

# Microbial ecological response of the intestinal flora of *Peromyscus maniculatus* and *P. leucopus* to heavy metal contamination

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## Abstract

Heavy metal contamination negatively affects natural systems including plants, birds, fish and bacteria by reducing biodiversity at contaminated sites. At the Tri-State Mining District, efforts have been made to remediate sites to mitigate the detrimental effects that contamination has caused on human health. While the remediation effort has returned the site to within federal safety standards, it is unclear if this effort is sufficient to restore floral and faunal communities. Intrinsic to ecosystem and organism health is the biodiversity and composition of microbial communities. We have taken advantage of recent advances in sequencing technology and surveyed the bacterial community of remediated and reference soils as well as the intestinal microbial community of two ubiquitous rodent species to provide insight on the impacts of residual heavy metal contamination on the ecosystem. Rodents found on the remediated site had reduced body mass, smaller body size and lower body fat than animals on reference sites. Using bar-coded, massively parallel sequencing, we found that bacterial communities in both the soil and *Peromyscus* spp. gastrointestinal tracts had no difference in diversity between reference and remediated sites but assemblages differed in response to contamination. These results suggest that niche voids left by microbial taxa that were unable to deal with the remnant levels of heavy metals on remediated sites were replaced by taxa that could persist in this environment. Whether this replacement provided similar ecosystem services as ancestral bacterial communities is unknown.

*Keywords:* gastrointestinal microbes, heavy metal, massively parallel sequencing, microbial ecology, *Peromyscus*

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## Introduction

Human-induced alteration of natural systems reduces ecosystem services (Cairns & Niederlehner 1996), promotes the spread of emerging infectious diseases (Daszak *et al.* 2001), reduces global biodiversity (Noss 1990) and alters community structure (Todd 1996). Environmental contaminants present a particularly challenging

problem, because they have a detrimental effect on biodiversity and function at all biological scales. Human intervention typically begins with environmental remediation for human health and often it is presumed that this mitigation is sufficient for environmental health, yet it is unclear how remediated sites function at the ecosystem or organism level.

It has long been known that microbial processes are affected by heavy metal contamination in soils (Lipman & Burgess 1914; Brown & Minges 1916). Microbial biomass is reduced in areas contaminated with heavy met-

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als (Frostegard *et al.* 1993, 1996; McGrath 1994; McGrath *et al.* 1995; Renella *et al.* 2005), bacterial community diversity and species richness can decrease in response to general disturbance (KLJ, JDC unpublished data) and these alterations in microbial community diversity and assemblage have important consequences on ecosystem function, biogeochemical properties in the soil and organism health (Giller *et al.* 1998; Nannipieri *et al.* 2003). While short-term studies of bacterial community response to heavy metal exposure are more commonplace (Ranjard *et al.* 2000; Shi *et al.* 2002; Gremion *et al.* 2004; Rajapaksha *et al.* 2004) and even long-term (chronic) effects of heavy metals on microbial communities have been studied (Pennanen *et al.* 1996; Kandeler *et al.* 2000; Sandaa *et al.* 2001; Renella *et al.* 2004; Frey *et al.* 2006), the effects of remediation of heavy metal contamination on microbial communities are largely understudied. In this study, we chose to study two separate, yet intertwined microbial communities at two sites remediated for heavy metal contamination, the soil microbial community and the intestinal microbial community of native rodents, to better understand the potentially cascading effect of residual heavy metal contamination at the Tri-State Mining District.

The Tri-State Mining District is a former heavy metal (lead, zinc and cadmium) mining area that encompasses 2500 square miles in southeastern Kansas, southwestern Missouri and northeastern Oklahoma. 100 years of mining in this area ended in the mid-1970s and clean-up efforts began in the early 1990s after unacceptably high blood lead levels were measured in children (EPA 2006). The sources of contamination were mining waste brought to the surface, groundwater that leached heavy metals into most watersheds and smelting operations that volatilized the ores. In populated areas, topsoil was removed and backfilled with clean soil. Remediation efforts have resulted in a 43% reduction in blood lead levels of children in Cherokee County, Kansas (Environmental Protection Agency 2006). Although sites have been remediated for human health, environmental contamination persists.

The residual contamination present in the environment of the Tri-County area has effects on aquatic communities (Roark & Brown 1996), bird communities (Beyer *et al.* 2004; Sileo *et al.* 2004), and plant communities (Dames & Moore 1995). Specifically, minnow populations have reduced genetic variability in areas with increased contamination (Roark & Brown 1996). Other members of the aquatic community are affected as well, including freshwater mussels, which are absent in the lower reaches of several metal contaminated rivers in the area, while they are present in high numbers in nearby non-contaminated sites (Clarke & Obermeyer 1996; Pope 2005; Angelo *et al.* 2007). Additionally,

amphibian species show decreased density of adults and tadpoles in contaminated sites in Cherokee County, Kansas, which is likely due to decreased hatching rates of eggs (Anderson & Arruda 2006). Significant effects of heavy metal exposure in the Tri-State Mining District are observed in various bird species including increased incidence of pancreatitis and toxic liver concentrations of zinc in waterfowl (Sileo *et al.* 2004) and significantly raised levels of lead in American Robins (*Turdus migratorius*) and Northern Cardinals (*Cardinalis cardinalis*) (Beyer *et al.* 2004). Additionally, plant communities have been affected by the mine tailings; for instance, percent ground cover and productivity were reduced according to proximity to mine-affected sites with areas closest to contamination having 23% reductions in productivity as compared to distant sites (Dames & Moore 1995).

These studies indicate that while the Tri-State Mining District has been remediated to within federal standards for safety, lingering contamination is at levels high enough to adversely affect wildlife. The US Fish and Wildlife Service is mandated by the Federal Migratory Bird Treaty Act to investigate the effects on migratory birds and their primary prey species. It is currently unknown how residual contamination affects small mammals, which are the primary prey of raptors. In this study, we investigate the effects of heavy metal contamination on the rodent community and physiology and the microbial communities associated with them. The importance of diversity and community structure in the microbiota of mammals has only recently been appreciated. Bacterial by-products have been found to be essential for health and intestinal microbes play an important role in nutrient absorption by intestinal epithelial cells. Commensal bacteria also play a role in protection from pathogenic microbes [reviewed in Hattori & Taylor (2009) and Mai & Draganov (2009)].

