The response of soil microbial diversity and abundance to long-term application of biosolids

Abdul-Wahab Mossa, Matthew J. Dickinson, Helen M. West, Scott D. Young*, Neil M. J. Crout

School of Biosciences, University of Nottingham, Sutton Bonington Campus, Leicestershire LE12 5RD, UK

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* Corresponding author, E-mail address: scott.young@nottingham.ac.uk

Abstract

The disposal of biosolids poses a major environmental and economic problem. Agricultural use is generally regarded as the best means of disposal. However, its impact on soil ecosystems remains uncertain. Biosolids can improve soil properties by supplying nutrients and increasing organic matter content but there is also a potentially detrimental effect arising from the introduction of heavy metals into soils. To assess the balance between these competing effects on soil health, we investigated soil bacterial and fungal diversity and community structure at a site that has been dedicated to the disposal of sewage sludge for over 100 years. Terminal restriction fragment length polymorphism (T-RFLP) was used to characterize the soil microbial communities. The most important contaminants at the site were Ni, Cu, Zn, Cd, and Pb. Concentrations were highly correlated and Zn concentration was adopted as a good indicator of the overall (historical) biosolids loading. A biosolids loading, equivalent to 700 – 1000 mg kg^{-1} Zn appeared to be optimal for maximum bacterial and fungal diversity. This markedly exceeds the maximum soil Zn concentration of 300 mg kg^{-1}permitted under the current UK Sludge (use in agriculture) Regulations. Redundancy analysis (RDA) suggested that the soil microbial communities had been altered in response to the accumulation of trace metals, `especially Zn, Cd, and Cu. We believe this is the first time the trade-off between positive and negative effects of long term (>100 years) biosolids disposal on soil microorganisms have been observed in the field situation.

Capsule: T-RFLP analysis indicates that microbial diversity responds to the application of biosolids and exhibits an optimal level due to the trade off between beneficial and toxic effects

Keywords: Biosolids; Microbial diversity; Heavy metals; T-RFLP, Long term effects on soil

1. Introduction

Municipal wastewater treatment results in large amounts of biosolids that require disposal and this is a major worldwide environmental concern. A number of disposal methods are used, including application to agricultural soils, dispose to landfill, and incineration (Sánchez-Monedero et al., 2004; Singh and Agrawal, 2008). Land application is suggested to be the best practicable environmental option and the most cost-effective method for the disposal of biosolids (Singh and Agrawal, 2008). This is because the practice supplies valuable nutrients and improves soil physical and biological properties by providing organic matter to soils (Coors et al., 2016; Singh and Agrawal, 2008). However, there are potentially damaging effects from long-term disposal of biosolids to soil including increased concentration of potentially toxic metals and organic compounds (Sánchez-Monedero et al., 2004; Singh and Agrawal, 2008), nitrate and phosphorus contamination of water (Joshua et al., 1998), and the introduction of pathogens to arable soils (Hong et al., 2009). It has been demonstrated that excessive application of biosolids to soil can cause stress to soil microorganisms. Macdonald et al. (2011) reported changes in the soil microbial community in response to 11 years exposure to sewage sludge enriched in Zn and Cu. Charlton et al. (2016) reported a 7-12% decrease in soil microbial biomass carbon in soils which had received sewage sludge contaminated with Zn and Cu over a period of 8 years. Understanding the effect of biosolids on soil microorganisms is important due to the critical role of microorganisms in biogeochemical processes and ultimately in ecosystem functions, such as decomposition of organic matter and nutrient cycling (Nannipieri et al., 2003).

The impact of biosolids application on soil microbiota has been explored previously (Chen et al., 2016; El Azhari et al., 2012; Kelly et al., 1999; Macdonald et al., 2011). However the time scale of most previous work was relatively short and therefore the resilience of microbiota to long-term exposure to extended successive biosolids application over time has not been assessed. To our knowledge there is little quantitative research that relates heavy metal contamination and biosolids loading to soil microbial diversity and community composition. This is because previous observations reported in the literature have been made at sites where the heavy metal concentrations were close to, or below, the regulatory limits and where the duration of disposal was short (ca. 10 years). Therefore our study site presents an excellent opportunity to investigate the impact of heavy metal loading in soils subject to biosolids applications over >100 yr and where the metal concentrations are above the regulatory limits

for normal arable land which does not have 'dedicated site' status (e.g. several thousand mg kg^{-1} Zn).

Several molecular techniques are available to characterise the soil microbial community and these can be used to monitor any potential changes in response to environmental stress. Techniques include ribosomal intergenic spacer analysis (RISA), denaturing and temperature gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) (Leckie, 2005). These techniques generally involve producing a fingerprint of the microbial communities based on amplifying genes, using polymerase chain reaction (PCR), extracted from the community. One of the most frequently used profiling techniques is T-RFLP, which depends on the variation of restriction sites within the sequences of different organisms (Schütte et al., 2008).

We hypothesized that prolonged application of biosolids to the soil will induce an increase in activity and diversity of soil microbial populations arising from increased humus content. We further hypothesized that biosolids, due to heavy metal loadings, will induce shifts in soil microbial community structure. To address these hypotheses we aimed to (i) study the effect of biosolids application on several soil chemical properties (pH, organic matter, and labile and soluble metal concentrations), (ii) assess the long-term impact of biosolids on soil microbial diversity, (iii) investigate soil bacterial and fungal community structure assessed by the T-RFLP fingerprinting technique. Our overall aim was to assess the balance of positive and negative effects on soil health arising from biosolids application and determine whether an optimum loading was apparent at our study site.

2. Materials and methods

2.1. Study site

The study was conducted on a dedicated sewage sludge disposal site, located adjacent to the River Trent, east of Nottingham, UK. The site comprises approximately 60 fields, some of which have been used for disposal of sewage sludge since 1880. The topsoils show a wide range of metal loadings ranging from background levels to high levels of metal contamination. The majority of the farm is now used to produce 'fodder maize' for an anaerobic digestion plant. It is important to note that the study location is a working site run by a water company for practical sewage disposal purposes. There are no records of biosolid disposal across the site, or control fields. The value of the site lies in its size and the range and duration of biosolids

disposal (for >100 year). It is the resulting variation in soil organic matter, phosphate and metal concentrations that provides the experimental basis for testing our hypotheses.

