]. Building off of this idea, this study attempts to address the following question: Will microbial communities, indigenous to soils with unique local environmental conditions, converge to a common community when exposed to the same selective pressure (i.e., metal contamination)? To answer this question, we compared the effects of metal stress on the structure and function of bacterial communities indigenous to three different soils, each with a history of background, moderate, or high levels of long-term metal contamination, respectively. Based on previous work in this area, we hypothesize that metal amendment will lead to shifts in both bacterial community structure and function. More specifically, we hypothesize that metal-associated selection pressure will cause the bacterial communities from each soil to converge to a common community, exhibiting similar structural and functional diversity. Finally, we hypothesize that the shifts in microbial community structure and function will be a direct effect of metal stress, rather than an artifact caused by acidification of soils from metal-salt amendments. Counter-ion and pH controls were included for each treatment to aid in delineation of metal, counter-ion, and pH effects. From this study, a better understanding of bacterial community responses to metal-associated selection pressure will be achieved.

Methods

Site Description and Sampling Procedure

Aerial and fluvial deposition of metals, following a century of mining and smelting activity, has resulted in widespread contamination of the soil surrounding the Anaconda Smelter site in Anaconda, Montana. Contaminants of concern at this site include copper (Cu), zinc (Zn), lead (Pb), cadmium (Cd), and the metalloid arsenic (As). Previous work at this site placed soils within a 24-km radius of the stack in the path of aerial metal deposition [36]. Two sites within 24 km of the smelter stack (a severely impacted smelter site and a moderately impacted smelter site [(N 46.1814, W 112.7633) and (N 46.09752, W 112.09207), respectively]) were selected based on the degree of total soil metal contamination [37]. Bacterial communities within each soil are assumed to have been exposed to either chronically high or moderate levels of metals, respectively. The vegetative type and cover within the two smelter-impacted sites has been characterized previously (percent vegetative cover, percent cover by grasses, forbs, live woody, dead woody, bare ground, gravel, and rocks) in 2000 using 1-m^2 Daubenmire quadrants. A third site, located 30 miles from the Anaconda Smelter site (N 45.75610, W 112.7639) was selected, and the bacterial community from this site represents a non-smelter impacted community. The nonimpacted site was chosen based on the similarity of vegetative cover and the background concentration of metals in the soil.

In August of 2005, soil from the severely and moderately impacted smelter sites and the nonimpacted site were sampled to a depth of 15 cm. The soil from each site was sieved in the field to remove the >2 mm size fraction, homogenized and stored at 4°C for 6 months prior to commencement of the laboratory dosing experiment.

Experimental Design

A plastic-lined cement mixer was used to homogenize 24 kg each of nonimpacted, moderately impacted and severely impacted soil, respectively. Metal-salt solutions [H_2NaAs0_4, $Pb(NO_3)_2$, $Cu(SO_4)$, $Cd(SO_4)$, and $Zn(SO_4)$ dissolved in ultrapure H_20(>18 MΩ cm)] were added to approximately 2,500 g of the homogenized soil from each site, to create a total of six metal-salt amended treatments (R_L, R_H, R_{2H}, L_H, L_{2H}, and H_{2H}, Table 1). Metal-salt solutions were mixed into soil treatments for 30 min with an electric five-speed food processor to ensure even distribution and uniform concentration. The stainless-steel mixer attachments and the plastic-lined cement mixer were rinsed for 30 min with 10% HNO_3 between treatments to prevent cross-contamination of soil metals. Unamended control treatments for nonimpacted, moderately impacted, and severely impacted soil (Treatments R, L, and H, respectively, Table 1) received equivalent amounts of ultrapure H_20 and were mixed for 30 min with an electric five-speed food mixer.

Results from a pilot study demonstrated that the addition of metal-salt solutions significantly decreased the pH of the amended soils. Soil pH in treatments receiving the highest concentrations of soil metals decreased 2 pH units and remained decreased relative to the unamended control treatments over time (data not shown). Because the effects of pH on soil microbial community structure and function were found to be confounding factors in the analysis and interpretation of results in previous studies [12, 16, 33, 43], six additional treatments (R_{LC}, R_{HC}, R_{2HC}, L_{HC}, L_{2HC}, and H_{2HC}, Table 1) were designed to control for the effects of increased concentrations of counter-ion or changes in soil pH. Counter-ions (added as $NaNO_3$ and $NaSO_4$) were mixed into 2,500 g of soil from each site, as described above. Additionally, 1 M HNO_3 was mixed into control soils until the control treatment soil pH was equal to that of its

Table 1 Nominal metal concentrations and corresponding day 7 soil pH (mean (SE)) of the nonimpacted reference site (Reference), moderately impacted (Low), and severely impacted (High) smelter site treatments, following amendment of soils

Soil	Treatment	Total metal concentration (ppm)	pH
Reference	R[a]	Background	7.11 (0.04)
Reference	R_L	Low	6.64 (0.04)
Reference	R_{LC}[b]	Background	6.73 (0.04)
Reference	R_H	High	5.34 (0.02)
Reference	R_{HC}[b]	Background	5.62 (0.04)
Reference	R_{2H}	2*High	4.78 (0.18)
Reference	R_{2HC}[b]	Background	4.98 (0.18)
Low	L[a]	Low	6.66 (0.06)
Low	L_H	High	5.02 (0.01)
Low	L_{HC}[b]	Low	5.17 (0.01)
Low	L_{2H}	2*High	4.53 (0.01)
Low	L_{2HC}[b]	Low	4.75 (0.02)
High	H[a]	High	8.16 (0.19)
High	H_{2H}	2*High	6.10 (0.19)
High	H_{2HC}[b]	High	6.19 (0.01)

[a] Unamended control treatments (no metal-salts added)
[b] pH and counter-ion control treatments (no metal-salts added)
Background—0 ppm Zn, 20 ppm Pb, 20 ppm As, 1 ppm Cd, 20 ppm Cu; Low—85 ppm Zn, 25 ppm Pb, 35 ppm As, 1 ppm Cd, 90 ppm Cu; High—1,500 ppm Zn, 800 ppm Pb, 800 ppm As, 30 ppm Cd, 1200 ppm Cu; High—3,000 ppm Zn, 1,600 ppm Pb, 1,600 ppm As, 60 ppm Cd, 2400 ppm Cu

corresponding metal-amended treatment (where treatments R_{LC}, R_{HC}, R_{2HC}, L_{HC}, L_{2HC}, and H_{2HC} correspond to metal-amended treatments (R_L, R_H, R_{2H}, L_H, L_{2H}, and H_{2H}, respectively).

Once each of the 15 metal-salt amended and control treatments were thoroughly mixed, 140 g of soil from each treatment were used to seed individual, sacrificial "conetainers" (Stuewe & Son, Corvalis, OR, USA). Each conetainer was modified to include a filter paper plug at the base and a polyfilm seal (perforated to allow air exchange) at the top, to prevent loss of soil through the base and excessive loss of soil moisture from evaporation. A total of 18 identical conetainers were created for each of the respective treatments and were stored at room temperature. Soil moisture was monitored in conetainers in each treatment over time, and ultrapure H_2O was added each week to maintain the soils between 12% and 20% moisture (based on dry weight).

Sampling and Analysis

On sampling days 7, 21, and 63 (after amendment of soils with metal salts), five replicate conetainers from each treatment were sacrificed. A 40-g subsample of soil from each conetainer was removed and stored at 4°C for up to 1 week prior to microbial analysis. Remaining soil from replicate containers in each treatment was composited and used to characterize the soil physiochemical, nutrient, and metal profiles. Additionally, soil physiochemical, nutrient, and metal profiles were preformed on day 0, using composited soil from each treatment.