Here we examined the effect of residual contamination at remediated sites in the Tri-State Mining District on rodent communities and the microbial communities found in the small intestines of two ubiquitous small mammals, *Peromyscus maniculatus* and *P. leucopus*, as well as in the soil. We utilized massively parallel sequencing (MPS) technologies to sequence bacterial 16S DNA amplified from the soil and mouse intestines to quickly and thoroughly survey microbial community diversity and assemblage.

## Materials and methods

### Study area

In June 2005 and 2006, we live-trapped for five nights each on two remediated and two reference sites in the

**Table 1** Summary of abundance data for trapping efforts on remediated and reference sites in the Tri-State Mining District in 2005 and 2006

	Remediated	Reference
<i>Peromyscus maniculatus</i>	24	8
<i>Peromyscus leucopus</i>	1	13
<i>Neotoma floridana</i>	11	29

Totals of *P. maniculatus*, *P. leucopus* and *N. floridana* caught in traps set in 2005 and 2006 in the Tri-State Mining District are shown by treatment (remediated vs. reference).

Tri-State Mining District. We trapped in each of these habitats to target sister rodent taxa, *Peromyscus maniculatus* on upland sites and *P. leucopus* on riparian sites (Nowak 1999). Remediated sites have previously been found to have between 270 and 732 mg/kg of Pb (Oklahoma Department of Environmental Quality 2000); heavy metal content was not measured on reference sites, but was assumed to be lower than on remediated sites. Remediated and reference areas were located approximately 40 km apart in similar habitat.

#### Rodent community and organismic measures

We compared small mammal diversity and abundance among remediated and reference sites during 2005 and 2006 (Table 1). For assessment of animal health, we used measures from 10 *P. maniculatus*, five from each habitat type collected in 2006. All animals were weighed to the nearest 0.1 g, hind foot length was measured as a proxy for body size, and body condition was evaluated categorically on a scale from 1 (low body fat) to 5 (high body fat). Morphometric data were analysed with Student's *t*-tests assuming equal variance and body condition was compared with a Mann-Whitney *U* test.

#### Sample collection and DNA extraction

Using 25 trap stations per site, we live trapped a representation of the small mammal community. In 2006, we trapped 10 *P. maniculatus* and 6 *P. leucopus* adults (Table 2), which were euthanized and necropsied in the field (Kansas State University Institutional Animal Care and Use Protocol No. 2389). For the 16 individuals that were euthanized, a 1 cm portion of colon containing faeces was removed and placed into a sterile 2 ml vial, and frozen. This sampling method has been shown to give accurate representation of the original bacterial community present (Roesch *et al.* 2009). We also collected 10–20 g of soil within 100 cm of the trap location of each euthanized animal. Soil was placed in a sterile

**Table 2** Summary of mouse and soil samples collected

Species	Sample ID	Habitat	Treatment	No. of sequences	
<i>Peromyscus maniculatus</i>	326-1	Riparian	Reference	1069	
	367-1	Upland	Reference	1225	
	369-1	Upland	Reference	718	
	365-1	Upland	Reference	1519	
	367-2	Upland	Reference	712	
	402-1	Upland	Remediated	360	
	419-1	Upland	Remediated	1104	
	403-1	Upland	Remediated	1025	
	423-1	Upland	Remediated	1064	
	<i>Peromyscus leucopus</i>	304-1	Upland	Reference	1426
		351-1	Riparian	Reference	925
		374-1	Riparian	Reference	622
		377-1	Riparian	Reference	696
374-2		Riparian	Reference	1257	
430-1		Riparian	Remediated	1125	
Soil		304	Upland	Reference	1638
	365	Upland	Reference	1905	
	367	Upland	Reference	1670	
	369	Upland	Reference	668	
	326	Riparian	Reference	1083	
	374	Riparian	Reference	64	
	377	Riparian	Reference	1527	
	402	Upland	Remediated	1267	
	403	Upland	Remediated	190	
	404	Upland	Remediated	1875	
	419	Upland	Remediated	751	
	423	Upland	Remediated	1209	
	430	Riparian	Remediated	956	

Mouse fecal and soil samples were collected in the Tri-State Mining district in Kansas and given Ids according to the trap they were collected in.

Whirlpak and frozen. DNA was extracted from faeces and soil using a soil DNA extraction kit (UltraClean Mega Soil DNA Kit, MoBio). Upon extraction DNA samples were diluted to 5 ng/μL and immediately stored at –80 °C.

#### 16S rDNA amplification and 454 sequencing

Universal bacterial primers that amplify the V3 region of the 16S rDNA gene (U341F and U533R, Watanabe *et al.* 2001) were modified by the addition of mass parallel sequencing primers (Margulies *et al.* 2005) as well as a unique 5 bp sequence specific to each sample which facilitates bioinformatics analysis (Table S1). bMPS primers were randomly assigned to samples and the bacterial community from each sample was amplified independently. bMPS primers allowed for random sequencing from mixtures of bar-coded bacterial sequences as amplicons representing different samples could be pooled and sequenced and reassigned to par-

ticular samples after sequencing. PCR reactions [one unit Amplitaq Gold LD polymerase (Applied Biosystems), 1x Amplitaq Gold polymerase reaction buffer, 3.75 mM MgCl<sub>2</sub>, 200 µM dNTP, 0.5 µM for each, forward and reverse bMPS V3 16S primers, and 5 ng extracted sample genomic DNA] were run for 25 cycles (95 °C for 1 min., 55 °C for 1 min., 74 °C for 1 min.) on an iCycler IQ real-time thermocycler (Bio-Rad Laboratories). Addition of SYBR green reagent was used to verify that PCR was stopped in log-phase. PCR reactions were performed in triplicate before pooling and cleanup with AMPure PCR cleanup kit (Agencourt Bioscience). For sequencing, 100 ng of PCR product from each uniquely identifiable sample was added in a pool and sequenced by 454 Life Sciences (Bradford, CT). Samples from this project were run simultaneously with those from other projects (data not shown) taking advantage of blocking that is available as part of the sequencing run. Raw sequences generated for this project are freely available in the Short Read Archive at NCBI (accession number SRA009883.5).