2.2. Sample collection

Soil samples were collected from 17 fields sown with maize. Four bulk soil samples within rows were taken from a 1 m^2 area from the upper 20 cm and combined into one composite sample. Four plant roots from maize were uprooted and the loosely adhered soil shaken off the roots in order to conserve the remaining adhering soil as 'rhizosphere soil'. Soil samples from bulk sampling and the rhizosphere (n=34) were sieved to <4 mm and then split into two sub-samples for analysis of microbial diversity, using T-RFLP assays, and soil chemical properties. The subsamples for molecular analysis were initially stored at 4 °C for 24 hours before storing at -20 °C; soil for chemical analysis was air dried and a subsample (c. 20 g) was agate ball milled (Retsch, Model PM 400) prior to acid digestion.

2.3. Soil chemical analysis

2.3.1. Soil characteristics

Total soil elemental concentration was determined using ICP-MS (Thermo-Fisher Scientific X-Series^{II}; Thermo Fisher Scientific Inc., Waltham, MA, USA), following acid digestion of 200 mg of finely ground soil with 2 mL of concentrated HNO_3 and 1 mL of $HClO_4$. Elemental analysis included 25 elements: Mg, K, Ca, Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Ag, Cd, Sn, Sb, Cs, Ba, Pb and U. Soil organic matter was estimated from loss on ignition (LOI); approximately 5 g of air dried soil (< 4 mm) was ignited in a muffle furnace at 550 °C for 8 h. Soil pH was measured in a suspension (1:2.5 m/v) of air dried soil (< 4 mm) and Milli-Q water after shaking for 30 minutes on an end-over-end shaker. Dissolved organic and inorganic carbon were measured, using a Shimadzu TOC –Vcp analyser, in the solution phase of soil suspensions in 0.01 M $Ca(NO_3)_2$ (3 g: 30 mL) equilibrated on an end-over-end shaker for 3 days, followed by centrifugation (2200 g) and syringe filtration (< 0.22 μm). Dissolved trace and major elements were measured in the same supernatant solutions using ICP-MS.

2.3.2. Isotopically exchangeable metal (E-values) in soil

The concentrations of isotopically exchangeable Ni, Cu, Zn, Cd and Pb were measured in all soil samples. Four replicates of each soil were suspended in 0.01 M $Ca(NO_3)_2$ (3 g: 30 mL) and pre-equilibrated on an end-over-end shaker for 2 days, then an aliquot (0.4 mL) of multi element isotopic spike solution enriched with $^{62}Ni^{2+}$, $^{65}Cu^{2+}$, $^{70}Zn^{2+}$, $^{108}Cd^{2+}$ and $^{204}Pb^{2+}$, was

added to two of the suspensions and the other two were left as controls. The level of spike concentration was determined after conducting a preliminary experiment testing a range of spike levels on two soils with contrasting (low and high) metal concentrations to ensure measureable differences in isotopic ratio between spiked and un-spiked samples. After spiking, the suspensions were shaken for a further 3 days to attain isotopic equilibrium (Marzouk et al., 2013a). Samples then were centrifuged (2200 g) for 15 minutes and syringe-filtered (<0.22 μm) and the isotopic ratios ($^{62}Ni/^{60}Ni$, $^{65}Cu/^{63}Cu$, $^{70}Zn/^{66}Zn$, $^{108}Cd/^{111}Cd$, $^{204}Pb/^{206}Pb$, $^{204}Pb/^{207}Pb$, and $^{204}Pb/^{208}Pb$) were measured in the filtered (< 0.22 μm) supernatant by ICP-MS. The isotopic abundance (IA) of the spike isotope was calculated from the isotopic ratios listed above, and the concentrations of 'isotopically exchangeable' Ni, Cu, Zn, Cd, and Pb (E value, mg kg^{-1}) were calculated from Equation 1 (Atkinson et al., 2011; Marzouk et al., 2013a).

$$E = \left(\frac{M_{soil}}{W}\right)\left(\frac{C_{spike}V_{spike}}{M_{spike}}\right)\frac{(^{Iso1}IA_{Spike} - {}^{Iso2}IA_{Sike}R_{SS})}{(^{Iso2}IA_{Soil}R_{SS} - {}^{Iso1}IA_{Soil})} \quad (1)$$

where M_{soil} and M_{spike} are the average atomic masses of the metal in soils and spike solutions respectively, W is the mass (kg) of soil used in the assay, C_{spike} is the concentration (mg L^{-1}) of the metal in the spike solution, V_{spike} is the spike volume added (L), IA represents the isotopic abundance, R_{ss} is the isotopic abundance ratio in the spiked soil solution. The same experimental procedure was repeated for all soils using 10^{-5} M Na_2EDTA as the suspending solution instead of 0.01 M $Ca(NO_3)_2$. This was in response to unsatisfactory results for Pb due to its extremely low solubility of Pb in the test soils which have a high phosphate content.

2.4. Soil solution speciation

Nickel, Cu, Zn, Cd and Pb speciation were resolved using the geochemical model, WHAM VII (Tipping, 1994). Inputs to the model included cation and anion concentrations and pH value in the $Ca(NO_3)_2$ extract. The dissolved organic carbon (DOC) was converted to fulvic acid concentration by assuming (i) a carbon content of 50% and (ii) that fulvic acid constituted 65% of DOC (Buekers et al., 2008; Marzouk et al., 2013b)

2.5. Microbial community structure

T-RFLP was used to investigate soil microbial community structure. Briefly, DNA was extracted from 0.25 g of each soil sample using a Power Soil DNA extraction kit (Mo Bio, Carlsbad, CA, USA) as instructed by the manufacture. Extracted DNA was amplified targeting the bacterial 23S ribosomal subunit gene and fungal ITS-2 region. For bacterial DNA, (5′-GCG ATT TCY GAA YGG GGR AAC CC-3′) primer and the reverse primer (5′ TTC GCC TTT

