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Loss of microbial diversity in soils is coincident with reductions in some specialized functions

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Summary

Loss of microbial diversity is considered a major threat because of its importance for ecosystem functions, but there is a lack of conclusive evidence that diversity itself is reduced under anthropogenic stress, and about the consequences of diversity loss. Heavy metals are one of the largest, widespread pollutant types globally, and these represent a significant environmental stressor for terrestrial microbial communities. Using combined metagenomics and functional assays, we show that the compositional and functional response of microbial communities to long-term heavy metal stress results in a significant loss of diversity. Our results indicate that even at a moderate loss of diversity, some key specialized functions (carried out by specific groups) may be compromised. Together with previous work, our data suggest disproportionate impact of contamination on microbes that carry out specialized, but essential, ecosystem functions. Based on these findings, we propose a conceptual framework to explicitly consider diversity of functions and microbial functional groups to test the relationship between biodiversity and soil functions.

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Introduction

Microbial communities are the most diverse and dominant group of organisms, and they play a key role in ecosystem functions including biogeochemical cycling. They determine ecosystem health and play an important role in shaping the earth's climate as well as the structure and function of plant and animal communities (DeLong *et al.*, 2006; Dinsdale *et al.*, 2008; Galand *et al.*, 2009). Understanding microbial community structure, diversity and metabolic potential is essential for both the fundamental knowledge of these functions and for applied research to protect and manage ecosystem services as well as to discover useful biotechnology products (Dinsdale *et al.*, 2008). It is crucial, therefore, to understand how terrestrial microbial communities will respond to anthropogenic stressors in order to understand the long-term consequences of management of our land for sustaining ecosystem services. In addition, the effect of different stressors can alter the community. Many stressors such as organic and inorganic contaminants vary in their bioavailability over time as a result of ageing and equilibration effects. Therefore, it is essential to evaluate the sensitivity of the microbial community to stressors in long-term experimental set-ups under typical field conditions.

Heavy metals are one of the largest widespread pollutant types globally, and because of their known toxicity to micro-organisms at high concentrations, metal pollution represents a significant environmental stressor for terrestrial microbial communities. Significant research has been carried out to assess the impacts of heavy metals on soil microbial communities and some processes (see review by Giller *et al.*, 2009), but little is known on the quantitative impact on soil biodiversity (Torsvik and Ovreas, 2002) and the consequences for key ecosystem functions. In a landmark study, Gans and colleagues (2005) suggested that more than a million distinct bacterial genomes were

present in pristine soils, and this diversity was reduced by 99.9% as a result of heavy metal pollution. The authors utilized DNA re-association data from previous work (Sandaa *et al*., 1999) and power law to best describe the abundance distribution. They concluded that previous work had substantially underestimated species diversity. However, these papers are the only studies that used the rigour of statistical and computation power to show loss of species richness because of an anthropogenic stress, and even here, the results of Gans and colleagues are contested (Volkov *et al*., 2006). Recent studies have also highlighted the limitations and uncertainties of available estimates of species richness, and it was argued that these uncertainties are because, largely, of the way the statistical tools are used in biodiversity studies (Hong *et al*., 2006). The advances in high throughput sequencing have started to confirm an immense and unseen majority of microbial communities within soils. However, such approaches mainly target the 16S rRNA gene for diversity work which has limited the ability to predict functions. It has been argued that functional community composition is probably a more sensitive determinant of stress response than phylogenetic surveys (Cowan, 2009). A combined approach to shotgun sequencing for whole metagenome analysis has also been attempted with varying degrees of success (Hemme *et al*., 2010; Temperton and Giovannoni, 2012).

Although important to understand the portion of the microbial diversity that is affected, there remains an extensive debate about how such losses in diversity are related to resistance, resilience and redundancy of microbial functions (Bell *et al*., 2005; Allison and Martiny, 2008). Relationships between microbial diversity and soil functions remain a topic of debate. Functional redundancy in soil micro-organisms are considered prevalent; however, some functions such as pesticide degradation are known to be limited to only in a few specialized functional groups. Therefore, it is argued that even a moderate loss of diversity may impact key specialized functions. However, experimental evidence to support this in field conditions is lacking (UK National Ecosystem Assessment, 2011). Here, we combined metagenomic, molecular, biochemical and physiological approaches to understand the long-term ecological response of microbial communities to environmental stressors by analysing samples from two long-term (11-year-old) experiments which have received the same treatments and heavy metal inputs but differed in soil properties and consequently heavy metal bioavailability. Our aims were to: (i) identify the magnitude of impact from long-term metal contamination on soil microbial diversity, (ii) examine which soil functions were compromised because of modest loss in biodiversity and (iii) explore if specialized microbial groups were disproportionately impacted by the loss of diversity.

Results and discussion

To assess the impact of metal stressors on the diversity and resilience of microbial communities over the long term, we investigated two field sites: Hartwood (HW) and Auchincruive (AC). At each site, the same wastewater sludge, rich in different heavy metals, had been applied in identical replicated designed experiments.

We chose these sites because (i) they have been comprehensively characterized as part of a larger study throughout the UK (Gibbs *et al*., 2006) and have been the focal point of annual response measures since 1997. This has provided high-quality chemical, physical and biological data and an opportunity to present our genomic studies in an appropriate context. (ii) As land application of wastewater sludge has been a common practise in the past and is accelerating because of pressures on sea disposal, the sites typifies many of the trade offs we have to make in land management. On one hand, there are significant ecological benefits in recycling the organic matter and nutrients, especially when many nutrients are becoming scarce (e.g. phosphorus). On the other hand, if unregulated, anthropogenic contaminants such as heavy metals and organic contaminants that are often present in sludge can build up to harmful levels in soil. (iii) Previous reports from these sites have suggested a significant decrease in a soil function, namely symbiotic nitrogen fixation (Chaudri *et al*., 2008). (iv) There has been extensive debate within the scientific community on the sensitivity of microbial communities to distinct environmental pressure compared with temporal variability and spatial heterogeneity under typical field conditions (Black *et al*., 2008). We have evaluated the sensitivity of microbial communities to environmental stressors at the sites to differentiate the microbial response from spatio-temporal heterogeneity under field conditions by sampling quarterly (seasonally) within a year for response stability. This provided strong evidence that the soil micro-organisms response to large-scale environmental gradients was consistent and easily interpretable from the minor spatial and temporal variability (Black *et al*., 2011).