A subset of composited soil from each treatment was oven-dried at 105°C for 48 h to determine soil oven-dry weight equivalent (ODE) and the percent moisture. Soil nutrient [nitrate (NO_3), phosphorus (P), and percent base saturation of the elements (potassium (K), magnesium (Mg), calcium (Ca)], and physical [pH, % organic matter, cation exchange capacity (CEC), particle size distribution] profiles were analyzed by Waters Agricultural Laboratories, Owensboro, KY, USA. Total extractable metals were digested following EPA method 3050B. Soluble soil metals were extracted by shaking soil in a 1:10 (*w*/*v*) 0.01 M $CaCl_2$ solution for 16 h, as described previously [17]. Total extractable and soluble metals (Zn, Pb, As, Cd, and Cu) were analyzed by inductively coupled plasma atomic emission spectroscopy (Texas Tech University, GeoAnalytical Laboratory).

Soil Bacterial Analysis

Biolog

Biolog GN microtiter plates (BIOLOG, Hayward, CA, USA) were used to assess bacterial activity and carbon substrate utilization patterns (SUPs), as described previously [14, 45, 48]. Briefly, Biolog plates were inoculated using 10 g ODE of soil from each sacrificed conetainer (n=five per treatment, per timepoint). Soil was blended with 0.2% water agar using an electric food processor and then serially diluted using sterile water. The 96-well plates were inoculated and incubated at 25°C for 72 h. Absorbance was analyzed every 12 h at a wavelength of 590 nm. Bacterial activity represents the total amounts of carbon substrate utilized on each Biolog plate (i.e., the sum of the total absorbance). Carbon SUPs represent the identities of substrates utilized on each Biolog plate.

Biomass

A modified chloroform-fumigation-extraction method was used to determine the effects of metal contamination of soil microbial biomass, as described previously [26]. Biomass was measured in duplicate, and the average microbial biomass in each sacrificed container was used for statistical comparison (n=5 per treatment, per timepoint).

DGGE

UltraClean Soil DNA Extraction kits, were used to extract genomic DNA from each sacrificed conetainer (n=5 per

treatment, per timepoint), following manufacturer's instructions (MO BIO Labs, Carlsbad, CA, USA). DNA extracted from 1 g soil was polymerase chain reaction (PCR)-amplified using the bacterial primer 341 f with a GC-clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′) and the universal primer 519r (5′-ATT ACC GCG GCT GCT GG-3′) (Integrated DNA Technologies, Coralville, IA, USA). Extracted DNA (1 μl) was PCR-amplified as follows: each 25 μl reaction contained: 20 pmol of each primer, 0.4 mM of each dNTP, 1× buffer (containing 2 mM $MgCl_2$), 1.5 U Taq (Takara Bio, Japan), and sterile water. Thermocycler conditions: initial denaturation at 94°C for 2 min; followed by 35 cycles of denaturation, annealing, and extension (at 98°C for 10 s, 54°C for 40 s, 72°C for 1 min, respectively); followed by a final extension at 72°C for 10 min. PCR products were stored at −20°C prior to DGGE analysis.

The effect of metal contamination on species richness and bacterial community structure was assessed via denaturant gradient gel electrophoresis (DGGE) of 16 S rDNA. Previously established protocols [24, 29] were modified and optimized for use on the BioRad DCode DGGE system as follows: an 8% *w*/*v* polyacrylamide gel containing a denaturant gradient ranging from 35% to 36% [where 100% denaturant contains 40% formamide (Sigma) and 7 M Urea (BioRad Laboratories, Richmond, CA, USA) was used to separate PCR-amplified 16 S rDNA. Amplicons were initially pulsed through the wells at 90 V for 15 min and then ran at 60 V for 16 h at a constant temperature of 60°C. A standard marker containing a mixture of *Pseudomonas aeruginosa, Shewanella putefaciens*, *Sphingomonas* sp., *Ralstonia* sp., *Desulfovibrio* sp., was run in the beginning, middle, and end lanes of each gel. After being stained in ethidium bromide, polyacrylamide gel were digitally photographed under UV light (Kodak Imaging) and analyzed using GelComparII software.

Data Analysis

Differences in the soil physiochemical profile and the soil metal profile between the moderately and severely impacted smelter sites (L and H, respectively) were analyzed by analysis of variance and compared to the nonimpacted site (R) using Dunnett's multiple comparison test. Bacterial activity, a measure of the amounts of substrates utilized on Biolog plates, was calculated based on raw difference data (i.e., the absorbance registered in the control well subtracted from the absorbance registered in each of the 95 substrate-containing wells [14]). Carbon substrate utilization patterns (SUPs) were generated with respect to the identities of substrates utilized.

Gel CompareII professional software (Applied Maths, Austin, TX, USA) was used to analyze PCR-DGGE banding profiles. All gels were normalized using identical standards run in multiple lanes on each gel. Bands within each lane were manually identified and quantified using a best-fit Gaussian curve. All gel images were compiled, and band lanes were assigned (optimization was adjusted to 0.50 and position tolerance was adjusted to 1.00) [Heather Christensen, Applied Maths, personal communication]. Quantitative band tables, representing both the presence/absence and intensity of bands in each lane, were generated.

Multivariate statistics were used to analyze Biolog SUPs and community DGGE banding profiles as follows: (1) To test the hypothesis that metal-amendment would cause shifts in bacterial community structure and function, canonical correspondence analysis (CCA) was used to explain the differences in bacterial community SUPs and DGGE banding profiles, with respect to soil pH and/or total extractable soil metal concentration (i.e., direct gradient analysis). Ellipses, representing the standard error of the multivariate means, were used to characterize the differences among treatments. Eigenvalues, which estimate the percent of variation in the data that is accounted for by environmental variables, is denoted by subscripts. (2) To test the hypothesis that unique bacterial communities would converge on a common community following metal-amendment, the SUPs and DGGE banding profiles supported by bacterial communities in the 2*High treatments (R_{2H}, L_{2H}, and H_{2H}) were compared by correspondence analysis (CA). First, bacterial SUPs and DGGE banding profiles on sample days (7, 21, and 63) were analyzed independently, and differences within treatments on each sample day were compared. Next, SUPs and DGGE banding profiles that were analyzed from each sample day were analyzed jointly (i.e., all three treatments and all three sample days analyzed on the same CA plot) to visualize convergence over time. All statistical tests were conducted using R [32].

Results

The nonimpacted site (R), the moderately impacted smelter site (L), and the severely impacted smelter site (H) are significantly different in terms of their soil physiochemical profiles (Table 2). The soils from each site are classified as sandy loam and share similar soil texture; however, the soil physiochemical profiles among the sites are variable. Notably, soil pH, cation exchange capacity (CEC), and organic matter levels were highest in the severely contaminated soil and lowest in the moderately contaminated soil. The nonimpacted site, moderately impacted smelter site, and the severely impacted smelter site were also significantly different in terms of their total metal profiles (Table 3

Table 2 Soil physiochemical parameters [mean (SE)] of the nonimpacted reference site (R) and the moderately impacted (L), and the severely impacted (H) sites (n=6 per site)

		R	L	H
Physiochemical parameters	pH	6.8 (0.1)	6.5 (0.0)*	7.6 (0.0)**
	CEC (μmol C g^{-1})	16.8 (2.1)	11.1 (0.6)*	28.2 (0.4)**
	Organic matter (%)	2.1 (0.2)	1.6 (0.2)	2.8 (0.2)*
	P (μg g^{-1} soil)	208.7 (3.2)	115.7 (3.8)**	146.0 (10.0)**
	K (%)	12.5 (1.2)	9.4 (0.5)*	2.3 (0.2)**
	Mg (%)	11.9 (1.0)	14.4 (0.2)*	5.9 (0.2)**
	Ca (%)	58.2 (4.7)	60.7 (0.4)	85.6 (0.3)**
	NO_3 (μg g^{-1} soil)	12.3 (3.8)	11.1 (0.6)	7.2 (2.0)
Particle size distribution	Sand %	64.2	74.6	65.8
	Silt %	11.0	5.0	15.0
	Clay %	24.8	20.4	19.2

Significant differences of smelter-impacted sites relative to the nonimpacted site are denoted *(p<0.05) and [b] (p<0.001) based on Dunnett's test

and 4). Metal concentrations in severely impacted site were significantly elevated relative to the metal concentrations in the moderately and nonimpacted sites.