#### Bioinformatics and OTU designation

Raw 454 sequences were searched for the occurrence of the primer barcode immediately preceding the 16S primer sequence within each sequence. The barcode was removed and the unique identifier was incorporated into the sequence name. A total of 61 406 sequence reads were generated for use in this project. Sequences were not considered for further analyses if they did not contain a valid primer or barcode sequence removing 2538 sequences. Additionally, sequence reads that were shorter than 95 bp or longer than 140 bp were removed with 30 512 remaining and this should drastically reduce the number of sequences that contain errors (Huse *et al.* 2007). Finally, sequences that contained more than one ambiguous base were removed reducing the total by 556 sequence reads with a total of 29 948 passing quality filters. For the sequences that passed quality control, CAP3 (Huang & Madan 1999) was used to align sequences at each of 19 sequence identity levels (80%–98%) using default parameters and a minimal overlap of 75 bp as in Miller *et al.* (2009). In order to investigate sequencing depth, OTU relative abundance at 98% SIL from each sample was used for calculation of rarefaction curves with the program Analytic Rarefaction 1.3.

#### Diversity indices

Overall taxonomic richness ( $S$ ) was calculated by summing the number of OTUs, including singlets, which occurred within each plot. Simpson's Dominance ( $\sum p_i^2$ ), Simpson's Diversity ( $1/\sum p_i^2$ ), and Shannon's

Diversity  $\{-e\sum p_i[\ln(p_i)]\}$  were calculated for each plot, where  $p_i$  was the relative abundance of each OTU. Evenness was calculated as the ratio of Shannon's Diversity and richness  $[e\sum p_i(\ln(p_i)/S)]$ . A final index of diversity, Fisher's alpha log-series (Fisher *et al.* 1943), was calculated by iterating the equation  $S/N = [(1 - x)/x][-\ln(1 - x)]$ , where  $S$  is richness and  $N$  is the total number of sequences within the plot. Once  $x$  was solved, the diversity index alpha ( $\alpha$ ) was calculated as  $N(1 - x)/x$ . Differences in diversity across treatments were analysed using the GLM procedure in SAS (SAS Institute Inc.) assuming equal variance and normal distribution of diversity values.

#### Taxon-specific analyses

Using the 98% sequence identity level determined OTUs, we performed t-tests to determine whether OTU relative abundance was significantly greater than zero ( $P < 0.05$ ) for inclusion in the analysis. Using those OTUs that had significantly non-zero relative abundance, we performed analysis of variance (ANOVA) with the mixed procedure in SAS (SAS Institute Inc.) to determine the overall effect of treatment and habitat type on relative abundance of OTUs. Specifically, we tested soil OTUs for significant differences due to habitat type (riparian vs. upland), treatment (remediated vs. reference) and their interaction; *P. maniculatus* intestinal OTUs for treatment effects (remediated vs. reference) and we tested for differences between the intestinal OTUs in *P. maniculatus* and *P. leucopus*. A log<sub>2</sub> transformation was performed on relative abundance data for input into the statistical software and ANOVAs were done for each bacterial taxa. We used two significance thresholds to determine statistical significance. First we used a less conservative threshold of  $P < 0.01$ . Second we used a more conservative significance threshold that corrected for increased experiment-wide error rate associated with multiple tests, where False Discovery Rate ( $q$ -values) was calculated using QVALUE software (Storey 2003) and we applied the false discovery rate threshold of  $q < 0.1$  to identify significance. The collection of sequences within each OTU was used to produce a consensus sequence and taxonomic identities of those consensus sequences were then explored via BLAST to the Ribosomal Database Project (<http://rdp.cme.msu.edu/>).

## Results

#### Study design

We sampled dissected intestinal contents from ten *P. maniculatus* and six *P. leucopus* trapped at pairs of sites

(remediated contaminated site and nearby non-contaminated reference site) in the Tri-State Mining District of the United States. A total of 16 mice were euthanized in this study and corresponding soil samples were taken from the trap site (Table 2) for determination of microbial communities (see 'Methods'). One sample from *P. maniculatus* failed to produce usable DNA. Of the remaining nine *P. maniculatus* that were euthanized, eight were from upland trap sites and one was from the riparian site, while *P. leucopus* were more frequently trapped in the riparian site with five trapped and only one trapped at the upland site. *Peromyscus maniculatus* was sampled roughly equally in the remediated and reference sites while *P. leucopus* was sampled predominantly in the reference sites. In two cases, two mice were caught in the same trap and in both cases the two were of the same species. Inclusion of both mouse species and soil from different locations allowed for multiple comparisons including contamination treatment effects on mouse and soil bacterial communities, effect of habitat type on soil bacterial communities and differences in intestinal bacterial communities between the two mice species.

#### *Peromyscus* community structure, animal health and body condition

Overall, we trapped 15 *P. maniculatus* in 2005 and 17 in 2006. Over the 2 years of the project, *P. maniculatus* were more abundant on remediated sites than on reference sites; however, *P. leucopus* and *Neotoma floridana* were more abundant on the reference sites than the remediated sites (Table 1). *Neotoma floridana* abundance was variable across years. In 2005, only one animal was caught in the remediated area. Despite higher abundance on remediated sites, *P. maniculatus* were significantly leaner on remediated ( $n = 5$ ) vs. reference sites ( $n = 5$ , mean  $\pm$  SE body condition score  $2.2 \pm 0.04$  vs.  $3.4 \pm 0.2$ , on a scale of 1–5,  $Z = 4.6$ ,  $P = 0.06$ ). In addition, on remediated sites *P. maniculatus* had marginally lower body mass ( $14.8 \pm 0.1$  g vs.  $20.8 \pm 8.8$  g,  $t = 2.0$ ,  $P = 0.06$ ), and significantly shorter hindfeet ( $17.2 \pm 1.1$  mm vs.  $19.8 \pm 1.2$  mm,  $t = 2.4$ ,  $P = 0.02$ ) than on reference sites.