The details of field sites and treatments are provided in the supplementary information. Briefly, at HW and AC, field plots received annual additions of Cu- and Zn-contaminated sludge to achieve two target concentrations (50 and 200 mg kg^{-1} for Cu and 150 and 450 mg kg^{-1} for Zn) from 1994 to 1997. Appropriate controls [i.e. no sludge treatment (Blank) and uncontaminated (raw and digested) sludge] were maintained throughout the project. Soils were sampled ($n = 42$) in 2008 and analysed for physiological (respiration and pesticide mineralization as measures of general and specialized functions, respectively), biochemical [phospholipid fatty acid (PLFA)], molecular [terminal restriction fragment length polymorphism (TRFLP)]

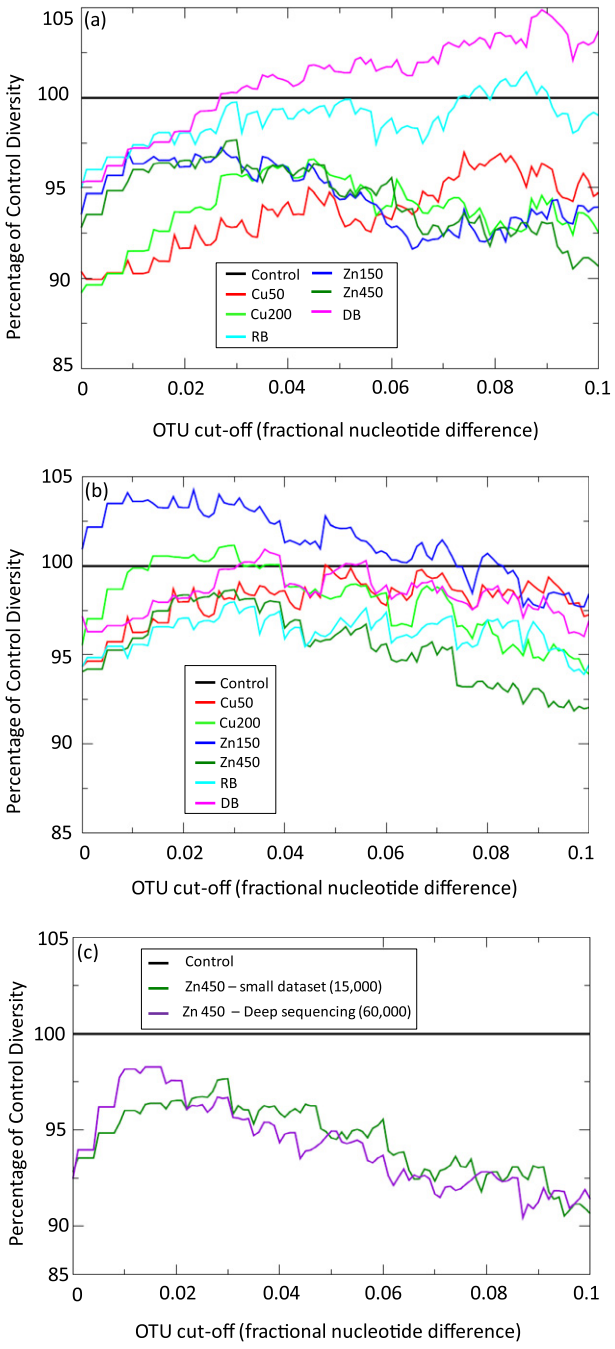


Fig. 1. Impact of heavy metal (Zn and Cu)-contaminated sludge on soil microbial diversity based on 454 sequencing of 16S rRNA genes. Observed OTU number as a function of fractional sequence difference as a percentage of the control OTU number (black line) for the contaminated soil data Cu50 is 50 mg kg^{-1} Cu (red), Cu200 is 200 mg kg^{-1} Cu (light green), Zn150 is 150 mg kg^{-1} Zn (dark blue), Zn450 is 450 mg kg^{-1} Zn (dark green), raw blank is in light blue) and digested blank is in magenta. For (A) Hartwood ($n = 21$), reduced species richness is observed in contaminated soils across all cut-offs. For (B) Auchincruive ($n = 21$), reduced species richness was observed at smaller OTU cut-offs. (C) Hartwood Zn450 deep-sequenced data from which results are subsampled to 60 000 reads (purple) which gave remarkably similar results for the reduction in diversity as a function of cut-off for the original samples.

and metagenomic (GeoChip and 454 pyrosequencing) response of microbial communities. Additionally, a range of soil abiotic properties including total and extractable metal concentrations was measured (see Supporting Information Appendix S1).

Pyrosequencing data were generated and processed for all samples (Supporting Information Appendix S1). The number of reads and number of observed operational taxonomic units (OTUs) at each resolution were obtained (Supporting Information Table S1). To correct for the different read numbers, we calculated the mean observed OTU number at each cut-off and subsampled to 15 000 reads (Supporting Information Table S2). Data analysis suggested that the effect of treatment was site specific, but the trend was similar at both sites. For HW soils, there was a small but statistically significant and consistent (5–10%) reduction in OTU richness in metal-contaminated soils at the 1.5% nucleotide cut-off. For the OTUs at higher cut-offs, some reduction in number was still observed but at a smaller magnitude. Similarly for AC soils, a reduction in unique OTU diversity under treatment was evident for all except the Zn150 treatment, but it was not consistent for all cut-off levels. (Fig. 1). For HW, the contaminated soils showed reduced OTU richness consistently across all cut-offs with a reduction that was greater than for the digested controls (Fig. 1A). For site AC, the reduction was observed mainly at small cut-offs and with a magnitude that was not statistically significantly different to the blank samples (Fig. 1B).

For comparing the OTU number with a diversity index, we express the Shannon entropy in number equivalents, S(H) = exp(H), the number of OTUs which if evenly distributed would have the same Shannon entropy as the sample (Supporting Information Table S3). Interestingly, for the HW site, we found a reduction in Shannon entropy with metal contamination just as for OTU number, but it was at a slightly larger magnitude, suggesting that contamination reduced not only the number of taxa (richness) but resulted in a more uneven distribution of those that remain. For the AC site, we also found a consistent reduction in Shannon entropy across OTU cut-offs with metal contamination for all except Zn150. This suggested that although taxa richness was not reduced significantly for this site, evenness was. These conclusions were confirmed by plotting the effect of cut-off on H(S) graphically (Supporting Information Fig. S1). We recognized that our sample size would not be large enough to capture the total OTU number in these communities, as confirmed by the rarefaction curves shown for the 3% and 5% OTUs (Supporting Information Fig. S2). Therefore, to estimate the true number of OTUs present in each sample, we used parametric methods as these are less sensitive to small sample sizes (Hong *et al.*, 2006) than non-parametric estimators such as Chao's (Chao *et al.*, 1993). We fitted four different types

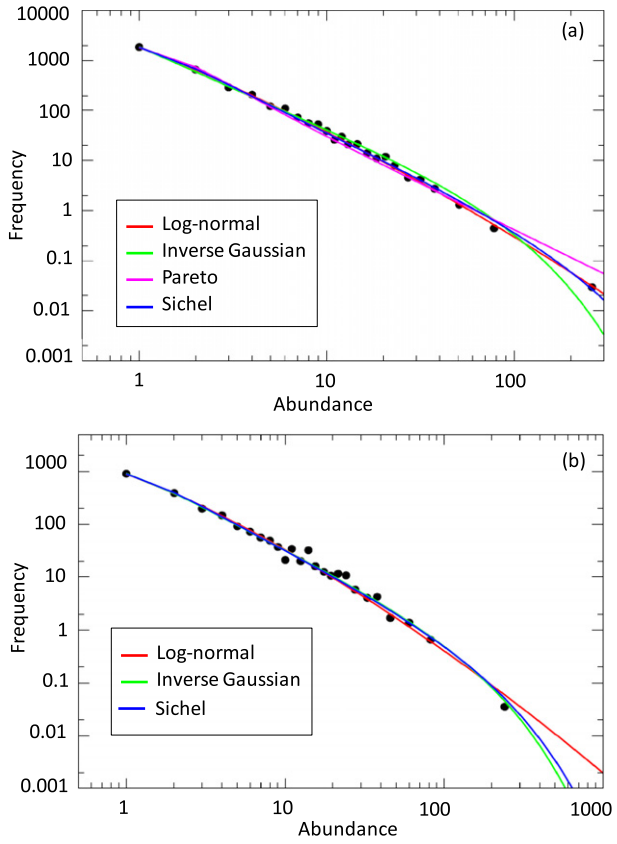


Fig. 2. Frequency–abundance curves for treatment 1 (HW – Control) (A) 3% OTUs on log–log axes; (B) 5% OTUs on log–log axes. Data at high abundances are aggregated to a count of at least 20 to reduce noise. Fits are means of the posterior distribution of expected abundances for log-normal (red line), inverse gaussian (light green line), Pareto (magenta line) and Sichel (blue line) parametric models.

of distribution to the abundances (log-normal, Inverse Gaussian, Pareto and Sichel) and extrapolated to the total diversity. The choice of the four parametric forms for the abundance distributions was based on previous observations that the log-normal and Sichel can fit complete microbial abundance distributions (Quince *et al.*, 2008), the inverse Gaussian, which is a special case of the Sichel, was included because it has been used in other studies (Hong *et al.*, 2006), and the Pareto to allow comparison with previous work (Gans *et al.*, 2005).