CCC TCA CGG TAC T-3′), labelled with the blue WellRED dye D4 (Sigma–Proligo, Gillingham, UK), were used (Martin et al., 2012). The fungal DNA was amplified using 5.8S for (5′-GCA TCG ATG AAG AAC GCA GC-3′) and FITSrev (5′- ATA TGC TTA AGT TCA GCG GGT-3′), labelled with the green WellRED dyeD3 (Sigma–Proligo, Gillingham, UK) (Martin et al., 2012). Prior to fragment analysis, PCR products were digested with restriction enzymes in 20 µL reactions made of 10 µL PCR product, 2 µL10x RE buffer (New England BioLabs, Hitchin, Hertfordshire, UK), 7 µl de-ionised water, and 1 µL of restriction enzyme. For bacteria, T-RFLP double digest (0.5 µL) was carried out using both MseI and HindIII enzymes (New England BioLabs, Hitchin, Hertfordshire, UK). For fungal T-RFLP only HaeIII enzyme (New England BioLabs, Hitchin, Hertfordshire, UK) was used. Digests were incubated at 37°C for 2 hours to complete the digestion followed by deactivation of enzymes by heating to 80 ºC for 20 min. Digestion products were verified on 1% of agarose gel containing ethidium bromide in 1X TBE buffer. Gels were run for 1 hr at 90V and then viewed under UV light. Both bacterial and fungal digests were then mixed in a 2:1 ratio prior to fragments analysis to accommodate differences in signal strength, because different dyes were used for each. One µL aliquots of the digest mix were loaded into a 96 well plate with each well containing 38.5 µL of GenomeLab sample loading solution and 0.5 µL internal 600 base pairs (bp) standard ladder (Beckman Coulter Inc.) to allow fragments between 60-640 bp to be considered. The samples were loaded with mineral oil to prevent oxidation of the sample and then separated by electrophoresis using a CEQ 8000 DNA analysis system (Beckman Coulter Inc, HighWycombe, UK).

2.6. Data analysis

Numerical data were obtained from transforming T-RFLP using CEQ 8000 (Beckman Coulter Inc, High Wycombe, UK). Only T-RFs larger than 60 bp were included in the analysis to avoid any dimmer signals. Data were further analysed using the T-REX platform (Culman et al., 2009) for noise reduction (Abdo et al., 2006) and fragments with a band width of 1.25 bp or less were binned (Martin et al., 2012). The relative abundance of individual T-RFs was calculated as the percentage of total peak height in a given T-RFLP profile. Only those T-RFs with a relative abundance of greater than 1% were considered (Azziz et al., 2016). These refining techniques increase the independent integrity of the T-RFLP profiles and enable comparisons to be made across soil samples. Nevertheless, individual TRFs will include more than one species.

The Vegan package (Oksanen et al., 2016) in R (R Core Team, 2015) was used to conduct all statistical analyses. The compositions of T-RFLP profiles were visualized with non-metrical multidimensional scaling (NMDS), using vegan's *metaMDS()* function, based on Bray–Curtis dissimilarity matrix of Hellinger transformed T-RFLP profiles (Legendre and Gallagher, 2001). Relationships between soil properties and soil microbial communities were further analysed with Redundancy Analysis (RDA); total, isotopically exchangeable, dissolved and the free ion activities of Ni, Cu, Zn, Cu, and Pb in addition to the total elemental concentrations were included to calculate the explained variance in RDA. The most appropriate statistical model for RDA was determined using forward selection as implemented in vegan's *ordistep ()* and *anova*() function. Only variables with $P<0.05$ were included in the selected model; to avoid any collinearity, the variance inflation factor (VIF) was calculated and only variables with VIF < 10 were included in the model (Borcard et al., 2011).

T-RF richness refers to the number of peaks, whilst Simpson's Diversity Index was calculated as implemented in R Vegan's *diversity()* function (Oksanen et al., 2016) using equation 2, which accounts for T-RF abundance in addition to number.

$$D = 1 - \sum_{i=1}^{S} \left(\frac{n_i}{N}\right)^2 \qquad (2)$$

Where n_i is the abundance of an individual TRF, N is the total abundance of all TRFs, s is the total number of TRFs.

3. Results and discussion

3.1. Soil characteristics

The general characteristics of soils used in the study are given in Table 1. Loss on Ignition (%LOI), ranged from 3.5% to 23 %. Soil pH showed near neutral values with very little variation across the 17 fields (soil pH= 6.9–7.6). Total N ranged between 0.4 – 1.2 %.

The soil metal concentrations covered a wide range, in some cases spanning over one order of magnitude (Table 1). This is the product of variable biosolids application rates over the operational time period of the site. A Pearson correlation matrix (Table 2) showed very strong relationships between individual trace metals in addition to strong correlations between the expected indicators of biosolid application such as soil organic matter (LOI), total P content, and metal loading. It should be noted that (i) the site has been exclusively used for biosolids disposal, (ii) the original soils would have similar background metal concentrations, and (iii)

the site consists of free draining soil used for arable agriculture. Therefore, the observed LOI reflects different levels of biosolids application, rather than individual field soil properties. Based on the above observations and, in the absence of past historical records of disposal, it is reasonable to assume that any of the metals could be used as a proxy measure of biosolids application. In this work Zn was adopted as an indicator for biosolids application because of the highly significant correlation with other biosolids tracers i.e. soil organic matter and phosphate content (Table 2). Furthermore, very similar results were obtained when different metals e.g. Cd or Cu were used instead of Zn (data not shown).

Table 1.
Descriptive statistics of the main properties of soils sampled.

	Mean	Minimum	Median	Maximum
pH	6.9	6.4	6.8	7.6
LOI	12.5	3.5	10.3	23.4
N (%)	0.7	0.4	0.5	1.2
P (mg kg^{-1})	6330	1510	5130	13700
Cr (mg kg^{-1})	656	67	483	1670
Ni (mg kg^{-1})	171	28	122	415
Cu (mg kg^{-1})	311	44	172	766
Zn (mg kg^{-1})	861	122	570	2050
Cd (mg kg^{-1})	16.8	1.4	8.4	48.6
Pb (mg kg^{-1})	353	70	270	688

With the exception of Pb, isotopically exchangeable metal concentrations (Cu, Zn, Cd, Ni) strongly correlated with their respective total concentration in soil ($r>0.98$, $P<0.001$). Copper was the most labile metal (31 - 44 %), whereas Pb had limited lability (1.5 – 8.4 %) (Fig. 1) as also determined for this site by Atkinson et al., (2011) and Mao et al., (2014). The isotopically exchangeable metals of studied elements did not show any obvious pH dependence although this is probably due to the restricted range of pH values. The comparison between bulk soil and rhizosphere soil did not show any rhizosphere effect on the lability of Ni, Cu, Zn, and Cd. However, Pb lability was significantly higher ($P<0.001$) in the rhizosphere compared to the bulk soil (Fig. 1).