Soils from the nonimpacted, moderately impacted, and severely impacted sites were amended with metal salts and counter-ions to create a total of 15 treatments. The total extractable metal concentrations corresponded with the nominal concentrations in each treatment, with few exceptions (Table 3). Compared to total extractable metals, the $CaCl_2$ extractable metal concentrations were significantly lower in all treatments (Table 4). Metal concentrations in pH-control treatments (R_{LC}, R_{HC}, R_{2HC}, L_{HC}, L_{2HC}, and H_{2HC}) were not significantly different in terms of total extractable metal concentrations, relative to their respective unamended treatments (R, L, and H, respectively) (data not shown). On the other hand, several pH-control treatments (L_{HC}, L_{2HC}, and H_{2HC}) had increased levels of $CaCl_2$ extractable metal concentrations relative to their respective unamended treatments (L and H), likely due to soil acidification.

The level of activity supported by bacterial communities indigenous to the nonimpacted (R) and moderately impacted soil (L) was elevated compared to the activity supported by bacterial communities native to the severely impacted soil (H) (Fig. 1). As seen by the decrease in bacterial activity supported by microbial communities following metal-amended, high concentrations of soil metals negatively affected bacterial activity in all three soils. For example, levels of activity were significantly decreased in R_{2H}, L_H, L_{2H}, and H_{2H}, relative to their respective unamended control treatments (R, L, and H). Because the level of activity supported within pH control treatments (R_{LC}, R_{HC}, R_{2HC}, L_{HC}, and H_{2HC}) remained similar to the level of activity supported by bacterial communities, the unamended treatments (R, L, and H, respectively), the observed decrease in activity is likely a result of metal-associated selection

Table 3 Total extractable metal concentrations (mean (SE))

Treatment (n=6)	Metals (μg g^{-1}soil)				
	Zn	Pb	As	Cd	Cu
R[a]	47 (3)	17 (1)	23 (2)	<1	19 (1)
R_L	107 (2)	51 (1)	49 (1)	7 (0)	91 (4)
R_H	1,467 (55)	746 (25)	773 (28)	24 (1)	1,143 (42)
R_{2H}	2,563 (112)	1,388 (55)	1,441 (55)	40 (1)	2,117 (95)
L[a]	71 (2)	19 (1)	32 (1)	<1	73* (2)
L_H	1,484 (28)	763 (10)	34 (14)	23 (0)	1,164 (19)
L_{2H}	2,799 (83)	1,482 (58)	1,473 (48)	42 (1)	2,229 (69)
H[a]	1,661** (49)	816** (22)	868** (23)	28** (1)	1,276** (35)
H_{2H}	3,120 (69)	1,522 (46)	1,648 (62)	47 (1)	2,406 (58)

Significant differences between the nonimpacted site (R) relative to the two smelter-impacted sites (L and H) to the are denoted *(0.1<p<0.05) and ** (p<0.001) based on Dunnett's test.

[a] Total extractable metal concentrations in the pH and counter ion control treatments (R_{LC}, R_{HC}, and R_{2HC}; L_{HC} and L_{2HC}; and H_{2HC}) were not significantly different from their respective unamended control soils (R, L, and H, respectively) (data not shown).

Table 4 $CaCl_2$ extractable metal concentrations (mean (SE))

Treatment (n=6)	Metals (μg g^{-1}soil)				
	Zn	Pb	As	Cd	Cu
R[a]	<1	<1	<1	<1	<1
R_L	4 (0)	<1	6 (0)	<1	<1
R_H	498 (31)	<1	132 (5)	10 (1)	26 (3)
R_{2H}	673 (112.3)	3 (0)	168 (6)	22 (2)	194 (26)
L	<1	<1	<1	<1	<1
L_H	536 (61)	1 (0)	105 (5)	11 (1)	56 (6)
L_{HC}	3 (0)	<1	<1	<1	<1
L_{2H}	857 (90)	5 (0)	154 (5)	22 (0)	316 (46)
L_{2HC}	5 (0)	<1	<1	<1	<1
H	3* (0)	<1	17* (0)	<1	<1
H_{2H}	376 (16)	1 (0)	59 (0)	7 (0)	7 (0)
H_{2HC}	50 (4)	1 (0)	26 (2)	3 (0)	4 (3)

Significant differences between the non-impacted site (R) relative to the two smelter-impacted sites (L and H) are denoted * (p<0.001) based on Dunnett's test.

[a] $CaCl_2$ metal concentrations in the pH and counter ion control treatments (R_{LC}, R_{HC}, and R_{2HC} were not significantly different observed in the unamended control soil (R) (data not shown).

pressure rather than due to changes in soil pH. The exception to this trend appears to be treatment L_{2HC}, which supported decreased activity as a result of soil acidification. Notably, the level of bacterial activity in L_H, L_{2H}, and H_{2H} metal-amended treatments remained depressed throughout the course of the experiment; whereas bacterial activity in the R_{2H} treatment returned to pre-exposure levels by day 63.

Microbial biomass was decreased in the smelter-impacted sites (L and H), relative to the nonimpacted site (R) (Fig. 2). As seen by the decrease in biomass in treatments following metal-amendment (R_L, R_H, R_{2H}, L_H, L_{2H}, and R_{2H}), relative to the unamended treatments (R, L and H, respectively), high concentrations of soil metals negatively affected biomass in all three soils. Biomass was also significantly affected by soil pH, as was seen by the decreased biomass in treatments R_{LC}, R_{HC}, R_{2HC}, and H_{2HC}, relative to their unamended soil treatments (R and H, respectively). Bacteria in treatments L_{HC} and L_{2HC} did not support decreased biomass as a result of soil pH. In all