We used a Bayesian fitting method, which allowed realistic predictions of uncertainty in our inferred total OTU numbers. Examples of the fits to OTUs at the 3% and 5% cut-off for treatment 1 (AC – Control) are shown in Fig. 2. From these, it is apparent that the best fits are obtained using the Sichel and log-normal distributions. This is supported by the deviance information criterion values (Supporting Information Table S4) from which we observed that in the majority of treatments, the best-fitting distribution was the log-normal but that it was mostly not significantly superior to the Sichel distribution. There was still an effect of metal contamination on microbial diversity even at 3% and 5% cut-off levels (Supporting Information Table S5) which confirmed our above observation. To estimate how much more sampling might be necessary to capture the majority of the diversity and definitively determine the OTU number in these treatments, we calculated the sample sizes necessary to obtain 90% (Supporting Information Table S6), and 50% (Supporting Information Table S7) of the total diversity for OTUs at the 3% and 5% cut-off levels. Unlike the total diversities which varied by a factor of two or three between the two distributions, these estimates showed much more variation with the order of a million reads necessary to get 90% at the 3% OTU diversity in the case of the log-normal. If the Sichel applies, then perhaps only half a million reads might be needed. These numbers reduced substantially if coverage included only 50% of the OTUs or at the 5% cut-off.

To better determine the absolute OTU numbers in the soil samples and verify the observed reduction in OTU number in the HW samples, we re-sequenced samples from the HW control and Zn450 treatments in greater depth. Sixty thousand reads (Supporting Information Table S8) from each treatment were used for further analyses. This gave remarkably similar results for the reduction in diversity as a function of cut-off (Fig. 1C) as we observed for our original samples, giving us confidence in those predictions. Although a significant reduction in OTU number was observed in metal-contaminated soils, it was nowhere near as dramatic as the 99% reduction in taxa number proposed earlier (Gans *et al.*, 2005) in soils contaminated with similar concentrations of heavy metal.

Response of microbial biomass, structural and functional community composition to environmental stressors

Each OTU was assigned phylogenetic affiliation using the Ribosomal Database Project (RDP 10) database for each replicate. Data (from Phyla to Genera level) were analysed across sites and treatments by (analysis of variance) ANOVA as well as using multivariate approaches [principal component (PCA) and canonical variate analysis (CVA)]. These analyses confirmed that the community composition of the two sites was very different. Effects on individual taxa (from phyla to genera levels) were also tested. PCA demonstrated clear shifts in community composition across treatments for both sites across all phylogenetic levels (Supporting Information Table S9). At the phylum level, there was a little consistency in the direction or magnitude of response to treatment at both sites (Supporting Information Table S10). However, at higher levels of phylogenetic resolution, a number of

taxa responded similarly to treatments in both sites in terms and direction of effects (Supporting Information Table S10). For example, at the class level, *Acidobacteria GP1*, *GP2* and *GP3*; *Clostridia*; *Gemmatimonates*; and *Holophage* all showed a linear increase in relative abundance to Zn and to a lesser extent Cu at both sites, whereas others such as *Calidineae* and *Verrucomicrobia* were negatively impacted by HM at both sites (Supporting Information Table S10). At the order level, the relative abundance of *Gemmatimonadeles* and *Xanthomonadales* responded positively to both Zn and Cu at both sites, whereas *Syntrophobacteriales* was negatively impacted by HM (Supporting Information Table S12). At the family level, the relative abundance of *Peptostreptococcae* showed a linear increase to Zn (Supporting Information Table S10), and there was a large degree of response to metal treatment at the genus level (Supporting Information Table S10), indicating adaptation for some genera to high HM concentrations (Gubry-Rangin *et al.*, 2011). For example, the relative abundance of *Acidobacteria GP1*, *GP2* and *GP3* responded positively to both Zn and Cu treatment. In the case of GP1, the increase under Zn treatment was substantial (twofold). *Bradyrhizobium* increased in Zn-treated soils. This finding is interesting as significant decreases in symbiotic association between clover and *Rhizobium leguminosarum* (Chaudri *et al.*, 2008) and other *Rhizobium* species (Macdonald *et al.*, 2011a) have been reported from these sites. The Zn treatment had a negative impact on several taxa (e.g. *Caldilinea*, *Longilinea*, *Rhodoblastus*). To differentiate treatment effects on individual OTUs from compositional effects, we used two approaches. First, we used the SparCC (Friedman and Alm, 2012) approach which suggested a mild compositional effect mainly because of high alpha diversity in our dataset. Secondly, we used relative abundance of dominant taxa as an additional predictor in a multiple regression model with the taxon of interests as response (data not included). Both approaches supported results from ANOVA. These observations were further supported by multivariate analysis, which demonstrated a clear effect of treatment on community composition as assessed at the genus (Fig. 3A and B) and class level (Fig. 3C). These results also illustrate that there were stronger treatment impacts on community composition rather than diversity per se.

Terminal restriction fragment length polymorphism (T-RFLP) data generally agreed with 454 sequencing. There were significant and consistent changes in bacterial community composition in metal-treated soils (Supporting Information Fig. S3) with a strong influence of metal on community composition as demonstrated by regression analysis of PC scores against environmental variables which is supported by previous observations from long-term sites with comparable level of metal contamination