Table 2.

Pearson correlation matrix for soil total metal concentration, soil organic matter (LOI) and soil total carbon content

LOI								
0.98	P							
0.98	0.98	Cr						
0.95	0.97	0.98	Ni					
0.98	0.98	0.99	0.98	Cu				
0.98	0.98	0.99	0.98	0.998	Zn			
0.92	0.96	0.96	0.99	0.96	0.96	Cd		
0.97	0.95	0.96	0.92	0.97	0.97	0.89	Pb	
0.94	0.90	0.90	0.87	0.92	0.92	0.86	0.88	Total C

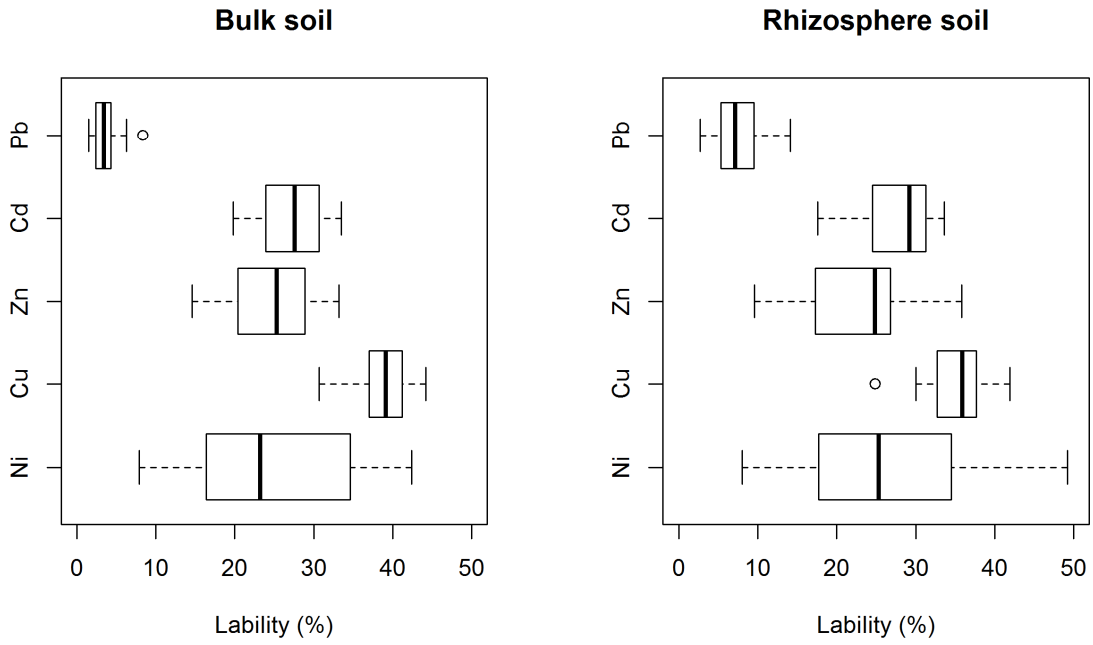

Figure 1.

Variation in metal lability (E-value) across the sampling sites expressed as a percentage of the total soil metal concentration

3.2. Soil microbial diversity

Figure 2 shows the relationship between soil Zn concentration, and the richness and diversity index of bacterial and fungal T-RFs, which revealed a range of trends. It is important to acknowledge that while our data span a broad range of Zn concentrations the middle range is less well represented. Bacterial diversity increased slightly with increasing soil Zn and peaked around 500-700 mg kg^{-1} before declining at higher Zn concentrations. As shown in Fig. 2, a significant polynomial model was found for bacteria suggesting an optimal biosolids application for bacterial diversity and richness. Thus moderate biosolids applications may increase the diversity of the soil ecosystem i.e. additional organic matter and nutrient input supports the growth of microbial populations and increases diversity. However, for soils with greater than 1000 mg kg^{-1} Zn, diversity steadily decreased with increasing Zn concentration. This suggests that, above a certain level of biosolids accumulation, any beneficial effects of the additional organic matter inputs are offset by the deleterious impact of high metal contamination on soil microorganisms. The toxic response appears to be the dominant factor at Zn >1000 mg kg^{-1}. These observations are in qualitative agreement with those of El Azhari et al. (2012) who reported that while 10 t ha^{-1} sewage sludge application resulted in higher bacterial diversity than that observed in control and farmyard manure treatments, at a higher dose, 100 t ha^{-1}, the lowest diversity was observed and any beneficial effect of sewage sludge was lost.

Bacteria were affected by the presence of biosolids to different degrees compared to fungi. Bacterial populations had a limited response to biosolids application rates as the diversity index across the studied soils did not vary widely and typically remained in the range 0.80-0.90. This may indicate that bacterial communities are resistant over these ranges of metal concentration. In contrast, fungi appeared to be more sensitive and whilst low application rates promoted an increase in diversity as described above, high application rates (>1000 mg kg^{-1}) caused a major decrease in diversity index from 0.8 to 0.4. These findings are consistent with the observations reported by (Garcia-Sánchez et al., 2015; Pennanen et al., 1996), although other authors have reported that fungi are more tolerant to heavy metal stress than bacteria (Bååth et al., 1998; Kandeler et al., 2000; Stefanowicz et al., 2008). Fungal diversity decreased linearly with increasing biosolids application (Fig. 2) in contrast to the curved response of bacteria which appeared to pass through a maximum value.