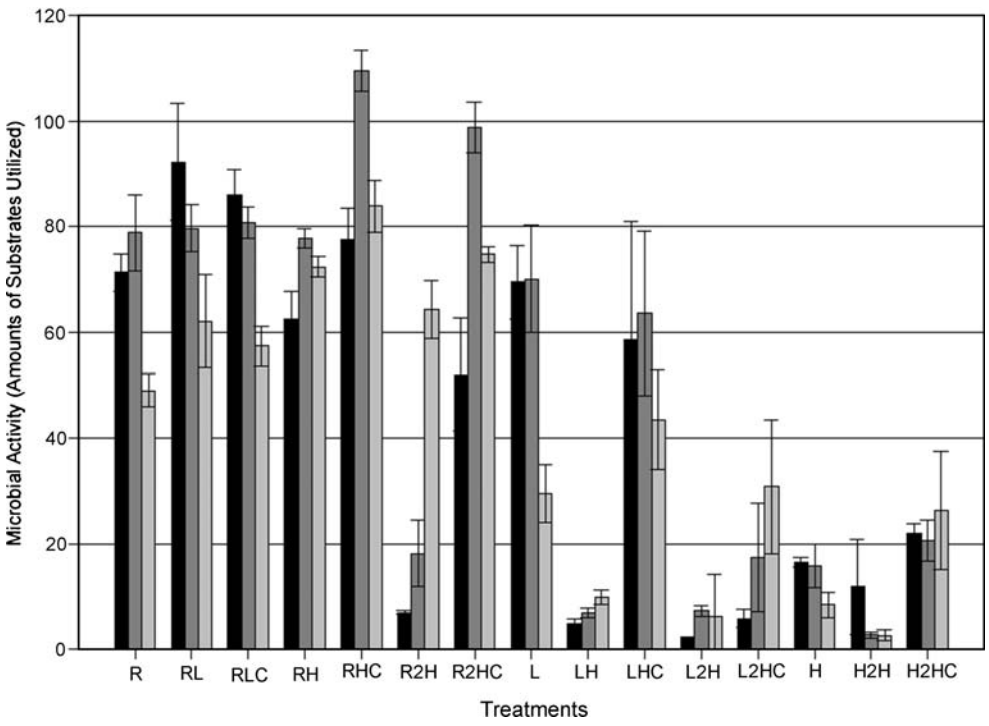

Figure 1 Total microbial activity, relative to the amounts of substrates utilized on Biolog plates, supported by bacterial communities within each treatment, following amendment. (Refer to Table 1 for description of treatment amendments). The *black*, *dark grey*, and *light grey bars* represent the total activity on sample days 7, 21, and 63, respectively. *Error bars* correspond to the mean±SE for each treatment (n=6)

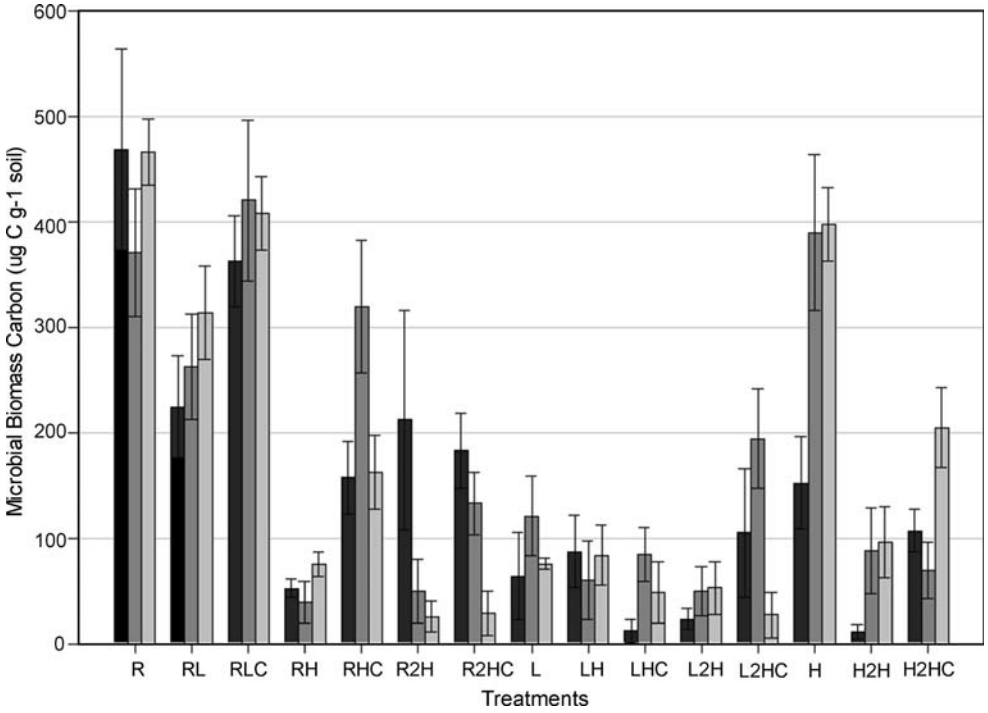

Figure 2 Total microbial biomass supported by bacterial communities within each treatment, following amendment (Refer to Table 1 for description of treatment amendments). The *black*, *dark grey*, and *light grey bars* represent the total activity on sample days 7, 21, and 63, respectively. Error bars correspond to the mean±standard error for each treatment (n=6)

soils, the decreased biomass observed as a result of metal-amendment was more pronounced than the decrease in biomass as a result of soil pH (i.e., compare R_L to R_{LC}, R_H to R_{HC}, R_{2H} to R_{2HC}, etc).

The bacterial communities indigenous to the nonimpacted (R) and smelter-impacted (L and H) sites were unique in terms of their structural diversity, estimated by canonical correspondence analysis (CCA) of community DGGE-banding patterns (Fig. 3a) and functional diversity, estimated by CCA of SUPs (Fig. 3b). In these soils, bacterial community structure and function were highly correlated with soil metal concentration, where metal concentration accounted for 51% and 37.6% of the total variation in the data sets, respectively. Amendment of the soils with increasing concentrations of metals caused significant shifts in both bacterial community structure and function (Fig. 4). Acidification of the soils also was observed to cause shifts in both structural and functional diversity (Fig. 4). For instance, as seen by their proximity in two-dimensional space, on day 63, the structural diversity of the microbial community within the metal-amended treatments (R_L, R_H, and R_{2H}) and the pH control treatments

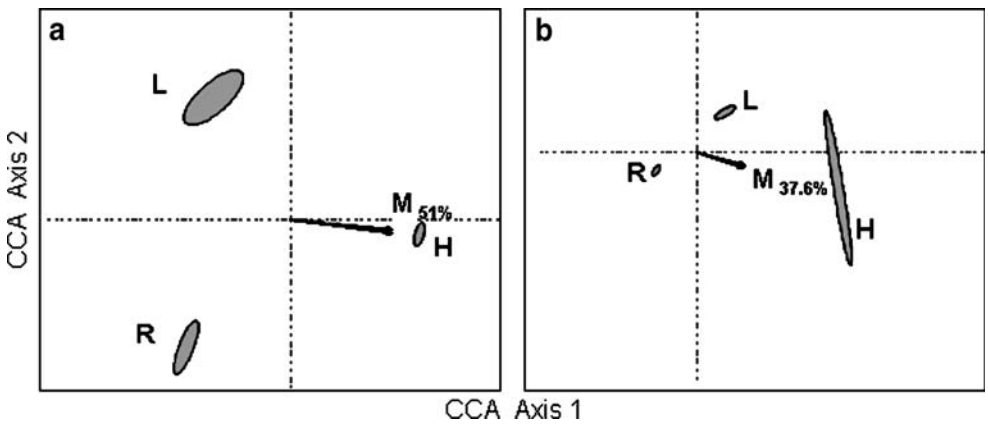

Figure 3 Direct gradient analysis of bacterial communities native to the nonimpacted reference site (R), moderately impacted smelter site (L), and severely impacted smelter site (H) on sampling day 63. Variation in community DGGE banding profiles (**a**) and substrate utilization profiles (**b**) are projected onto two axes via canonical correspondence analysis (CCA). *Ellipses* represent the SE of the means of each treatment. *Arrows* represent significant correlations of total extractable metal concentration (Cd, Cu, As, Pb, and Zn) with ordination axes and is denoted (*M*). Eigenvalues, which estimate the percent of variation in the data that is accounted for by metal concentration, is denoted by subscripts