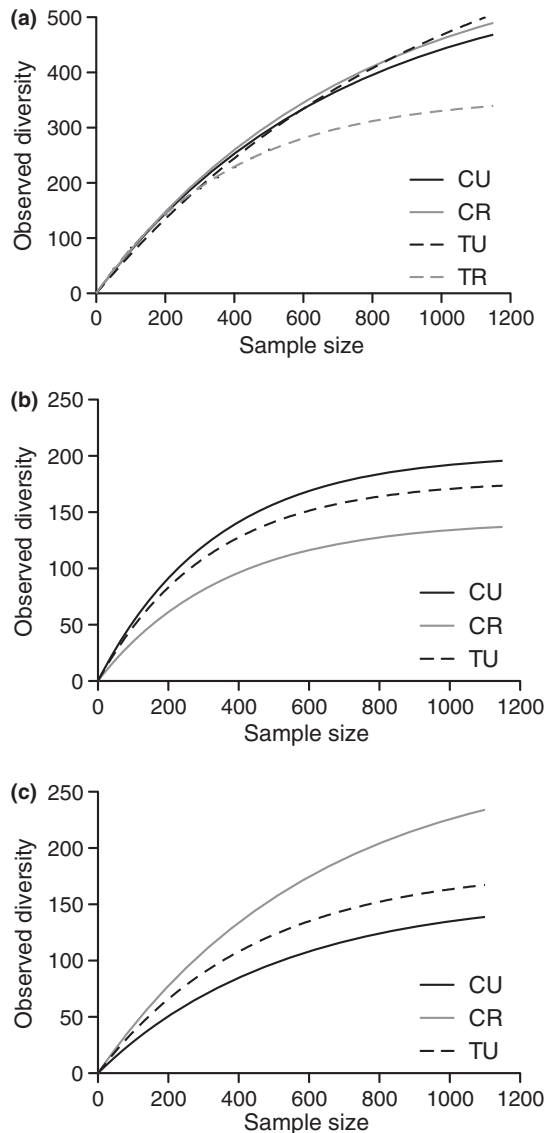
#### Sequencing, bioinformatics and OTU relative abundance

From each of the 15 mouse intestinal contents and the corresponding 13 soil samples from which we extracted usable DNA, we used pooled amplified V3 16S segments for first generation 454 sequencing. Generated sequences went through quality control to remove short reads and long sequencing reads resulting in a total of

29 948 sequences used in downstream analyses. There were an average of 1059 reads per sample showing moderate sequencing depth and sequences were on average 100 base pairs in length. Using CAP3 software (Huang & Madan 1999; Acosta-Martinez *et al.* 2008), sequence alignments were made and operational taxonomic units (OTUs) were determined by percent sequence identity level (% SIL) as in Miller *et al.* (2009), clustering at each level from 80 to 98% sequence identity. Relative abundance was then determined for the all OTUs at each of 19% SILs (80–98), for each of 28 soil and mouse fecal samples. To investigate sampling depth we performed rarefaction analysis (Fig. 1) on OTU relative abundance for each unique mouse intestinal or soil bacterial sample type at 98% SIL. Figure 1 shows that plots approached the plateau phase but did not reach it. This graphical representation suggests that we underestimated the number of unique bacterial OTUs. Despite the likely underestimate of richness, the sampling intensity from mouse intestinal and soil samples appears to be in line with other recent work; however, both the intestinal and soil bacterial communities had far less richness than environmental samples from other published studies of soil and seawater microbial communities (Fig. 1b–c, Sogin *et al.* 2006; Roesch *et al.* 2007; Acosta-Martinez *et al.* 2008) but more than that found in alkaline hot springs (Miller *et al.* 2009). This diminished diversity suggests that the soil system in our study is less complex than predicted. We expected and observed that intestinal bacterial communities had low species richness. The rarefaction analysis predicted that further sampling efforts would yield greater estimates of species richness and suggested that the design of future experiments using massively parallel sequencing should consider additional sequencing to fully capture the entire diversity of most systems.

#### Microbial community diversity response to heavy metal exposure

Using the relative abundance data collected using massively parallel sequencing we calculated diversity indices including species richness, alpha log series diversity, Shannon diversity, Simpson's dominance, inverse of Simpson's dominance and evenness at each of 19 sequence identity levels (Figs 2, 3, S1, S2). These diversity indices were used to understand how the bacterial community responds to different environmental conditions. When we compared the intestinal bacterial community diversity of *P. maniculatus* to that of *P. leucopus* we found no significant difference at any % SIL tested (Fig. 2). To further investigate *Peromyscus* intestinal microbial responses, comparisons were made of intestinal bacterial communities of *P. maniculatus* from



**Fig. 1** Rarefaction curves for microbial communities. Rarefaction analysis for each sample type was performed on relative abundance information for OTUs identified at the 98% SIL with the program Analytic Rarefaction 1.3. Rarefaction curves for (a) soil bacterial communities, (b) *P. maniculatus* bacterial communities, and (c) *P. leucopus* bacterial communities are shown. CU = control upland, CR = control riparian, TU = contaminated upland, TR = contaminated riparian.

remediated and reference sites using the aforementioned diversity indices (Fig. 3). We found no significant effect of heavy metal exposure on *P. maniculatus* intestinal bacterial community diversity. Comparisons of remediated vs. reference sites were restricted to *P. maniculatus*, as sample sizes were not large enough for comparisons of heavy metal exposure using *P. leucopus*. Comparisons were not made between the different habitat types for either species as only one *P. maniculatus*

sample was obtained from riparian habitat and only one *P. leucopus* sample was collected from upland areas (Table 2) precluding statistical analysis.