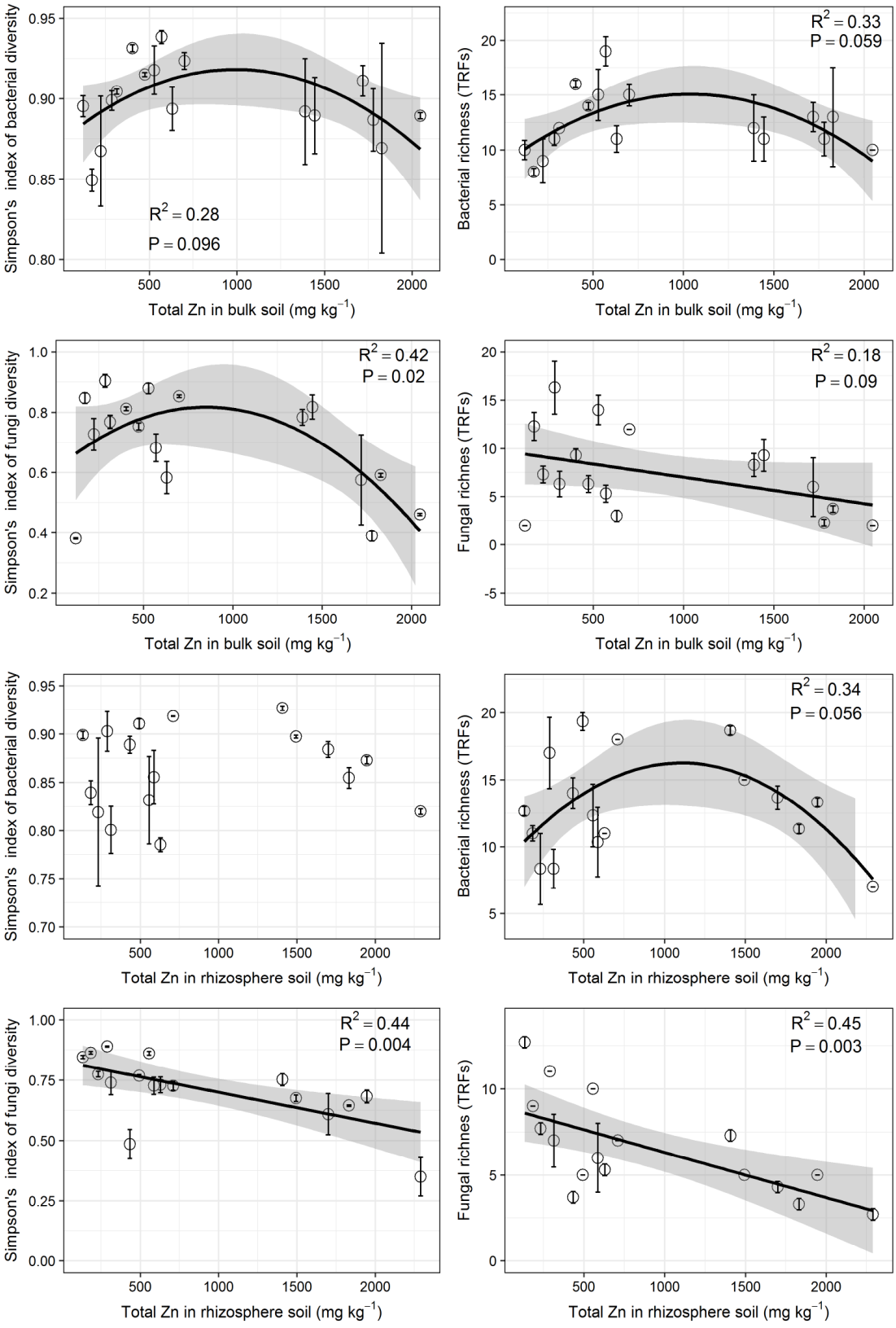

Figure 2. The relationship between total Zn concentration and soil microbial diversity, estimated using the Simpson index (left), and richness (right), defined as the number of T-RFs in each soil profile. The solid line is the regression model fit. The grey shaded areas represent the 95% confidence interval.

3.3. Effects of biosolids on soil microbial abundance

Figure 3 visualizes the differences in soil microbial communities. A Bury-Curtis dissimilarity matrix was calculated from T-RFLP profiles, which was then coordinated into two dimensions with nMDS. Each point in the figure represents a field (n=17); fields that ordinated close to one another are similar in term of microbial abundance. The differences in soil microbial abundance are evident across the studied fields across the sewage disposal site. In bulk soil, the bacterial communities showed greater similarity in the fields with high concentrations of Zn, clustered at the right of the nMDS biplot, in clear contrast to the fields with low Zn concentration (Fig. 3).