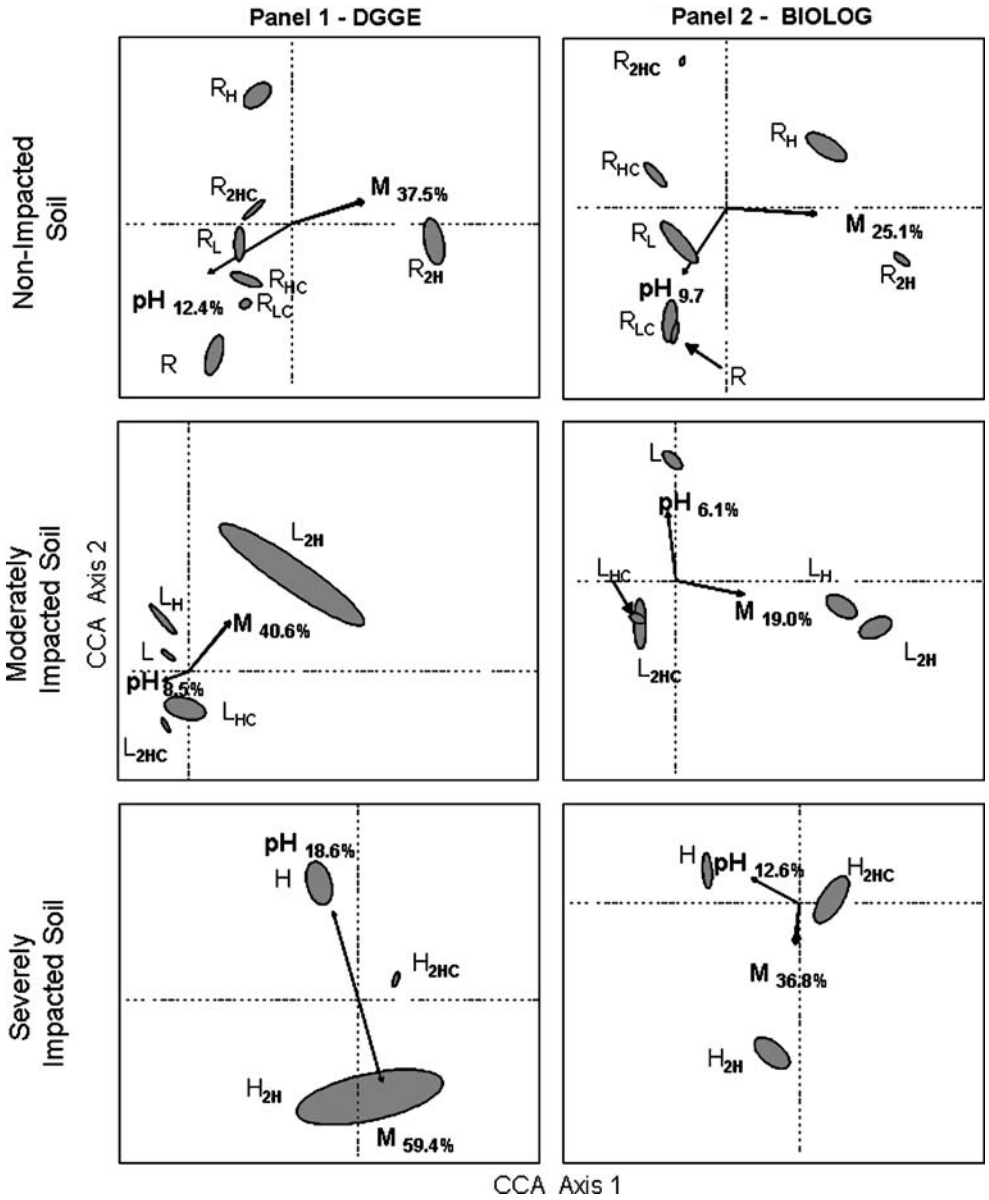

Figure 4 Direct gradient analysis of the shifts in structural and functional diversity of bacterial communities in each soil, following amendment (Refer to Table 1). Variation in community DGGE banding profiles (**Panel 1**) and variation in substrate utilization profiles (**Panel 2**) are projected onto two axes via canonical correspondence analysis (CCA). *Ellipses* represent the standard error of the means of each treatment. *Arrows* represent significant correlations of soil pH and total extractable metal concentration (Cd, Cu, As, Pb, and Zn) with ordination axes and is denoted (pH or M, respectively). Eigenvalues, which estimate the percent of variation in the data that is accounted for by the environmental variables, is denoted by subscripts

(R_{LC}, R_{HC}, and R_{2HC}) were significantly shifted relative to the unamended control treatment (R) (Fig. 4, Panel 1). Soil pH and soil metal concentration accounted for 45.5% of the total variation observed, indicating that the structural diversity of microbial communities within the nonimpacted smelter site is sensitive to both metal stress and changes in soil pH. However, the structural diversity of the microbial communities within the metal-amended treatments (R_L, R_H, and R_{2H}) were also significantly shifted relative to their respective pH-control treatments (R_{LC}, R_{HC}, and R_{2HC}). Soil metal concentrations alone accounted for 37.5% of the total variation, indicating that metal contamination had a direct effect on soil microbial community structure, above and beyond that of pH only. The carbon use profile of the microbial community in the nonimpacted reference site was also observed to be sensitive to changes in soil pH and metal contamination, where metal contamination and soil pH accounted for 25.1% and 9.7% of the total variation, respectively (Fig. 4, Panel 2). These effects were most pronounced in the most contaminated and acidified treatments (R_H, R_{2H}, and R_{2HC}). Treatment R_{LC} was not significantly different than the control treatment, as seen by their overlapping ellipses.

Similarly, the structural and functional diversity of the bacterial communities indigenous to the smelter-impacted soils followed the trends previously observed in the nonimpacted soil (Fig. 4, panels 1 and 2, respectively). Bacterial communities from these soils were sensitive to both decreased soil pH and increased metal stress; however, as shown by the percent of the total variation accounted for by each environmental variable, the effects of soil metal concentrations superseded the effects of soil pH in both

soils. For example, metal concentration accounted for 40.6% and 19.0% of the total variation observed in DGGE profiles and SUPs of microbial communities from the moderately impacted soil (L_H and L_{2H}), respectively, whereas soil pH only accounted for 8.5% and 6.1% of the total variation in this soil. Similarly, metal concentration accounted for 59.4% and 36.8% of the total variation observed in DGGE profiles and SUPs of microbial communities from the severely impacted soil (H_{2H}), respectively, whereas soil pH only accounted for 18.6% and 12.6% of the total variation in this soil.

To test the hypothesis that the extreme levels of soil metal contamination would select for structurally and functionally similar microbial communities in each soil, DGGE banding profiles and SUPs of microbial communities within the most highly metal-contaminated treatments (R_{2H}, L_{2H}, and H_{2H}) were compared using CA. The SUPs and DGGE banding profiles within each community were generated following 7, 21, and 63 days of incubation. SUPs and DGGE banding profiles from each sample day were analyzed independently and jointly to determine if the three communities converged during the course of the incubation. As seen by their proximity in two-dimensional space, when the three sample days are analyzed independently, convergence of treatments R_{2H}, L_{2H}, and H_{2H} is not observed (Fig. 5). Bacterial communities from the three treatments were structurally distinct on day 7 and did not converge to a structural community by day 63 (Fig. 5, panel 1), and, bacterial communities from each site failed to converge on a functionally similar community by day 63, despite