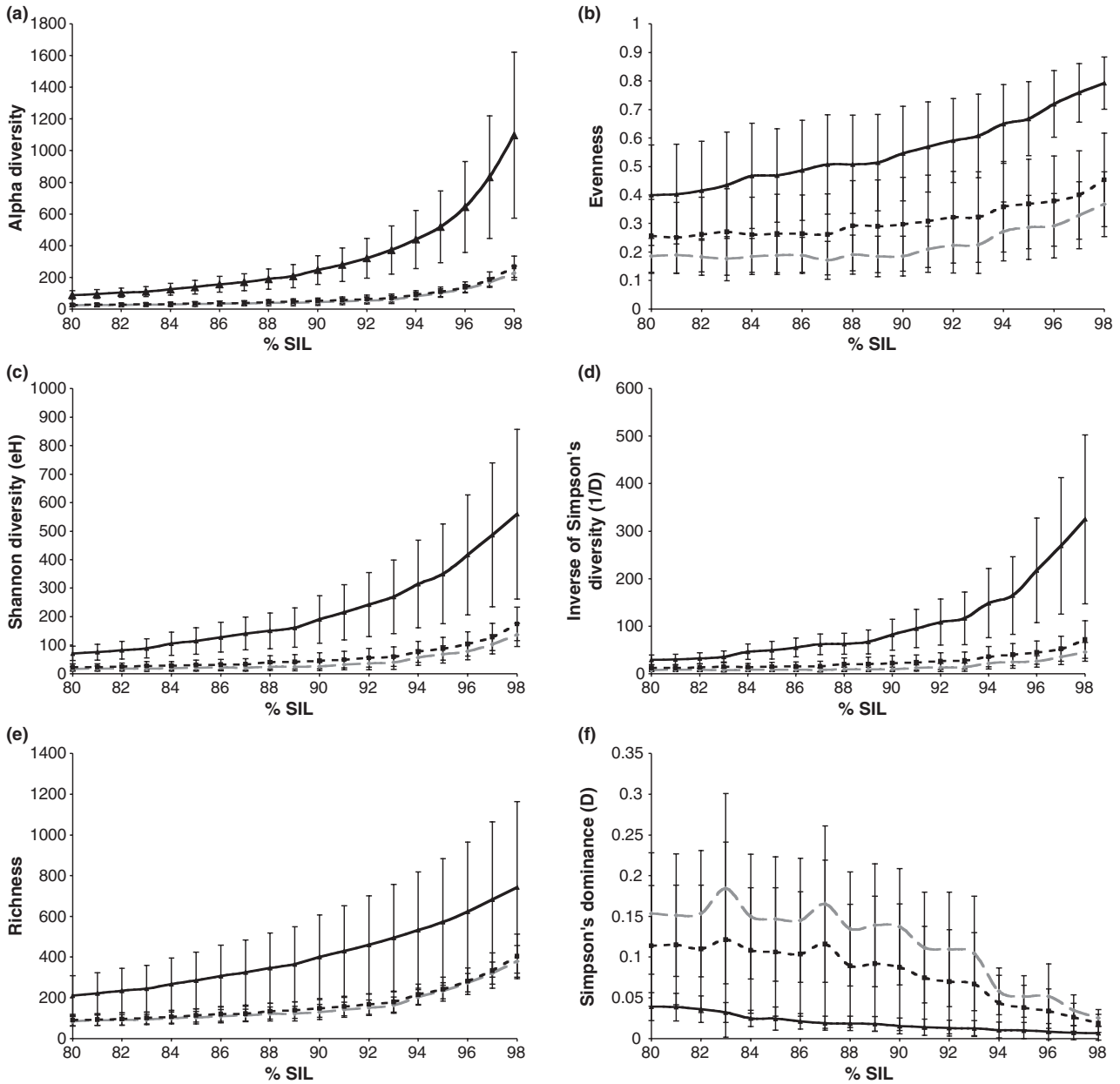
We calculated the same diversity indices for the corresponding soil bacterial communities from remediated and reference sites. We found no significant difference in any diversity index at all %SIL (Fig. S1). Additionally, we compared soil bacterial communities from upland and riparian sites and again found no significant differences for any diversity indices at every %SIL (Fig. S2). Thus results were similar between remediated and reference sites for both soil and mouse intestinal communities.

#### *Microbial community assemblage response to perturbation*

In order to determine taxon-specific responses, we analysed changes in OTU relative abundance at 98% SIL. This level of taxonomic organization is the most exclusive and allows for fine resolution differences among community assemblages.

When we compared the relative abundance of intestinal bacterial species between *P. maniculatus* and *P. leucopus*, we identified three taxa that differed significantly using the less conservative significance threshold ( $P < 0.01$ ) between the two species, all of which had higher relative abundance in *P. maniculatus* than in *P. leucopus* intestinal samples (Table 3). The first identified taxon was an uncultured member of the *Ruminococcaceae* family, which belongs to the class Clostridia. It was identified as the closest match to the sequence obtained by massively parallel sequencing (SAB = 1.00) when compared to the Ribosomal Database Project (RDP, <http://rdp.cme.msu.edu/>). The second taxon matched an unclassified member of the *Porphyromonadaceae* family, and the final taxon best matched an unclassified bacterial sequence in RDP, potentially representing a previously undescribed species of bacteria. When we used the more conservative significance threshold (false discovery rate  $q < 0.01$ ) none of the three bacteria were found to differ significantly between *P. maniculatus* and *P. leucopus* (Table 3).

When we tested the effect of residual heavy metal contamination on bacterial taxa in *P. maniculatus* intestinal communities we identified five bacteria that differed significantly using the less conservative significance threshold (Table 4). Of the five bacterial taxa identified as significantly different, four matched best to uncultured species in the *Tannerella* genus. Of the four *Tannerella* spp., two had significantly greater relative abundance in mouse intestines sampled from the remediated site and the other two had significantly higher relative abundance in the reference samples. The final