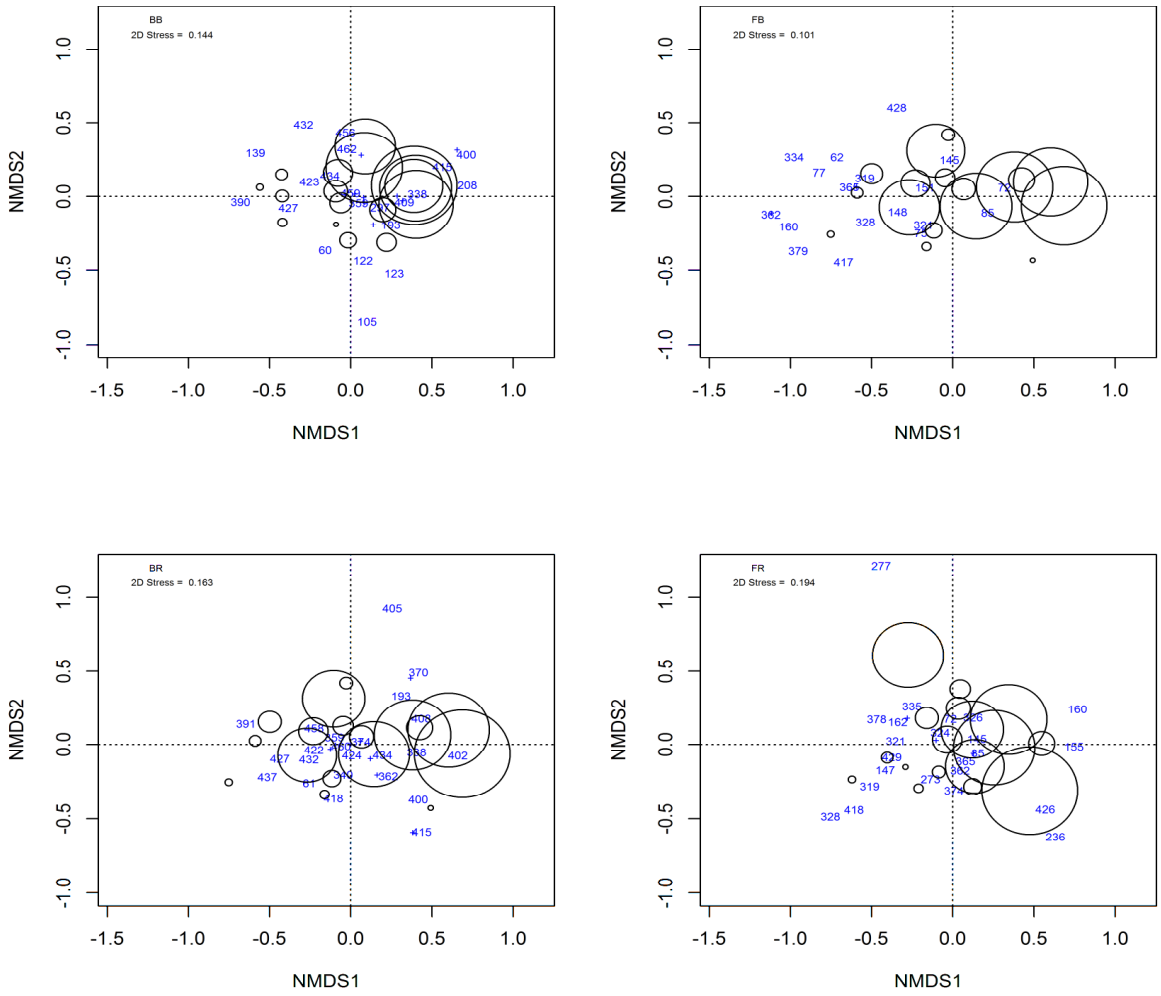

Figure 3. nMDS ordination biplot showing bacterial (left panels) and fungal (right panels) T-RFLP community composition in both bulk and rhizosphere soil (BB: bacteria in bulk soil, FB: fungi in bulk soil, BR: bacteria in rhizosphere soil, FR: fungi in rhizosphere soil). Circles represent the studied fields, the size of the circles reflects total soil Zn concentration (mg kg^{-1}). 2D stress represents the level of confidence in the ordination.

In common with bacteria, differences in fungal community structure were evident in the nMDS biplots. Although the trend was less obvious than that observed for bacteria, the fields enriched in metals tended to cluster together. Moreover, the clustering of the fields with high metal loading was more apparent in the rhizosphere than the bulk soil (Fig. 3). This pattern suggests an effect of the rhizosphere on soil fungal abundance.

Overall, these results show a clear impact of historical biosolids application on soil microbial community structure. This agrees with the findings of Macdonald et al., (2011), who reported a shift in the structure of soil microbial communities in response to sludge-derived metal. Tipayno et al., (2012) also found a change in bacterial communities in metal contaminated sites.

3.4. Effect of soil characteristics on soil microbial abundance

Redundancy analysis (RDA) was used to identify the main soil variables that explained the variance in soil microbial structure generated from T-RFLP. In the case of the five metals assayed for E-values and free ion activities, it was observed that isotopically exchangeable, dissolved and free ion activity concentration explained more variation in T-RFLP profiles and hence microbial community structure, than the total soil metal concentration. This supports the notion that total soil metal concentration is a poor estimate of bioavailability and subsequent toxicity of metals in soil (Scheckel et al., 2009). RDA showed that LOI, C:N ratio, total As and Ag, significantly affected the structure of the soil microbial community. In bulk soil, RDA explained 40% ($P<0.001$) of the variation in both bacterial and fungal T-RFs respectively, while in rhizosphere soil, the corresponding figures were 30 and 23.9 % ($P<0.001$) respectively. The RDA showed that isotopically exchangeable Zn, dissolved Cu, and free ion activity Cd^{2+} were significant in explaining the distribution of bacterial T-RFs, while the free ion activity Zn^{2+}, dissolved Cu, and C:N ratio were significant in explaining fungal community composition (Fig. 4). Bacterial T-RFs 400, 402, and 415 correlated positively with Cd free ion activity and these T-RFs were only present in fields with high metal loadings (> 1500 mg kg^{-1} Zn). In contrast, bacterial T-RFs 390, 427, and 423 negatively correlated with Cd free ion activity and these T-RFs were found in fields with low metal loadings (<570 mg kg^{-1} Zn). Most of the fungal T-RFs correlated negatively with LOI and hence metal loading (Fig. 4).