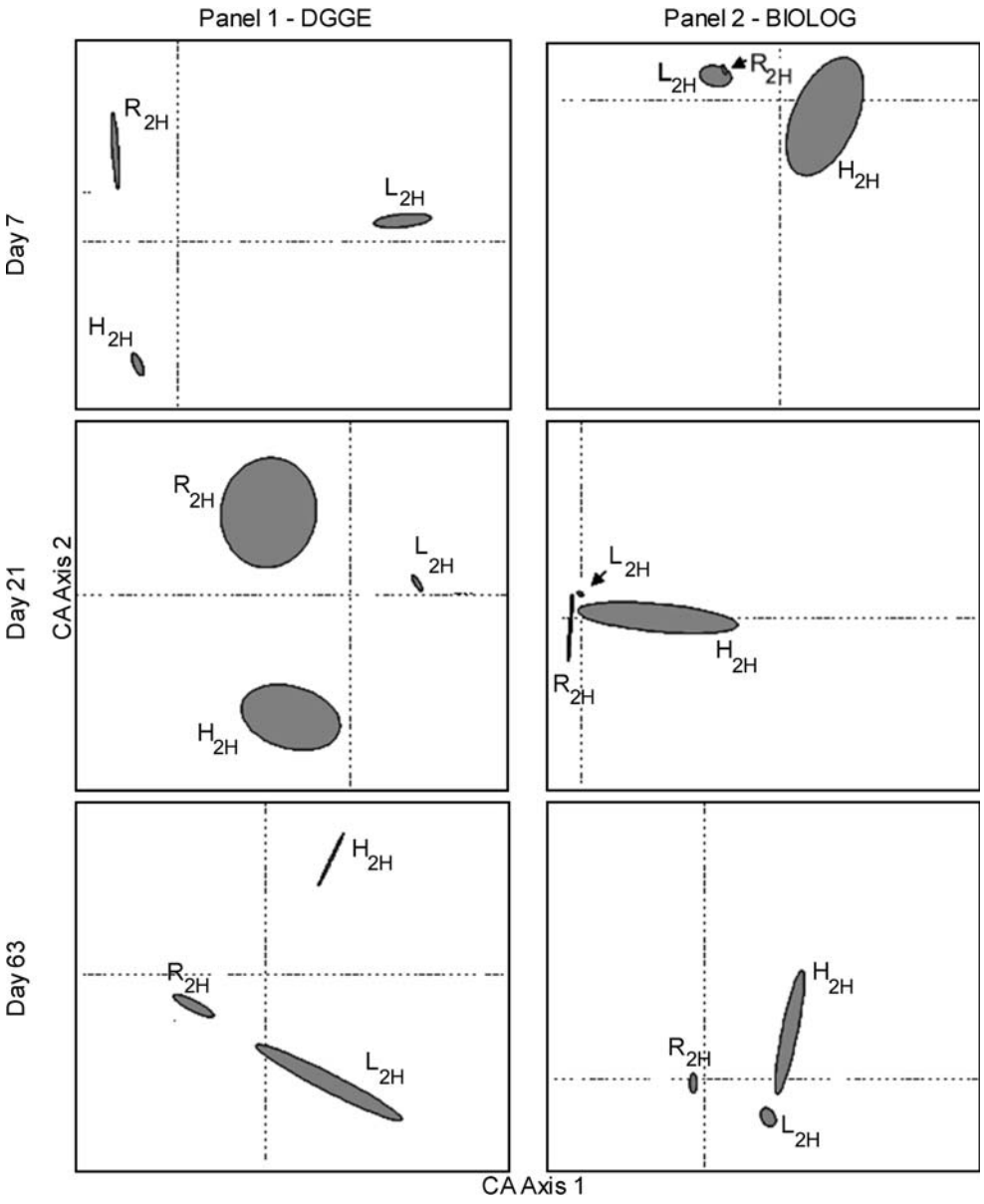

Figure 5 Direct gradient analysis of the shifts in structural and functional diversity of bacterial communities native to each site following amendment with the highest nominal metal concentration (2*High). Variation in community DGGE banding profiles (**Panel 1**) and variation in substrate utilization profiles (**Panel 2**) are projected onto two axes via correspondence analysis (CA). *Ellipses* represent the standard error of the means of each treatment

evidence of overlapping carbon use profiles on day 7 (Fig. 5, panel 2). Likewise, when all three treatments and all three sample days were analyzed jointly on the same CA plot, convergence of community DGGE and SUP profile was not observed (data not shown).

Discussion

Through the selection of tolerant species and selection against sensitive species, soil metals have the capacity to shape the structural and functional diversity of microbial communities in soils [18]. The strong selective pressure of metal contamination is well documented, and soils exposed to long-term metal contamination have been shown to support metal-tolerant communities. For instance, microbial communities chronically exposed to a gradient of Zn concentrations in field-contaminated soil, were found to have increased tolerance to Zn, as measured via the pollution-induced community tolerance method (PICT) [6, 38]. Likewise, microbial communities exposed to long-term metal-contaminated sewage sludge exhibited an increase in community tolerance to metals [46], and forest soils exposed for centuries to naturally elevated soil lead concentrations were observed to support an abundance of lead-tolerant species [1]. Due to the diversity, ubiquity, and heterogeneity of bacterial species in soils [34], it is likely that microbial communities native to pristine soil environments support species inherently tolerant to metal insults. For example, lead-tolerant species were isolated from previously uncontaminated forest soils [1], and metal-tolerant communities were established following the artificial contamination of pristine soils with Zn, Cd, Cu, and Ni [9].

Still, questions remain regarding the capacity for metals to shape microbial communities in soils and the uniformity of microbial community responses to metal stress across soil types. In this study, three soils in the vicinity of a copper smelter, previously exposed to background, low or high concentrations of metals, were artificially contaminated with metal-salts, and the shifts in the structural and functional diversity of each community as a result of metal stress was compared. Based on the assumption that microbial communities are shaped by the selective forces in their local environments, we question whether metal stress will cause initially distinct microbial communities to shift toward structurally and functionally similar communities. The idea of microbial communities with unique structural profiles converging to a similar community profile, as a result of strong selection pressure, has been addressed previously. While in one study, indigenous groundwater microbial communities were seen to converge on a common community in bioreactors treating aromatic hydrocarbon-contaminated groundwater [23], microbial communities from different soils did not converge on a similar hydrocarbon-degrading community when exposed to diesel contamination [5], and unique soil communities did not converge on a similar community profile following fumigation with chloroform [10]. To our knowledge, this is the first study to characterize the convergence following metal stress.

In this study, the nonimpacted reference site supported a bacterial community with inherently higher levels of activity and biomass, relative to the smelter-impacted sites. These results are consistent with previously reported results, in which microbial communities from metal-contaminated sites were shown to support decreased levels of biomass relative to uncontaminated sites [12, 18]. Following amendment with metal-salts, bacterial communities from each site supported decreased levels of activity, and biomass, indicating that the communities were similarly sensitive to metal stress. However, while the levels of activity remained depressed in soil communities native to the smelter-impacted sites, the levels of activity supported by the bacterial community from the nonimpacted smelter site (R_{2H}) returned to preexposure levels throughout the course of the study. Previously reported findings of pollution-induced community tolerance (PICT), suggest that the previously exposed communities (i.e., communities from the smelter-impacted sites) would be more tolerant to additional metal stress than previously unexposed communities [6]. The observed "resiliency" of the community in treatment R_{2H} is likely related to PICT, where the initial high concentrations of metals killed off the majority of metal-sensitive species, which then served as substrate for the surviving community [9, 13].

In addition to differences in activity and biomass, the bacterial communities native to each site were significantly different in terms of their structural and functional diversity. Following amendment with the same nominal concentrations of metals-salts, the bacterial communities native to the nonimpacted, moderately impacted, and severely impacted smelter sites failed to converge on a functionally or structurally similar community. The structural and functional differences in the microbial communities from each site following similar levels of metal stress indicate that the cumulative effects of all environmental variables (i.e., soil pH, organic matter, metal bioavailability, and/or the initial composition of the community) may outweigh the effects of a single environmental stressor in governing bacterial community succession.