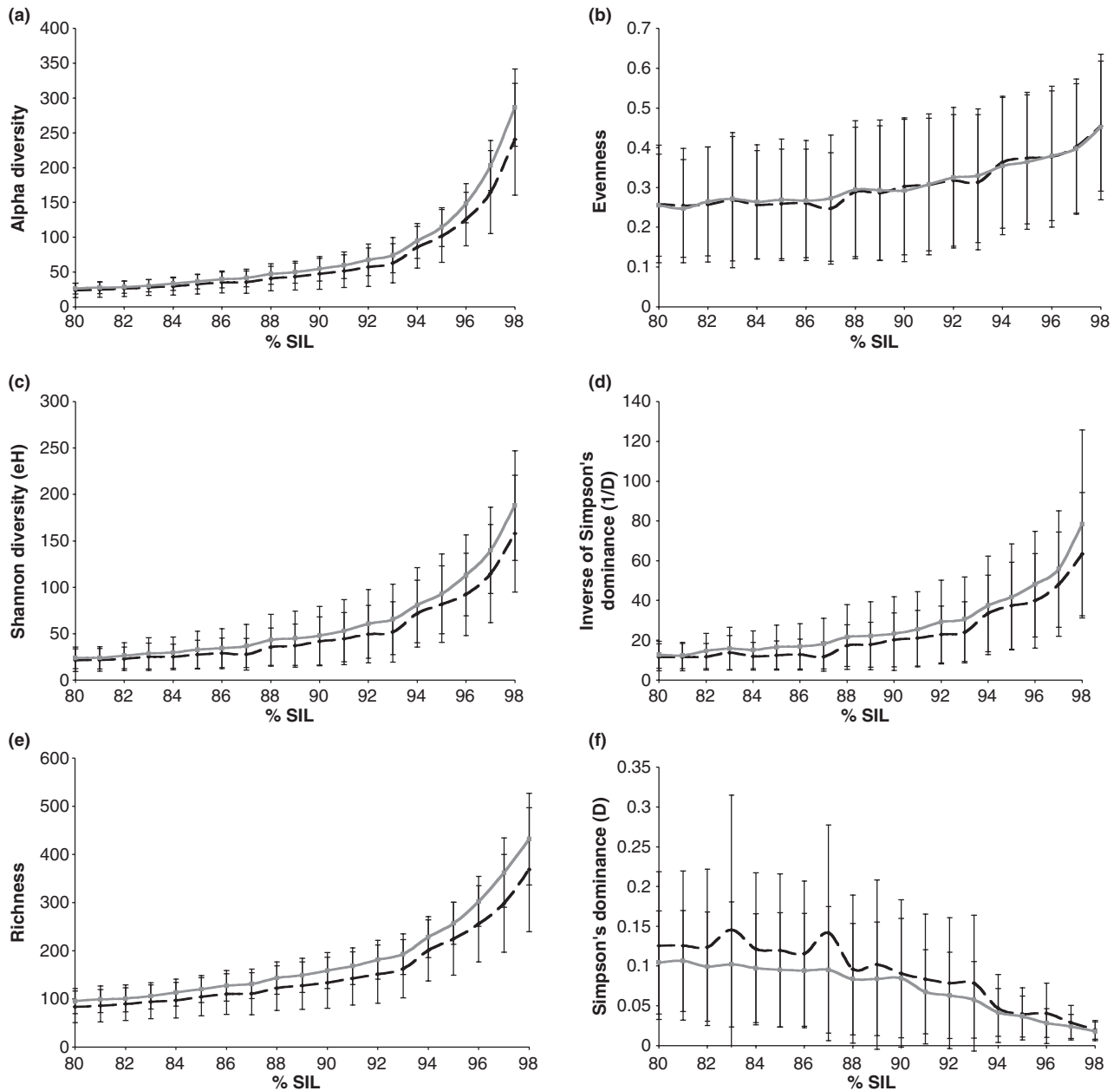


**Fig. 2** Bacterial community diversity in *P. maniculatus* (black dashed), *P. leucopus* (gray dashed) and soil (black solid). Diversity indices calculated from relative abundance data for OTUs at each % SIL used in this study (80–98% SIL) are shown. Means from *P. maniculatus*, *P. leucopus* and soil are included. Diversity indices are (a) alpha diversity, (b) species evenness, (c) Shannon diversity, (d) inverse of Simpson's dominance, (e) species richness and (f) Simpson's dominance. SDs are indicated with error bars.

bacterial taxon, identified as significantly different between reference and remediated sites, matched best to an uncultured *Ruminococcaceae* and was found at a higher relative abundance in reference sites. When we applied the more conservative significance threshold only two bacterial taxa remained significant, both were *Tannerella* sp. (OTUs 305 and 614, Table 4). Of the two *Tannerella* spp., one had significantly greater relative

abundance in mouse intestinal samples from the remediated site and the other had significantly higher relative abundance in the reference samples.

The analysis of soil bacteria identified seven taxa that were significantly affected by one of the main effects (treatment or habitat type) or their interaction using the less conservative significance threshold (Table 5). An OTU that best matched *Rhodococcus fascians* (SAB =



**Fig. 3** Comparison of *P. maniculatus* fecal bacterial community diversity in remediated and reference sites. Diversity indices calculated from relative abundance data for OTUs at each % SIL used in this study (80–98% SIL) are shown. Means from *P. maniculatus* collected in remediated (black dashed) and reference (gray) sites are included. Diversity indices are (a) alpha diversity, (b) species evenness, (c) Shannon diversity, (d) inverse of Simpson's dominance, (e) species richness and (f) Simpson's dominance. SDs are indicated with error bars.

0.949) was found to have two interaction terms that were significant, with approximately six-fold higher relative abundance in reference riparian sites than in either remediated riparian (6.1-fold) or reference upland sites (6.6-fold, Table 5). Also, an OTU that best matched a *Geodermatophilus* sp. had two significant interaction terms. This *Geodermatophilus* species relative abundance was significantly lower in reference upland soils than in

both reference riparian and remediated upland soils (Table 5). Another OTU, which best matched *Phenylbacterium immobile* (SAB = 0.952), had two significant interaction terms and had higher relative abundance in reference riparian soils than in both reference upland (2.2-fold) and remediated upland soils (1.5-fold). Interestingly, an uncultured *Acidobacteriaceae* GP4 sp. was found to significantly differ by treatment with higher



**Table 3** Bacterial species that differ significantly between *Peromyscus maniculatus* and *Peromyscus leucopus* fecal samples

OTU	Bacterial species	SAB score	Direction of difference	Fold difference	<i>P</i>	<i>q</i>
11	Uncultured Ruminococcaceae	1.00	M > L	0.8019	0.0085	NS
791	Unclassified Porphyromonadaceae	0.917	M > L	1.9011	0.0057	NS
1038	Unclassified Bacteria	0.87	M > L	1.8382	0.0055	NS

Using a mixed model analysis of variance on OTUs from the 98% SIL we have identified bacterial taxa that differ significantly ( $P < 0.01$ ) between *P. maniculatus* and *P. leucopus* fecal samples. Identity of the best hit from BLAST analysis querying consensus sequences for each OTU (at 98% SIL) against the ribosomal database (RDP), and corresponding % similarity score (SAB score) are shown. Direction of difference and fold difference between *P. leucopus* (L) and *P. maniculatus* (M) is indicated and corresponding *P*-values (*P*) from ANOVA and False discovery rate (*q*) are shown (NS =  $q > 0.1$ ).

**Table 4** Bacterial species that differ significantly between *Peromyscus maniculatus* fecal samples from reference and remediated sites

OTU	Bacterial species	SAB score	Direction of difference	Fold difference	<i>P</i>	<i>q</i>
305	Uncultured <i>Tannerella</i> sp.	0.805	Ref > Rem	2.9064	0.0002	0.024
614	Uncultured <i>Tannerella</i> sp.	0.755	Rem > Ref	3.6512	0.0012	0.072
665	Uncultured <i>Tannerella</i> sp.	0.821	Rem > Ref	0.4812	0.0092	NS
1171	Uncultured Ruminococcaceae	0.870	Ref > Rem	0.5641	0.0078	NS
1239	Uncultured <i>Tannerella</i> sp.	0.728	Ref > Rem	0.6147	0.0072	NS

Using a mixed model analysis of variance on OTUs from the 98% SIL we have identified bacterial taxa that differ significantly ( $q < 0.1$ ) between *P. maniculatus* fecal samples from reference and remediated sites. Identity of the best hit from BLAST analysis querying consensus sequences for each OTU (at 98% SIL) against the ribosomal database (RDP), and corresponding % similarity score (SAB score) are shown. Direction of difference and fold difference between remediated (Rem) and reference (Ref) is indicated and corresponding *P*-values (*P*) from ANOVA and False discovery rate (*q*) are shown (NS =  $q > 0.1$ ).