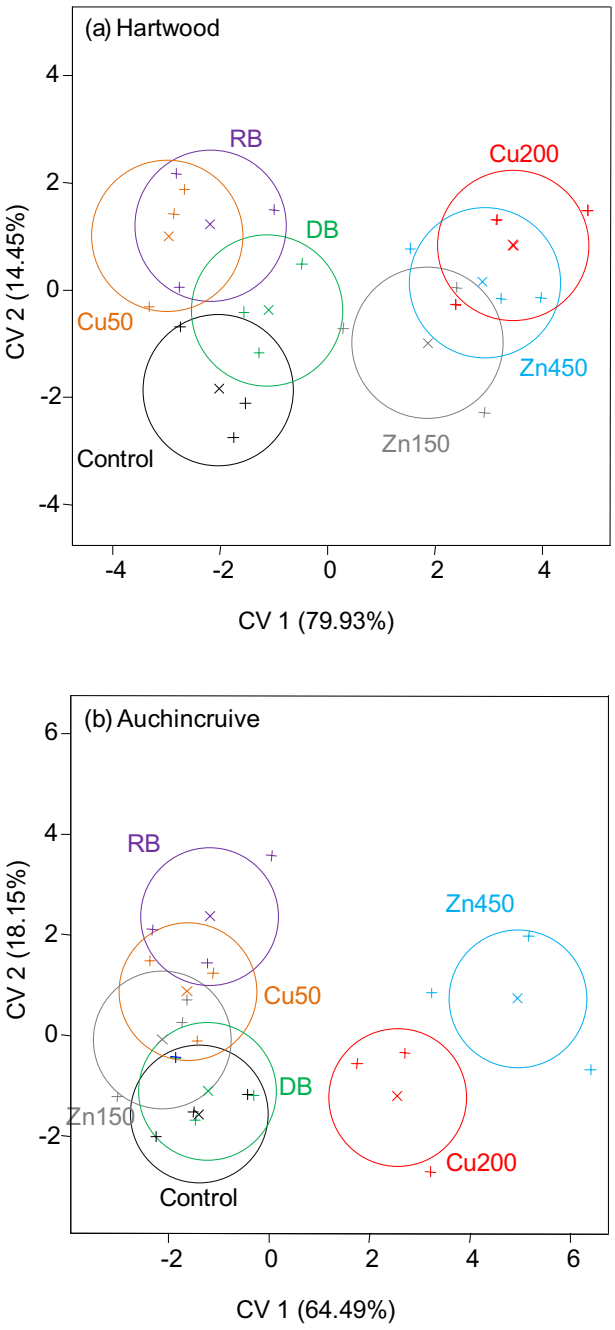
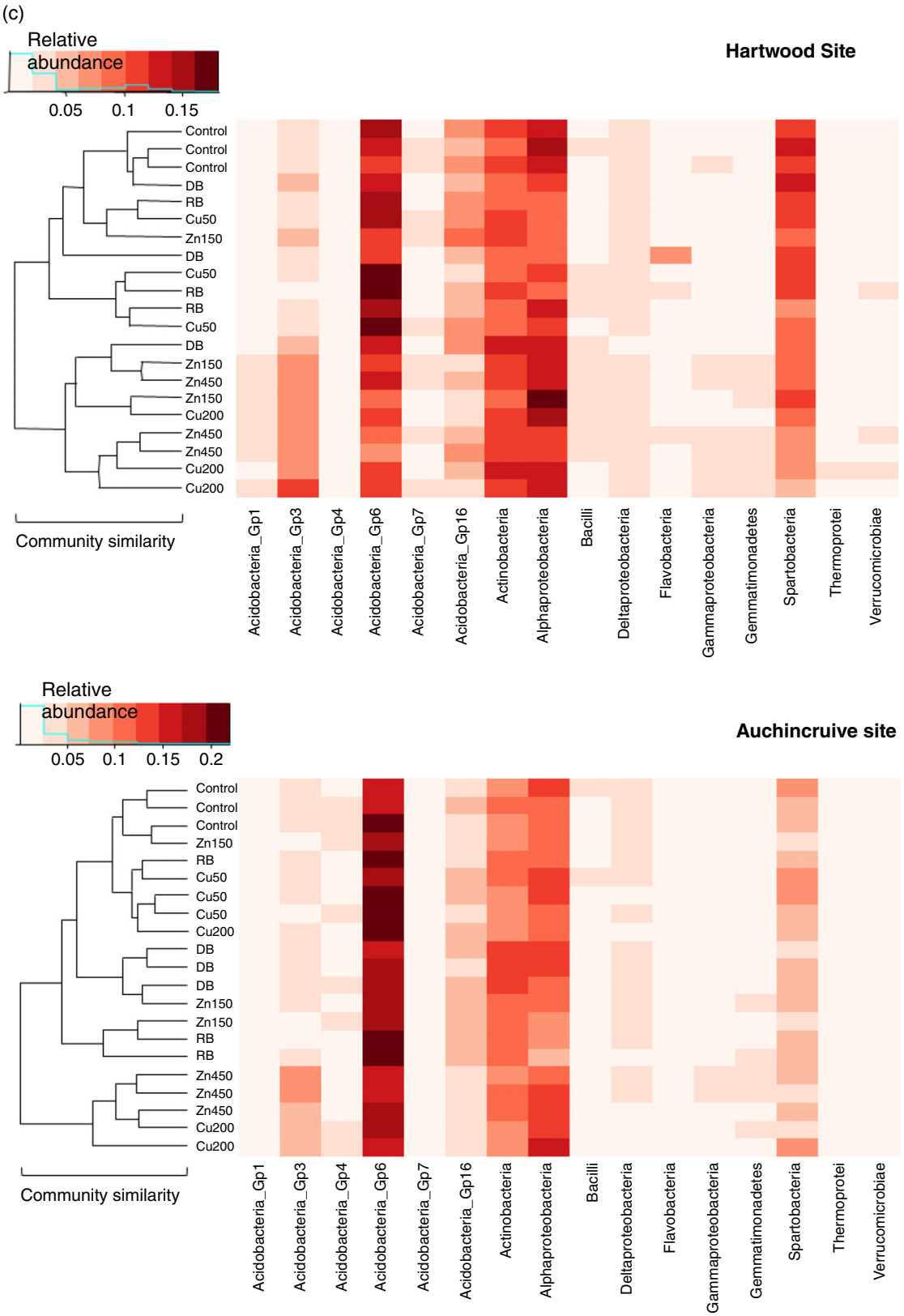


Fig. 3. Impact of heavy metal (Zn and Cu)-contaminated sludge on soil bacterial community structure based on 454 sequencing of 16S rRNA gene. Data are presented as ordination plots of canonical variates (CV) generated by CV analysis of genus level pyrosequencing data (A) Hartwood ($n = 21$) and (B) Auchincruive ($n = 21$) exposed different levels of sludge associated Zn and Cu stress. Control is no-sludge control (black); DB is digested blank sludge (green); RB is raw blank sludge (purple); Cu50 is 50 mg kg^{-1} Cu (orange); Cu200 is 200 mg kg^{-1} Cu (red); Zn150 is 150 mg kg^{-1} Zn (grey); Zn450 is 450 mg kg^{-1} Zn (blue). Percentages in parenthesis are percentage variance explained by each CV. Circles are 95% confidence intervals. (C) Dendrogram showing the impact of heavy metals on soil bacterial community at class level, and relative abundance of each class is displayed in heat map representation.