The Biolog technique has become widely used in literature, as a measure of bacterial community function, with respect to carbon substrate utilization [14, 21, 22, 45, 48]. As a culture-based technique, however, Biolog is inherently biased due to selective enrichment [2], which

may ultimately serve to misrepresent the indigenous populations [6]. These selection biases have been addressed previously [20]. Additional bias in the Biolog technique, which may be relevant to this study, is related to pH. Biolog plates are buffered at a pH of 6.5, and highly acidic soils have been shown to affect Biolog results [47]. The soil pH in our treatments range from 8.11 (H) to 4.53 (L_{2H}). While we made every effort to control for the confounding effects of soil pH on the bacterial communities in this study, we did not control for the potential bias associated with acidic soil pH in the Biolog technique. Therefore, results should be interpreted with caution.

Potential Biases Associated with Metal-Salt Amended Mesocosms

Soils artificially contaminated with metal salts (nitrate, sulfate, or chloride salts) have been used extensively in the past to characterize the dose–response relationships of various microbial endpoints, including activity [8, 9, 12, 33, 40], respiration [27, 33, 39, 40], composition (using PFLA) [12, 33], nitrification [27, 39, 40, 43], N-mineralization [39], biomass [12, 21, 44], and tolerance [8, 9] to metal stress. While artificially contaminated microcosm studies have notable advantages over field studies (i.e., they allow for control over the confounding soil parameters (e.g., soil organic matter, soil type, soil cation exchange capacity, soil moisture) and associated stresses (e.g., temperature fluctuations, precipitation, mixed pollution) that can vary between sites in natural systems [27], they introduce their own set of confounding variables [43]. Amendment of soils with metal salts is often accompanied by significant decreases in soil pH [27, 42, 43], which cannot only affect bacterial community structure, activity, biomass, and substrate utilization [25], but is also a driving factor controlling metal bioavailability [7], mobility [28, 40], speciation [16], and toxicity. Because the overall change in soil pH as a result of metal-salt hydrolysis is largely dependent on the concentration of metal-salt amendments, the nature of the soil (e.g., percent organic matter, cation exchange capacity, clay content) and the inherent buffering capacity of the soil [41], the degree to which metal-salt amendments lead to changes in soil pH may vary between studies. Several studies have noted the confounding effects of pH on the microbial endpoints tested in artificially spiked soils [12, 16, 33, 43]; however, a few studies have included pH controls, and the effects of soil acidification on microbial communities is rarely considered when interpreting results [41].

In this study, a series of pH-control treatments were used to account for the changes in soil pH as a result of metal-salt amendment. Decreases in soil pH were observed to decrease bacterial biomass and activity and lead to shifts in both microbial structural and functional diversity. As a general trend, bacterial community function, with respect to the identities of substrates utilized on Biolog plates, seemed to be less sensitive to decreases in soil pH, than community structure in the nonimpacted and moderately impacted site communities (as seen by the overlapping functional profiles of R and R_{LC} and L and L_{HC}, respectively), which may indicate that bacterial community functional diversity endpoints have a higher resilience to changes in pH than bacterial community structure endpoints in these soils. Additionally, the bacterial community native to the non-impacted reference site appeared to be relatively robust in terms of its ability to tolerate changes in soil pH, whereas the moderately impacted and severely impacted soil communities appeared to be more sensitive.

In addition to artifacts caused by changes in soil pH, differences in the soil physiochemical profiles between the sites add further sources of bias, and the direct comparison of bacterial community responses between the three soil types may be hindered due to the differences in soil metal bioavailability. The $CaCl_2$ extraction method has been shown to provide a good estimate of the bioavailable fraction of metals in soils [11, 17, 31]. In this study, metals in the severely impacted site appear to be less bioavailable than metals in the nonimpacted site or the moderately impacted site, as seen by the decreased $CaCl_2$ extractable metal concentration in H_{2H} compared to R_{2H} or L_{2H}. The reasons for this decreased bioavailability may be twofold: (1) Because soils were dosed with metal-salt solutions to bring their total metal concentration to a nominal level, the soil from the severely impacted site, which had significantly higher levels of aerially deposited metals, received less metal salt, relative to the moderately impacted site. Metals in contaminated field soils are less bioavailable than metals in artificially spiked due to weathering, aging of the soils, and complexation of metals within the soil matrix [4, 27, 39]. (2) The severely impacted site had significantly elevated soil organic matter, likely due to decreased mineralization of organic matter by soil microbes, as was seen previously in soils surrounding metal-contaminated smelter sites [15]. The moderately impacted site, which supported the lowest levels of organic matter, appears to have the greatest bioavailable fraction of metals. Bioavailability of metals may be controlled by levels of organic matter in the soils [12, 18].

Conclusions

While the use of artificially spiked soil mesocosms for characterizing effects of soil metals on bacterial communities has noted advantages over field studies, the potential biases associated with these studies need to be considered when interpreting metal effects. Controlling for changes in

soil pH was useful in this study to delineate pH-associated effects from metal effects; however, differences in soil metal bioavailability and toxicity between sites remains a potential confounding factor. Nevertheless, this study confirms the variation in soil bacterial community responses to selection pressure across different soil types and highlights the complexity of bacterial community responses to metal stress. Additionally, although the bioavailable concentrations of soil metals varied among treatments from different soil types due to differences in bioavailability, the general trend suggests that bacterial communities did not converge on a common community. Extension of these studies using soils with similar levels of bioavailability may help determine the potential for bacterial communities to converge as a result of metal stress.

Acknowledgements The authors would like to acknowledge significant contributions by the following persons: Bill Olsen, Kevin Reynolds, Pamela Bryer, Paul Story, and Toby McBride for assistance in the field with sample collection, Dr. Melanie Barnes and the Department of Geosciences at Texas Tech University for assistance with soil metal analysis, Jay Clarke and Jim Campbell for assistance with DGGE and PCR, and the Department of Environmental Toxicology at Texas Tech University. This work was sponsored in part by NIEHS ES04696.