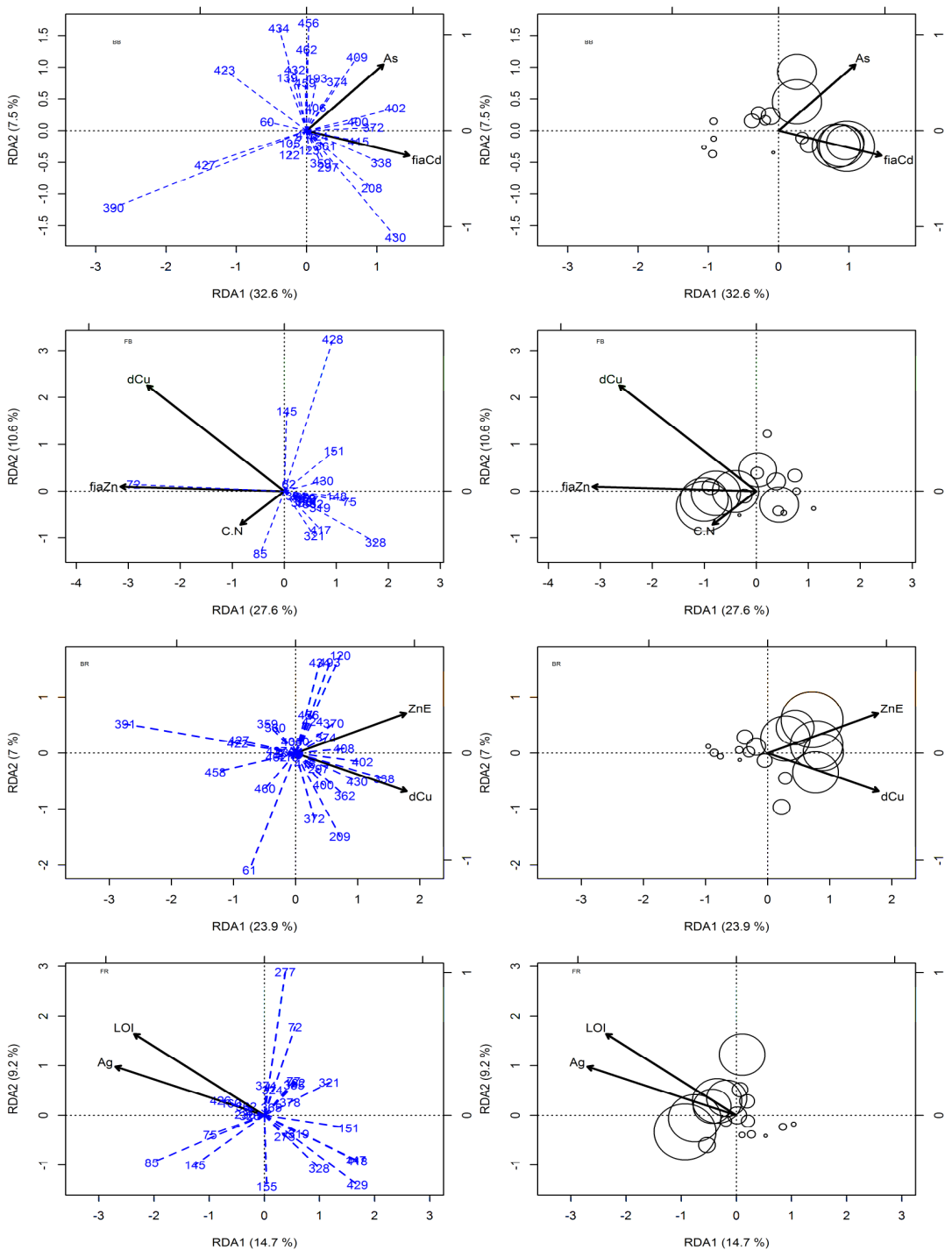

Figure 4. Ordination plot of the RDA results generated from T-RFLP profiles with constrained soil properties chosen by forward selection. T-RFs are shown in dashed blue lines and soil variables are shown in black arrows. (BB: bacteria in bulk soil, FB: fungi in bulk soil, BR: bacteria in rhizosphere soil, FR: fungi in rhizosphere soil, fia: free ion activity, d: dissolved, E: isotopically exchangeable, LOI: loss on ignition). Circles represent the studied fields, the size of the circles reflects total soil Zn concentration (mg kg^{-1}). The plots are scaled so that the angles between T-RFs and environmental variables reflect the correlations. For clarity, the triplot of RDA was split into species plots (left) and site plots (right)

Trends between the abundance of selected T-RFs and soil Zn concentration were shown by bacterial T-RFs 427 and 390 which were reduced or completely absent in fields with high Zn content (Fig. 5). In contrast, bacterial T-RFs 402 and 415 were dominant in these fields. This, combined with the results of RDA, suggests that the long-term application of biosolids induces a change in soil microbial community structure towards dominance of particular species and reduction or even the complete absence of sensitive species. This is in agreement with (Kelly et al., 1999) who found a 20-fold increase in Zn-resistant bacteria in response to biosolids application. Chen et al (2016) also found a substantial increase in the abundance of antibiotic resistant bacteria as a result of long-term application of sewage sludge.