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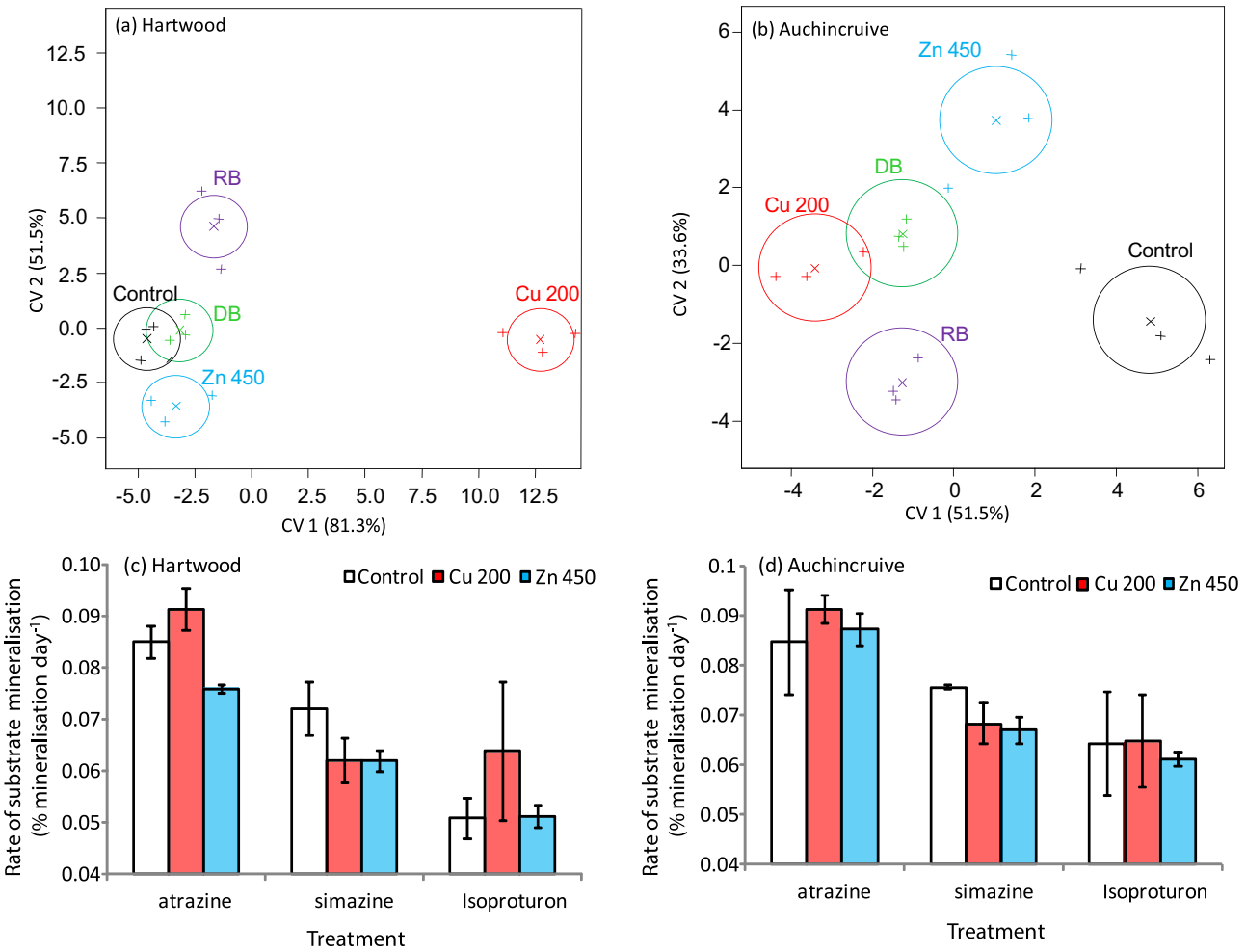

Fig. 4. Impact of heavy metal-contaminated sludge on soil bacterial functional community based on GeoChip analysis (A and B) and pesticide mineralization rates (C and D). Functional gene data are presented as ordination plots of canonical variates (CV) generated by CV analysis of functional genes (GeoChip) profiles in two grassland soils (A) Hartwood ($n = 15$) and (B) Auchincruive ($n = 15$). Control is no sludge control (black); DB is digested blank sludge (green); RB is raw blank sludge (purple); Cu200 is 200 mg kg^{-1} Cu (red); Zn450 is 450 mg kg^{-1} Zn (blue). Percentages in parenthesis are percentage variance explained by each CV. 'X' represent treatment means, '+' represents each replicate and circle represent 95% confidence intervals. The impact of Zn- and Cu-contaminated sludge additions on mineralization rates of the pesticides atrazine, simazine and Isoproturon in (C) Hartwood and (D) Auchincruive soils. Bars are ± one SE ($n = 3$).

(Macdonald *et al.*, 2007; 2008; 2011b). This provides direct evidence that these effects of metal contamination are consistent across time and space. For the AC site, the relationship between PC scores and metal concentrations was weaker, which is in accordance with the general observation that community response to metal stress at AC was not as strong as that observed for HW (Fig. 3C, 4A and B; Supporting Information Fig. S3). This may be explained by the lower concentration of available Zn in AC soils because of the inherent soil properties (Supporting Information Fig. S4). Nevertheless, the direction of change in both sites was similar. PLFA analysis suggested no effect of heavy metals or sludge treatment on the size of the microbial biomass, with the exception of a strong decline (> 50–80%) in the NFLA 16:1w5 in soils with highest metal treatments (Supporting Information Table S11). This is a significant finding as the NFLA 16:1w5 represents arbuscular mycorrhizal (AM) fungi, a functionally important species in grassland soils. Copper is a well-known fungicide (Pickering, 1912). Interestingly, our data suggest a comparable impact of Zn contamination on AM fungi, suggesting a disproportionate and selective impact of both HMs on this key functional group.

To examine the impact of treatment on functional communities, we used a functional gene array GeoChip 3.0 that covered 57 000 genes, including genes associated with carbon, nitrogen, phosphorus and sulphur cycles as well as antibiotic resistance, energy metabolism, antibiotic resistance and organic contaminant degradation (He *et al.*, 2010). The composition of functional genes

Table 1. Percentage variance explained by each principal component across different gene categories as assessed by GeoChip 3.0 functional gene array and MANOVA of principal component scores in two soils exposed to Zn- and Cu-contaminated sludge addition for two sites, Hartwood and Auchincruive.

		Site			
		Hartwood		Auchincruive	
Gene category	Principal component	% Variance explained by each PC	MANOVA of PC scores	% Variance explained by each PC	MANOVA of PC scores
All genes	1	45.06	$P < 0.001$	41.71	$P < 0.001$
	2	11.73		16.35	
	3	7.22		8.99	
Antibiotic resistance	1	33.73	$P < 0.001$	34.54	$P < 0.001$
	2	14.93		22.37	
	3	10.26		9.39	
Metal resistance	1	46.28	$P < 0.001$	45.24	$P < 0.001$
	2	11.12		15.97	
	3	7.59		8.31	
Aromatic compound degradation	1	47.98	$P < 0.001$	44.25	$P < 0.001$
	2	11.62		15.46	
	3	7.42		8.16	
Energy processes	1	36.50	$P < 0.001$	32.16	$P < 0.001$
	2	14.10		23.45	
	3	10.31		10.55	
C-cycling	1	47.04	$P < 0.001$	44.97	$P < 0.001$
	2	12.17		13.71	
	3	8.08		10.54	
N-cycling	1	48.52	$P < 0.001$	44.25	$P < 0.001$
	2	11.13		14.22	
	3	6.90		9.81	
P-cycling	1	55.79	$P < 0.01$	56.45	$P < 0.001$
	2	10.14		15.20	
	3	9.80		12.62	
S-cycling	1	48.15	$P < 0.001$	38.82	$P < 0.001$
	2	10.72		17.64	
	3	7.59		9.87	

MANOVA, multivariate analysis of variance.

responded similarly to structural community composition where treatment had a highly significant impact on the functional community (Fig. 4A and B) and mirrored the change in taxonomic composition where almost all functional groups (involve in C, N, P, S, antibiotic resistance, metal reduction/resistance) dramatically shifted in response to metal treatment (Supporting Information Figs S5 and S6, Table 1). This further confirms the above finding of a large impact on community composition.