References

1. Bååth E, Diaz-Ravina M, Bakken LR (2005) Microbial biomass, community structure and metal tolerance of a naturally Pb-enriched forest soil. Microb Ecol 50:496–505
2. Bååth E, Diaz-Ravina M, Frostegård Å, Campbell CD (1998) Effect of metal-rich sludge amendments on the soil microbial community. Appl Environ Microbiol 64:238–245
3. Bass Becking LGM (1934) Geobiologie of inleiding tot de milieukunde. The Hague. W.P. Van Stockum& Zoon (in Dutch), the Netherlands
4. Bossio DA, Scow KM (1995) Impact of carbon flooding on the metabolic diversity of microbial communities in soils. Appl EnvironMicrobiol 61:4043–4050
5. Bundy JG, Paton GI, Campbell CD (2002) Microbial communities in different soil types do not converge after diesel contamination. J Appl Microbiol 92:276–288
6. Davis MRH, Zhao F, McGrath S (2003) Pollution induced community tolerance of soil microbes in response to a zinc gradient. Environ Toxicol Chem 23:2665–2672
7. Del Val C, Barea JM, Azcon-Aguilar C (1999) Diversity of arbuscular mycorrhizal fungus populations in heavy-metal-contaminated soils. Appl Environ Microbiol 65:718–723
8. Diaz-Ravina M, Bååth E, Frostegård Å (1994) Multiple heavy metal tolerance of soil bacterial communities and its measurement by a thymidine incorporation technique. Appl EnvironMicrobiol 60:2238–2247
9. Diaz-Ravina M, Bååth E (1996) Development of metal tolerance in soil bacterial communities exposed to experimentally increased metal levels. Appl Environ Microbiol 62:2970–2977
10. Dickens HE, Anderson JM (1999) Manipulation of soil microbial community structure in bog forest soils using chloroform fumigation. Soil Biol Biochem 31:2049–2058
11. Feng M, Shan X, Zang S, Wen B (2005) A comparison of the rhizosphere-based method with DTPA, EDTA, CaCl2, and Nano3 extraction methods for prediction of bioavailability of metals in soil to barley. Environmental Pollution 137:231–240
12. Frostegård Å, Tunlid A, Bååth E (1993) Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. Appl Environ Microbiol 59:3605–3617
13. Frostegård Å, Tunlid A, Bååth E (1996) Changes in microbial community structure during long-term incubation in two soils experimentally contaminated with metals. Soil Biol Biochem 28:55–63
14. Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Appl Environ Microbiol 57:2351–2359
15. Giller KE, Witter E, McGrath SP (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biol Biochem 30:1389–1414
16. Ginocchio R, Sanchez P, De La Fuente LM, Camus I, Bustamante E, Silva Y, Urrestaazu P, Torres JC, Rodriguez P (2005) Agricultural soils spiked with copper mine wastes and copper concentrate: Implications for copper bioavailability and bioaccumulation. Environ Toxicol Chem 25:712–718
17. Houba VJG, Lexmond TM, Novozamsky I, Van der Lee JJ (1996) State of the art and future developments in soil analysis for bioavailability assessment. Science of the Total Environment 178:21–28
18. Kahn M, Scullion J (2000) Effect of soil on microbial responses to metal contamination. Environmental Pollution 110:115–125
19. Kandeler F, Kampichler C, Horak O (1995) Influence of heavy metals on the functional diversity of soil microbial communities. Biol Fert Soil 23:299–306
20. Konopka A, Oliver L, Turco RF (1998) The use of carbon substrate utilization patterns in environmental and ecological microbiology. Microb Ecol 35:103–115
21. Knight B, McGrath S, Chaudri A (1997) Biomass carbon measurements and substrate utilization patterns of microbial populations from soils amended with cadmium, copper, or zinc. Appl Environ Microbiol 63:39–43
22. Liao M, Xie X (2007) Effect of heavy metals on substrate utilization pattern, biomass, and activity of microbial communities in a reclaimed mining wasteland of red soil area. Ecotoxicol Environ Saf 66:217–223
23. Massol-Deya A, Weller R, Rios-Hernandes L, Zhou JZ, Hickey RF, Tiedje JM (1997) Succession and convergence of biofilm communities in fixed-film reactors treating aromatic hydrocarbons in groundwater. Appl Environ Microbiol 63:270–276
24. Muyzer G, De Waal E, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturant gradient gel electrophoresis analysis of PCR-amplified genes coding for 16s rRNA. Appl Environ Microbiol 59:695–700
25. Niklinska M, Chodak M, Laskowski R (2005) Characterization of the forest humus microbial community in a heavy metal polluted area. Soil Biol Biochem 37:2185–2194
26. Nunnan N, Morgan MA, Herlihy M (1997) Ultraviolet absorbance (280nm) of compounds released from soil during chloroform fumigation as an estimate of microbial biomass. Soil Biol Biochem 30:1599–1603
27. Oorts K, Bronckaers H, Smolders E (2006a) Discrepancy of the microbial response to elevated copper between freshly spiked and long-term contaminated soils. Environ Toxicol Chem 25:845–853
28. Oorts K, Ghesquiere U, Swinnen K, Smolders E (2006b) Soil properties affecting the toxicity of cucl2 and nicl2 for soil microbial processes in freshly spiked soils. Environ Toxicol Chem 25:836–844

29. Øvreås L, Forney L, Daae FL, Torsvik V (1997) Distribution of bacterioplankton in meromictic lake sea lenvannet, as determined by DGGE of PCR-amplified gene fragments coding for 16s rRNA. Appl Environ Microbiol 63:3367–3373
30. Pennanen T, Frostegård Å, Fritze H, Bååth E (1996) Phospholipid fatty acid composition and heavy metal tolerance of soil microbial communities along two heavy metal-polluted gradients in coniferous forests. Appl Environ Microbiol 62:420–428
31. Perez-de-Mora A, Burgos P, Madejon E, Cabrera F, Jaekel P, Schloter M (2005) Microbial community structure and function in a soil contaminated by heavy metals: Effects of plant growth and different amendments. Soil Biol Biochem 38:327–341
32. R Development Core Team (2007) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria ISBN 3-900051-07-0. http://www.R-project.org
33. Rajapaksha RMCP, Tobor-Kaplon MA, Bååth E (2004) Metal toxicity affects fungal and bacterial activities in soil differently. Appl Environ Microbiol 70:2966–2973
34. Ramette A, Tiedje JM (2006) Biogeography: an emerging cornerstone for understanding prokaryotic diversity, ecology and evolution. Microb Ecol 53:197–207
35. Reche I, Pulido-Villena E, Morales-Baquero R, Casamayor EC (2005) Does ecosystem size determine aquatic bacterial richness? Ecology 86:1715–1722
36. Redente EF, Zadeh H, Paschke MW (2002) Phytotoxicity of smelter-impacted soils in southwest Montana, USA. Environ Toxicol Chem 21:269–274
37. Reynolds KD, Schwarz MS, McFarland CA, McBride T, Adair B, Hooper MJ (2006) Norther pocket gophers (*Thomomys talpoides*) as biomonitors of environmental metal contamination. Environ Toxicol Chem 25:458–469
38. Seguin F, Le Bihan F, Leboulanger C, Berard A (2001) A risk assessment of pollution: induction of atrazine tolerance in phytoplankton communities in freshwater outdoor mesocosms, using chlorophyll fluorescence as an endpoint. Water Res 36:3227–3236
39. Smolders E, McGrath SP, Lombi E, Karman CC, Bernhard R, Cools D, Van den Brande K, van Os B, Walrave N (2003) Comparison of toxicity of zinc for soil microbial processes between laboratory-contained and polluted field soils. Environ Toxicol Chem 22:2592–2598
40. Smolders E, Buekers J, Oliver I, McLaughlin MJ (2004) Soil properties affecting toxicity of zinc to soil microbial properties in laboratory-spiked and field-contaminated soils. Environ Toxicol Chem 23:2633–2640
41. Speir TW, Kettles HA, Percival HJ, Parshotam A (1999) Is soil acidification the cause of biogeochemical responses when soils are amended with heavy metal salts? Soil Biol Biochem 31:1953–1961
42. Stevens DP, McLaughlin MJ, Heinrich T (2003) Determining toxicity of lead and zinc runoff in soils: Salinity effects on metal partitioning and on phytotoxicity. Environl Toxicol Chem 22:3017–3024
43. Struczynski TI, McCarty GW, Siebielec G (2003) Response of soil microbiological activities to cadmium, lead, and zinc amendments. J Environ Qual 32:1346–1355
44. Tandy S, Barbosa V, Tye A, Preston S, Paton G, Zhang H, McGrath S (2005) Comparison of different microbial bioassays to assess metal-contaminated soils. Environ Toxicol Chem 24:530–536
45. Willig MR, Moorhead DL, Cox SB, Zak JC (1996) Functional diversity of soil bacterial communities in the Tabonuco forest: Interaction of anthropogenic and natural disturbances. Biotropica 28:471–483
46. Witter E, Gong P, Bååth E, Marstorp H (2000) A study of the structure and metal tolerance of the soil microbial community six years after cessation of sewage sludge applications. Environ Toxicol Chem 19:1983–1991
47. Yao H, He Z, Wilson MJ, Campbell CD (2000) Microbial biomass and community structure in a sequence of soils with increasing fertility and changing land use. Microb Ecol 40:223–227
48. Zak JC, Willig MR, Moorhead DL, Wildman HG (1994) Functional diversity of microbial communities: a quantitative approach. Soil Biol Biochem 26:1101–1108