**Table 5** Bacterial species that differ significantly between soil samples according to treatment and habitat type

OTU	Effect	Bacterial species	SAB score	Direction of difference	Fold difference	<i>P</i>	<i>q</i>
257	T*H	<i>Rhodococcus fascians</i>	0.949	Ref-R > Rem-U	-6.0882	0.0003	0.08
257	T*H	<i>Rhodococcus fascians</i>	0.949	Ref-R > Ref-U	6.629	0.0005	0.09
369	T*H	<i>Phenylobacterium immobile</i>	0.874	Ref-R > Ref-U	2.2145	0.0027	NS
369	T*H	<i>Phenylobacterium immobile</i>	0.874	Ref-R > Rem-U	-1.5509	0.0065	NS
440	T	Uncultured <i>Acidobacteriaceae</i> GP4 sp.	0.952	Rem > Ref	3.3331	0.0009	0.09
440	T*H	Uncultured <i>Acidobacteriaceae</i> GP4 sp.	0.952	Rem-U > Ref-U	3.3331	0.0009	0.09
1006	T*H	<i>Geodermatophilus</i> sp.	0.893	Rem-U > Ref-U	2.216	0.0003	0.08
1006	T*H	<i>Geodermatophilus</i> sp.	0.893	Ref-R > Ref-U	1.7104	0.0003	0.08
1277	H	Uncultured <i>Opitutus</i> sp.	0.887	R > U	2.1746	0.0031	NS
1277	T*H	Uncultured <i>Opitutus</i> sp.	0.887	Ref-R > Ref-U	2.1746	0.0031	NS
1556	T*H	<i>Sphingomonas melonis</i>	0.967	Rem-U > Ref-R	1.8077	0.0065	NS
2120	H	Uncultured <i>Acidobacteriaceae</i> GP6 sp.	0.953	R > U	4.7801	0.0007	0.09
2120	T*H	Uncultured <i>Acidobacteriaceae</i> GP6 sp.	0.953	Ref-R > Ref-U	4.7801	0.0007	0.09

Using a mixed model analysis of variance on OTUs from the 98% SIL we have identified bacterial taxa that differ significantly ( $q < 0.1$ ) between soil samples from reference and remediated sites that are either riparian or upland habitat types. Identity of the best hit from BLAST analysis querying consensus sequences for each OTU (at 98% SIL) against the ribosomal database (RDP), and corresponding % similarity score (SAB score) are shown. Effect found significant Treatment (reference vs. remediated, T) and habitat type (riparian vs. upland, H). Direction of difference and fold difference between remediated (Rem), reference (Ref), Riparian (R) and Upland (U) is indicated and corresponding *P*-values (*P*) from ANOVA and False discovery rate (*q*) are shown (NS =  $q > 0.1$ ).

relative abundance in the remediated site soil, however this OTU also had a significant interaction of treatment and habitat type and it appears that the difference in treatment is driven by the interaction, as *Acidobacteriaceae* GP4 sp. had a threefold higher relative abundance in remediated upland than in reference upland soils. A similar trend was observed for an OTU that best matched an uncultured *Opitutus* species, which had a significant main effect of habitat with a two-fold higher relative abundance in riparian soil than in the upland habitat, however this was driven by the interaction where there was a two-fold higher relative abundance of this *Opitutus* species in reference riparian samples than in reference upland samples. Additionally, an OTU that best matched *Acidobacteriaceae* GP6 had exactly the same trend as *Opitutus* species, except that it was an almost five-fold difference between reference riparian samples and reference upland samples. Finally, an OTU that best matched *Sphingomonas melonis* had a significant interaction term in the ANOVA with a two-fold higher relative abundance of *Sphingomonas melonis* in remediated upland soil than in reference riparian soil (Table 5). Using the more conservative significance threshold four bacterial taxa (OTUs 257, 440, 1006, 2120) were significantly affected by treatment (reference vs. remediated), habitat type (riparian vs. upland) or their interaction.

## Discussion

The use of massively parallel sequencing has begun to drastically change the way in which we view microbial ecology. Genomic techniques have significantly increased the number of described bacteria species and shed light on the diversity inherent to bacterial communities. Here we utilized a bar-coded 454 massively parallel sequencing technique to investigate the effects of remaining contamination at the Tri-State Mining District on small mammal and bacterial communities. Specifically, we were interested in the effects of remnant heavy metal contamination (lead, zinc and cadmium) on rodent and soil bacterial communities and the bacterial communities present in the intestinal tracts of native rodents.

Residual contamination appeared to decrease rodent community species evenness. Although three rodent species were found each year of sampling, substantially fewer *P. leucopus* and *N. fucipes* were found on the remediated sites. The consistent and high abundance of *P. maniculatus* substantiates the results of Allen & Otis (1998) who attributed the higher abundance of *P. maniculatus* in contaminated habitat to its adaptive ability and affinity for highly disturbed habitats. Despite the high abundance, however, *P. maniculatus* exhibited poor

health conditions including lower body fat, as well as smaller body size and mass. While it is likely that direct effects of heavy metal toxicity played a role in the reduced fitness of animals on the remediated site, changes in the microbiota were apparent and could have also contributed to the overall decline in animal health.

Microflora community assemblage differed among remediated and reference sites and among rodent species. The identified bacteria were members of *Ruminococcaceae*, *Porphyromonadaceae*, Bacteroidetes and an unclassified bacterial taxon. These taxa are known to be common components of the gastrointestinal tract of mammals (Hertogh *et al.* 2006; Nam *et al.* 2008; Antonopoulos *et al.* 2009), and some members of *Ruminococcaceae* are known to contain genes whose expression promotes human health (Hattori & Taylor 2009). The decrease of *Ruminococcaceae* in animals from the remediated sites suggests that microbiota community structure could indeed influence the reduction in health and fitness of these animals. The fact that these bacteria would be present at significantly higher relative abundance in the gastrointestinal tract of *P. maniculatus* than in *P. leucopus*, may provide clues why *P. maniculatus* may be more resilient to contamination.