Response of soil functions to environmental stressors

Functional activities (basal respiration, glucose and pesticide mineralization) were tested using MicroResp™ (Macaulay Scientific Consulting Ltd, UK) (Campbell *et al*., 2003) (Supporting Information Appendix S1). Basal- and substrate-induced respiration was unaffected by HM at both sites (Supporting Information Table S12, Fig. S7) indicating that changes in diversity and community composition have not had long-term impact on the measured broad metabolic functions. For specialized functions, we used pesticide mineralization capacity of soils as these functions are often more limited to a few bacterial species. We chose three pesticides (atrazine, simazine and isoproturon) as those widely used in soils historically. Simazine mineralization was significantly impacted in Cu- and Zn-treated soils at both sites (Supporting Information Table S12, Fig. 4C and D), and there was a trend (but not statistically significant) towards reduced mineralization rates of atrazine in Zn-treated soils (Supporting Information Fig. S7) at the AC site, suggesting weak evidence of a negative impact of Zn on this specialized function. This decrease in mineralization ability was accompanied by a change in the pesticide-degrading communities measured by GeoChip (Supporting Information Fig. S8; Table S13). These findings provide direct evidence that general functions such as community respiration and broad metabolic function remained resilient in these stressed ecosystems, but some key specialized functions (that are more restricted within a few taxa) such as pollutant mineralization were compromised. Although statistically significant, our data were not consistent across the two sites. For example, the trend in triazine-degrading

functional genes showed a weak difference between Zn450 and control samples at the HW site but a significant difference at the AC site (Supporting Information Fig. S8), although functions were compromised at both sites. Therefore, we suggest further experiments with manipulated diversity may be needed to provide more conclusive evidence. Such experiments should explicitly consider: (i) serial dilutions of diversity and its relationship with ecosystem function (Crawford *et al*., 2012); (ii) the shape of the biodiversity and function relationship (i.e. linear or saturating) (Reich *et al*., 2012); and (iii) differences in bioavailability of stressors (Girvan *et al*., 2005). Nonetheless, our data suggest that some specialized function (simazine degradation) and biomass of a key functional and specialized group (AM fungi) were compromised. Together with previous reports of inhibition of symbiotic nitrogen fixation and Rhizobia populations from these sites (Chaudri *et al*., 2008; Macdonald *et al*., 2011a), our data suggest that HM contaminations may have had a disproportionally greater effect on some measured key functional groups with specialized functions.

Perspective and conclusions

Understanding the role of soil microbial communities in biodiversity, ecosystem function relationship is made more complex because of the diversity of functions mediated by microbes, whether functions are rare or common, whether they are mediated by specialized groups or general across the community, as well as differences in the properties of soils that control bioavailability of nutrients and toxins. For example, organic matter decomposition is carried out by a large number of microbial species, and community-level respiration often remains unchanged in diversely different communities, whereas symbiotic N_2 fixation/xenobiotic degradation capability is restricted to a few specialized species. Consequently, diversity loss will have a greater effect on some functions than on others. We find moderate but significant reductions in diversity because of long-term HM contaminations, which was linked to loss in some specialized functions. Genomic approaches used in this study provided strong evidence of clear shift in phylogenetic and functional microbial communities and synergy between structural and functional communities. There is a clear selection of some phylotypes over others and the apparent loss of some genera because of metal contamination, which is coincidental with changes in functional groups and measured functional processes. Both structural and functional communities at the two sites were remarkably different. Despite this, the relative abundance of certain phylogenetic taxa showed similar responses to HM at both sites, providing correlative evidence for consistent and generic niche adaptation in these microbial taxa. This study therefore provides some useful empirical data for testing and developing ecological theories for the relationship between microbial diversity and function in a manner more similar to plant and animal ecology (Tilman *et al*., 2006; Reich *et al*., 2012). Furthermore, our result demonstrates that overall, general gross metabolism (respiration) is relatively stable in both soils despite the impact of HM on the communities responsible for these functions. Additionally, we provide strong evidence that microbial communities show a low level of resistance to HM contamination, and even > 10 years after the initial perturbation, the communities show a distinct composition from non-perturbed communities, which support previous work (Allison and Martiny, 2008). Further, our results provide statistically significant evidence and suggest that some key specialized functions were compromised, even at a modest loss of diversity. Together with significant loss of AM fungal biomass and previous reports of loss of N_2 fixation and N_2-fixing communities, our data indicate a disproportionate impact on the microbes that carry out some specialized functions. Overall, this observation provides evidence that loss of diversity is linked to loss of some specialized functions. There is a need, therefore, to explicitly include functional groups and diversity of functions in future research where impacts on biodiversity and consequences for functions are investigated to support the integration of this knowledge in conservation and environmental policies.

Experimental procedure

Field sites

The two field sites, HW and AC, represent two ryegrass grasslands where plots received annual additions of heavy metal (Zn or Cu)-contaminated sludge between 1994 and 1997 (Gibbs *et al*., 2006). Full details of the sites can be found in Supporting Information Appendix S1. Briefly, experimental treatments were in triplicate in a randomized block design and included control (no sludge); a raw blank (RB) treatment representing uncontaminated sludge equivalent to the undigested Cu-rich sludge; two undigested Cu-rich treatments (Cu50 and Cu200) aimed at achieving soil concentrations of 50 and 200 mg Cu kg^{-1} respectively; and a digested blank (DB) treatment representing uncontaminated digested sludge equivalent to digested Zn-rich sludge; two digested Zn-rich treatments were aimed at achieving soil concentrations of 150 and 450 mg Zn kg^{-1} respectively.

Soil functional measurement

Basal respiration was determined using the MicroResp colorimetric detection system (Campbell *et al*., 2003). Glucose, atrazine and simazine mineralization were determined using the MicroResp radiolabel detection system (Campbell *et al*., 2003) (see Supporting Information Appendix S1).

Microbial community structure and function measurements

Microbial biomass and broad-scale community structure were assessed by PLFA and neutral lipid fatty acid (NFLA) analysis (White *et al*., 1979). Bacterial community structure was assessed by T-RFLP (Singh *et al*., 2006a; Singh and Thomas, 2006b). To measure bacterial diversity and community composition using fusion primers to amplify multiplexed bar-coded 16S rRNA gene sequences, 454 pyrosequencing was used (Berg *et al*., 2012). Microbial functional gene analysis was determined on control, RB, DB, Cu200 and Zn450 only for each site ($n = 30$) using GeoChip functional gene array (He *et al*., 2010). Full details of each method are detailed in Supporting Information Appendix S1.

Statistical analysis

Because of a dominant effect of site on microbial community structure, HW and AC data sets were analysed separately. ANOVA was used to determine the effect of treatment on functional parameters and on microbial functional and structural parameters. PCA, CVA, analysis of variance and multiple regression analyses were used to determine the influence of treatment on each set of data (PLFA and NFLA, T-RFLP, GeoChip and 454 pyrosequencing). Relative abundance values may cause spurious correlations. To explore this further, we used the SparCC (available at https:bitbucket.org/youatanf/sparcc) (Friedman and Alm, 2012) that estimates the correlations from compositional data and reports pseudo *P*-values representing the degree of correlation. All the 21 samples were collectively analysed for possible correlation. Pseudo *P*-values were assessed based on 1000 bootstrap replicates. All other analyses were carried out using GenStat v.14 (VSN International Limited, Hemel Hempstead, UK) and R3.0.2 (R development Core Team, 2008).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Observed Shannon entropy number equivalents (S(H)), based on 454 sequencing of 16S rRNA genes, as a function of fractional sequence difference as a percentage of the control H(S) (black line) for the contaminated soil data from (A) Hartwood ($n = 21$) and (B) Auchincruive ($n = 21$). Cu50 is 50 mg kg^{-1} Cu (red); Cu200 is 200 mg kg^{-1} Cu (light green); Zn150 is 150 mg kg^{-1} Zn (dark blue); Zn450 is 450 mg kg^{-1} Zn (dark green); RB is raw blank uncontaminated undigested sludge as a control for Cu (light blue); DB is digested uncontaminated sludge as a control of Zn (magenta). A consistent reduction in S(H) is seen across OUT cut-offs with metal concentration for all except Zn150.
Fig. S2. Rarefaction curves for the contaminated soil data (A) Hartwood, 3% OTUs; (B) Hartwood, 5% OTUs; (C) Auchincruive, 3% OTUs; (D) Auchincruive, 5% OTUs demonstrating 15 000 reads do not capture total OTU number in these communities. Control is no sludge (black line); Cu50 is 50 mg kg^{-1} Cu (red); Cu200 is 200 mg kg^{-1} Cu (light green); Zn150 is 150 mg kg^{-1} Zn (dark blue); Zn450 is 450 mg kg^{-1} Zn (dark green); RB is raw blank uncontaminated undigested sludge as a control for Cu (light blue); DB is digested uncontaminated sludge as a control of Zn (magenta).
Fig. S3. Impact of heavy metal (Zn and Cu) on soil bacterial community structure based on TRFLP analysis. Data are presented as ordination plots of canonical variates (CV) of bacterial TRFLP community structure in (A) Hartwood ($n = 21$) and (B) Auchincruive ($n = 21$) soils exposed to Cu and Zn sludge additions. Control is no sludge control (black); DB is digested blank sludge (green); RB is raw blank sludge (purple); Cu50 is 50 mg kg^{-1} Cu (orange); Cu200 is 200 mg kg^{-1} Cu (red); Zn150 is 150 mg kg^{-1} Zn (grey); Zn450 is 450 mg kg^{-1} Zn (blue). Percentages in parenthesis are percentage variance explained by each CV. X represents treatment means, plus symbols represent each replicate and circle represents 95% confidence intervals. For (A) Hartwood, there is clear separation in microbial community structure on the first CV for Zn-contaminated soils and for those with the highest level of Cu contamination. For (B) Auchincruive, there is significant differences between treatments.
Fig. S4. Extractable (A) Zn (mg kg^{-1}) and (B) Cu (mg kg^{-1}) in Hartwood ($n = 9$) and Auchincruive ($n = 9$) treated with metal-contaminated sludge. Values with different letters are not significantly different ($P < 0.05$). Control is no sludge; DB is digested uncontaminated sludge as a control of Zn; Zn450 is

450 mg kg^{-1} Zn; RB is raw blank uncontaminated undigested sludge as a control for Cu, and Cu200 is 200 mg kg^{-1} Cu. Bars are ± one SE ($n = 3$). Extractable Zn is significantly higher in Hartwood soils than Auchincruive soils in soils receiving the highest level of Zn-contaminated sludge.

Fig. S5. Impact of heavy metal (Zn and Cu)-contaminated sludge on microbial community functional gene structure based GeoChip analysis in Hartwood soils. Ordination plot of canonical variates (CV) generated by CV analysis of functional genes (GeoChip) profiles grouped into gene category (a–i) in Hartwood soil exposed to zinc and copper stress ($n = 15$). Control is no sludge control (black); DB is digested uncontaminated sludge as a control of Zn (green); RB is raw blank uncontaminated undigested sludge as a control for Cu [light blue (purple)]; Cu200 is 200 mg kg^{-1} Cu (red); Zn450 is 450 mg kg^{-1} Zn (blue). Percentages in parenthesis are percentage variance explained by each CV. X represents treatment means, plus symbols represent each replicate and circle represents 95% confidence intervals. Most functional groups (involved in C, N, P, S, antibiotic resistance, metal reduction/resistance) are dramatically impacted by metal. The magnitude and direction of response are different for Zn and Cu.

Fig. S6. Impact of heavy metal (Zn and Cu)-contaminated sludge on microbial community functional gene structure based GeoChip analysis in Auchincruive soils. Ordination plot of canonical variates (CV) generated by CV analysis of functional genes (GeoChip) profiles grouped into gene category (a–i) in Auchincruive soil exposed to zinc and copper stress ($n = 15$). Control is no sludge control (black); DB is digested uncontaminated sludge as a control of Zn (green); RB is raw blank uncontaminated undigested sludge as a control for Cu [light blue (purple)]; Cu200 is 200 mg kg^{-1} Cu (red); Zn450 is 450 mg kg^{-1} Zn (blue). Percentages in parenthesis are percentage variance explained by each CV. X represents treatment means, plus symbols represent each replicate and circle represents 95% confidence intervals. Most functional groups (involved in C, N, P, S, antibiotic resistance, metal reduction/resistance) are dramatically impacted by metal. The magnitude and direction of response are different for Zn and Cu.

Fig. S7. The impact of Zn- and Cu-contaminated sludge additions on soil (A) Basal respiration and (B) glucose mineralization in Auchincruive and Hartwood soils. Control is no sludge control; Cu200 is 200 mg kg^{-1} Cu; Zn450 is 450 mg kg^{-1} Zn. Bars are ± one SE ($n = 3$). Respiration is a little affected by metal treatment, whereas there is a consistent, but statistically not significant, trend of reduced substrate mineralization in metal-contaminated soils at Hartwood, and to a lesser extent, Auchincruive.

Fig. S8. The impact of Zn- and Cu-contaminated sludge addition on the community structure of genes involved in atrazine and triazine mineralization as assessed by GeoChip analysis of soils from two sites: Hartwood (A and B) and Auchincruive (C and D). Ordination plot of canonical variates (CV) generated by CV analysis of atrazine (A and C) and triazine (B and D) degrading genes. Control is no sludge control (black); DB is digested uncontaminated sludge as a control of Zn (green); RB is raw blank uncontaminated undigested sludge as a control for Cu [light blue (purple)]; Cu200 is 200 mg kg^{-1} Cu (red); Zn450 is 450 mg kg^{-1} Zn (blue). Percentages in parenthesis are percentage variance explained by each CV. X represents treatment means, plus symbols represent each replicate and circle represents 95% confidence intervals. For Hartwood soils, there is a clear separation of Cu-contaminated soils, and to a lesser extent, Zn-contaminated soils. For Auchincruive, there is a clear separation of Zn, and to a lesser extent, Cu-contaminated soils.

Table S1. Summary of the soil OTU numbers across the 14 treatments ($n = 42$) based on 454 sequencing of 16S rRNA genes for two soils, Hartwood (HW) and Auchincruive (AC), exposed to increasing levels of Cu and Zn contamination. Filtered number gives number of reads after initial prefiltering. Clean number following removal of PCR chimeras. Unique is the number of different reads present after de-noising. Clean unique after chimera removal. The percentage of clean reads as a fraction of clean number, and percentage of clean unique as a fraction of total unique is given in parentheses. OTUs were then constructed using a complete linkage algorithm and distances based on exact pairwise alignments and the number of OTUs at 1.5%, 3%, 5% and 10% nucleotide sequence difference given. Control is no sludge control; Cu50 is 50 mg Cu kg^{-1}; Cu200 is 200 mg kg^{-1} Cu; Zn150 is 150 mg kg^{-1} Zn; Zn450 is 450 mg kg^{-1} Zn; RB is uncontaminated undigested sludge as a control for Cu; DB is digested uncontaminated sludge as a control of Zn.