In addition to changes in abundance of *Ruminococcaceae*, two OTUs in the genus *Tannerella* (class Bacteroidetes) had reduced relative abundances in mouse intestines from remediated sites while two other OTUs in this genus increased in response to the same perturbation. These findings suggest that niche voids left by bacteria, which were unable to cope with the remnant levels of heavy metals were replaced by taxa that could persist in this environment. Thus diversity was conserved by replacement taxa, which filled the void left by the missing taxa. It is unknown if the change in community assemblage altered the health benefits derived from the microfloral community. Further studies are needed to resolve the relationship and possible mechanisms involved between heavy metal contamination and the role of microflora communities in the health and fitness of rodents.

Surprisingly we found no significant effects of heavy metal contamination or habitat type on soil or *Peromyscus* intestinal bacterial diversity. This result was not expected given previous studies on animals, plants and even microbes in the presence of heavy metal contamination. Additionally many studies of soil bacterial communities have shown that diversity is affected in response to multiple disturbances including prairie soil bacterial community response to nitrogen addition, altered burning regime, tilling for agriculture and deforestation (Baath *et al.* 1995; Marschner *et al.* 2003; Allison *et al.* 2005). In particular, other investigators have

also shown that bacterial diversity decreases as contamination increases (Waldron *et al.* 2009). Our findings illustrate that although diversity may not be affected by heavy metals, replacement of species may occur and drive changes in ecosystem assembly and function.

In addition to affecting ecosystem function, soil bacterial community can impact resident animal health in multiple ways. First, the environment is an important source of inoculants for beneficial and commensal intestinal bacteria (Belden & Harris 2007). Because soil bacterial communities are a source of infectious pathogens as well as beneficial microflora, differences in the environment could lead to different assemblages of microflora in the intestinal tracts of small mammals on sites with and without heavy metal contamination, leading to differential health of these animals. Second, bacteria are an important component of food webs and microflora in other organisms in the ecosystem, thus contaminate-induced changes to the soil bacterial community or the microflora of other community members could have adverse effects on the higher trophic levels through bottom-up effects.

Although soil bacterial community diversity did not differ between remediated and reference soil samples, we found significant differences in community assemblage of soil bacteria and identified seven bacterial taxa that responded to elevated levels of heavy metal contamination, habitat composition or their interaction (Table 5). Two genera of *Acidobacteriaceae* differed in relative abundance based on contamination levels and habitat types; most Acidobacteria are difficult if not impossible to culture so identifying the responses of these taxa is only possible through culture-independent techniques like the one described here. Additionally, we found that *Sphingomonas melonis* increased in response to higher levels of heavy metals. *S. melonis* is a known plant pathogen (Buonaurio *et al.* 2002) and could therefore have downstream effects on ecosystem health. Interestingly, all responsive bacterial taxa identified had significant interactions terms suggesting that the effects of remnant contamination depend on the specific environment in which they are present. This is an important observation and suggests that the effects of contamination are complex and their impact is not solely dependant on the concentration of the contaminant. Further studies as to the cause for the observed contamination by habitat interactions are needed to better understand these responses and for future management practices.

While responsive taxa were identified, some number of those identified could be false positives given the number of tests conducted and the relatively few taxa identified. It is possible that our study failed to identify more responsive bacterial taxa because we lacked the power to detect differences or that the bacterial commu-

nities are highly heterogeneous masking differences that exist. This is an important point for the design of massively parallel sequencing bacterial assessment studies. It is impossible to know beforehand the number of species and distribution of relative abundances of bacterial taxa, which are both important for proper depth of sequencing coverage and therefore sampling intensity. Additionally, there are important caveats to consider with any massively parallel sequencing study investigating microbial diversity and abundance. Importantly, a PCR amplification step is typically utilized to produce bar-coded sequences and this amplification could introduce biases, produce chimeric sequences or introduce sequence changes. And because of the magnitude of these studies removing these problems is not trivial and in general difficult to identify. Additionally, the method of alignment and assignment of OTUs can influence the results and because there are a multitude of methods and no consensus yet on the best way to do it, it is best to view the results of these studies cautiously. For studies like this one where short sequencing reads were obtained the above concerns are quite important as taxonomic assessment with short reads is difficult. Despite these shortcomings, bar-coded massively parallel sequencing assessment of bacterial communities has already begun to change the way microbial ecology is done. With further advances in the sequencing technology and methods for analysis more discoveries and understanding of the complex microbial world are soon to come.

This study combined emerging techniques with traditional ecological measures of community diversity to determine if there is a link between community, organismic and physiological responses to residual heavy metal contamination. Ecosystem services can be severely impaired by heavy metal toxicity as has been previously shown. Our study demonstrates that ecosystem integrity and function extends not only to community and organism level responses but also across ecosystems into the complex and still underexplored ecosystem of mammalian microflora.

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## Conflicts of interest

The authors have no conflict of interest to declare and note that the sponsors of the issue had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Comparison of soil bacterial community diversity in remediated and reference sites

Diversity indices calculated from frequency (of occurrence data for OTUs at each % SIL used in this study (80–98% SIL) are shown. Means from soil collected in remediated and reference sites are included. Diversity indices are A) Alpha Diversity E) Species Evenness C) Shannon Diversity D) Inverse of Simpson's Dominance E) Species Richness and F) Simpson's Dominance. SD is indicated with error bars.

**Fig. S2** Comparison of soil bacterial community diversity in riparian and upland sites Diversity indices calculated from frequency of occurrence data for OTUs at each % SIL used in this study (80 - 98% SIL) are shown. Means from soil collected in riparian and upland sites are included. Diversity indices are A) Alpha Diversity B) Species Evenness C) Shannon Diversity D) Inverse of Simpson's Dominance E) Species Richness and F) Simpson's Dominance. SD is indicated with error bars.

**Table S1.** Primers for bar-coded massively parallel sequencing (bMPS) were produced by adding unique barcode sequences

(underlined) between the "A" sequencing primer of Margulies *et al.* (2005) and the reverse 16S primer U529R (bold) of Watanabe *et al.* (2001). As sequencing was done in only the reverse direction, no barcode was necessary within the "B" construct.

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