Table S2. Summary of the mean soil OTU numbers across the 14 treatments subsampled to 15 000 reads based on 454 sequencing of 16S rRNA genes, for two soils (Hartwood [HW] and Auchincruive [AC]) exposed to increasing levels of Cu and Zn contamination ($n = 42$). We also give diversity as a percentage of the control in parentheses. Control is no sludge control; Cu50 is 50 mg Cu kg^{-1}; Cu200 is 200 mg kg^{-1} Cu; Zn150 is 150 mg kg^{-1} Zn; Zn450 is 450 mg kg^{-1} Zn; RB is uncontaminated undigested sludge as a control for Cu; DB is digested uncontaminated sludge as a control of Zn.

Table S3. Summary of the Shannon entropies H in numbers equivalents [S(H) = exp(H)], based on 454 sequencing of 16S rRNA genes, for two soils (Hartwood [HW] and Auchincruive [AC]) exposed to increasing levels of Cu and Zn contamination. We also give S(H) as a percentage of the control in parentheses. Control is no sludge control; Cu50 is 50 mg Cu kg^{-1}; Cu200 is 200 mg kg^{-1} Cu; Zn150 is 150 mg kg^{-1} Zn; Zn450 is 450 mg kg^{-1} Zn; RB is uncontaminated undigested sludge as a control for Cu; DB is digested uncontaminated sludge as a control of Zn.

Table S4. Deviance Information Criterion (DIC) values of abundance distribution fits to the (A) 3% OTU distributions and (B) 5% OUT distributions based on 454 sequencing of 16S rRNA genes for two soils (Hartwood and Auchincruive) exposed to increasing levels of Cu and Zn contamination. Smaller values indicate a better fit. We have indicated the best fit with a *1 or **1 if it is significantly better than the next best denoted *2. Significant was interpreted as a difference in DIC values greater than 7. Control is no sludge control; Cu50 is 50 mg Cu kg^{-1}; Cu200 is 200 mg kg^{-1} Cu; Zn150 is 150 mg kg^{-1} Zn; Zn450 is 450 mg kg^{-1} Zn; RB is uncontaminated undigested sludge as a control for Cu; DB is digested uncontaminated sludge as a control of Zn.

Table S5. Total species number estimates from abundance distribution fits to the (A) 3% OTU distributions and (B) 5% OUT distributions in soils with increasing Cu and Zn contamination at two sites, Hartwood (HW) and Auchincruive (AC).

Results are quoted as medians with 95% confidence intervals. These were calculated by sampling from the posterior distribution. Control is no sludge control; Cu50 is 50 mg Cu kg^{-1}; Cu200 is 200 mg kg^{-1} Cu; Zn150 is 150 mg kg^{-1} Zn; Zn450 is 450 mg kg^{-1} Zn; RB is uncontaminated undigested sludge as a control for Cu; DB is digested uncontaminated sludge as a control of Zn.

Table S6. Estimates of the sample size necessary to obtain 90% of the (A) 3% and (B) 5% OTU diversity determined from fits of the two best-fitting abundance distributions (log-normal and Sichel) to the soil treatments based on 454 sequencing of 16S rRNA genes for two soils (Hartwood [HW] and Auchincruive [AC]) exposed to increasing levels of Cu and Zn contamination. Results are given as medians with 95% confidence intervals. Control is no sludge control; Cu50 is 50 mg Cu kg^{-1}; Cu200 is 200 mg kg^{-1} Cu; Zn150 is 150 mg kg^{-1} Zn; Zn450 is 450 mg kg^{-1} Zn; RB is uncontaminated undigested sludge as a control for Cu; DB is digested uncontaminated sludge as a control of Zn.

Table S7. Estimates of the sample size necessary to obtain 50% of the (A) 3% and (B) 5% OTU diversity determined from fits of the two best-fitting abundance distributions (log-normal and Sichel) to the soil treatments based on 454 sequencing of 16S rRNA genes for two soils (Hartwood [HW] and Auchincruive [AC]) exposed to increasing levels of Cu and Zn contamination. Results are given as medians with 95% confidence intervals. Control is no sludge control; Cu50 is 50 mg Cu kg^{-1}; Cu200 is 200 mg kg^{-1} Cu; Zn150 is 150 mg kg^{-1} Zn; Zn450 is 450 mg kg^{-1} Zn; RB is uncontaminated undigested sludge as a control for Cu; DB is digested uncontaminated sludge as a control of Zn.

Table S8. Summary of the mean soil microbial diversities across the two deeply sampled treatments from Hartwood (HW) subsampled to 60 000 reads based on 454 sequencing of 16S rRNA genes. We also give diversity as a percentage of the control in parentheses. Control is no sludge control; Zn450 is 450 mg kg^{-1} Zn.

Table S9. *P*-values (ANOVA) and percentage variation explained (principal components analysis) by the first three principal components (PC) of microbial composition at different levels of phylogenetic resolution based on 454 sequencing of 16S rRNA genes, for in two soils (Hartwood and Auchincruive) exposed to increasing levels of Cu and Zn contamination ($n = 42$).

Table S10. *P*-values (ANOVA) for the effect of heavy metal treatment on microbial communities at the phylum, class, order, family and genus levels based on 454 sequencing of 16S rRNA genes for Hartwood and Auchincruive sites ($n = 42$). Values in bold are statistically different.

Table S11. Impact of heavy metal (Zn and Cu)-contaminated sludge on microbial abundance and community composition as assessed by PLFA (nmol g^{-1}) analysis of two sites, (A) Hartwood and (B) Auchincruive. Values with different letters are significantly different (ANOVA). NFLA is neutral lipid fatty acid; F : B is fungal to bacterial ratio; VAM is arbuscular mychorhizae; p : n is Gram positive to Gram negative ratio; P : E Is prokaryote to eukaryote ratio; Control is no sludge control; RB is uncontaminated undigested sludge as a control for Cu; Cu50 is 50 mg Cu kg^{-1}; Cu200 is 200 mg kg^{-1} Cu; DB is digested uncontaminated sludge as a control of Zn; Zn150 is 150 mg kg^{-1} Zn; Zn450 is 450 mg kg^{-1} Zn. NS is not significant.

Table S12. The impact of heavy metal (Zn and Cu)-contaminated sludge addition on basal respiration and substrate mineralization for Auchincruive and Hartwood sites. Results are presented as *P*-value result from two-way analysis of variance to test for the significance of treatment. Values in bold indicate a significant effect.

Table S13. *P*-values (ANOVA) and direction of impact for the effect of heavy metal treatment on the abundance of Atrazine- and Triazine-associated mineralization genes at Hartwood and Auchincruive as assessed by GeoChip analysis. Control is no sludge control; Cu is 200 mg kg^{-1} Cu; Zn is 450 mg kg^{-1} Zn; RB is uncontaminated undigested sludge as a control for Cu; DB is digested uncontaminated sludge as a control of Zn. ns is not significant.

Table S14. ^{14}C-labelled substrates and level of substrate used in mineralization tests to test the impact of heavy metal (Zn and Cu) on glucose and pesticide mineralization rates in two soils (Hartwood and Auchincruive) exposed to increasing metal-contaminated sludge additions.

Appendix S1. Detailed description of field sites, experimental treatments, and materials and methods used in this study.