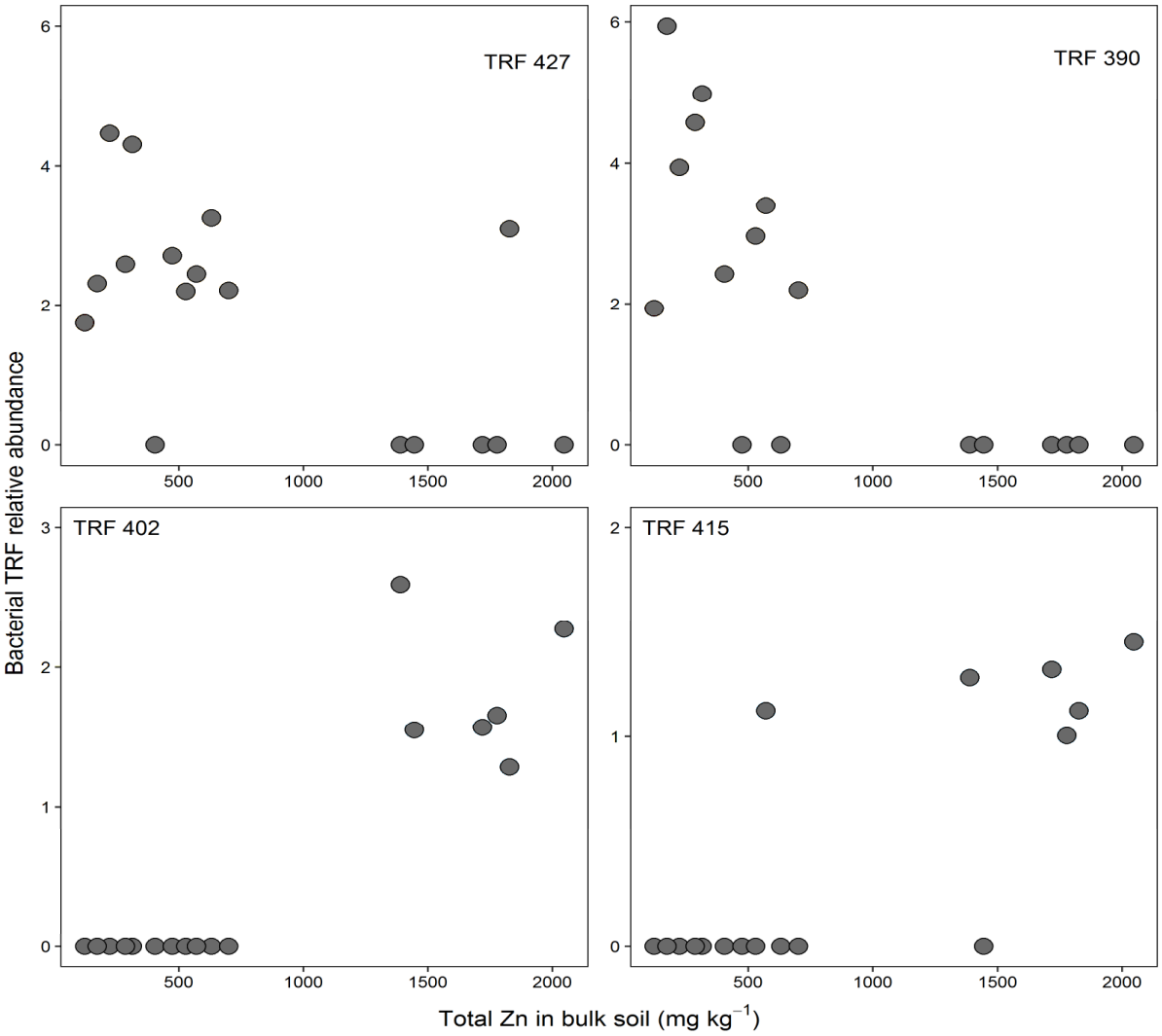

Figure 5. Trend in selected individual T-RF numbers with soil Zn concentration.

Our results indicate that a total sludge loading equivalent to accumulation in the soil of 180-220, 350-450, 700-1000, 20-30, 350-450 mg kg^{-1} of total Ni, Cu, Zn, Cd, Pb is optimal for (maximum) soil microbial diversity at the study site. This contrasts markedly with the maximum soil Zn concentration permitted under the current UK Sludge (use in agriculture) Regulations' of 135, and 300 mg kg^{-1} of Cu and Zn respectively (Charlton et al., 2016).

Regulations for biosolids disposal take into account a range of potential harmful effects, including phytoxicity, metal transfer to the human/animal food supply, and possible impacts on the soil invertebrate community etc. Our study, at a site subject to long-term biosolids disposal (>100 years) suggests that microbial diversity is unlikely to be the limiting factor for biosolids disposal rates. In addition, the relationship between accumulated soil metal and sludge loading will depend explicitly on the sludge composition and presumably on soil conditions governing decomposition rate. Thus, the relative importance of each causal influence must be considered before microbial diversity could be incorporated into policy making.

4. Conclusion

While low application rates of biosolids can increase soil microbial diversity, this trend is reversed at higher rates of application. This is probably due to increased concentration of heavy metals at higher application rates. Moreover in the current study, there was a change in the structure of soil microorganism communities with increasing application of biosolids. The trade-off between positive and negative effects may depend on the specific characteristics of a site and the biosolids applied. However, compared to current statutory standards, it appears that microbial diversity is unlikely to be the limiting factor for regulatory limits for biosolids disposal rates. In the current study, the maximum beneficial effect on microbial diversity occurred at a metal (Zn) concentration well in access of current regulations governing application of biosolids to agricultural land which address issues of phytotoxicity and transfer to the foodchain.

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References

Abdo, Z., Schüette, U.M.E., Bent, S.J., Williams, C.J., Forney, L.J., Joyce, P., 2006. Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. Environ. Microbiol. 8, 929–938. doi:10.1111/j.1462-2920.2005.00959.x

Atkinson, N.R., Bailey, E.H., Tye, A.M., Breward, N., Young, S.D., 2011. Fractionation of lead in soil by isotopic dilution and sequential extraction. Environ. Chem. 8, 493–500.

Azziz, G., Trasante, T., Monza, J., Irisarri, P., 2016. The effect of soil type, rice cultivar and water management on ammonia-oxidizing archaea and bacteria populations. Appl. Soil Ecol. 100, 8–17. doi:10.1016/j.apsoil.2015.11.009

Bååth, E., Díaz-Raviña, M., Frostegård, Å., Campbell, C.D., 1998. Effect of Metal-Rich Sludge Amendments on the Soil Microbial Community. Appl. Environ. Microbiol. 64, 238–245.

Borcard, D., Gillet, F., Legendre, P., 2011. Canonical Ordination, in: Numerical Ecology with R. Springer New York, New York, NY, pp. 153–225.

Buekers, J., Degryse, F., Maes, A., Smolders, E., 2008. Modelling the effects of ageing on Cd, Zn, Ni and Cu solubility in soils using an assemblage model. Eur. J. Soil Sci. 59, 1160–1170. doi:10.1111/j.1365-2389.2008.01053.x

Charlton, A., Sakrabani, R., Tyrrel, S., Rivas Casado, M., McGrath, S.P., Crooks, B., Cooper, P., Campbell, C.D., 2016. Long-term impact of sewage sludge application on soil microbial biomass: An evaluation using meta-analysis. Environ. Pollut. doi:10.1016/j.envpol.2016.07.050

Chen, Q., An, X., Li, H., Su, J., Ma, Y., Zhu, Y.-G., 2016. Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil. Environ. Int. 92–93, 1–10. doi:10.1016/j.envint.2016.03.026

Coors, A., Edwards, M., Lorenz, P., Römbke, J., Schmelz, R.M., Topp, E., Waszak, K., Wilkes, G., Lapen, D.R., 2016. Biosolids applied to agricultural land: Influence on structural and functional endpoints of soil fauna on a short- and long-term scale. Sci. Total Environ. 562, 312–326. doi:10.1016/j.scitotenv.2016.03.226

Culman, S.W., Bukowski, R., Gauch, H.G., Cadillo-Quiroz, H., Buckley, D.H., 2009. T-REX: software for the processing and analysis of T-RFLP data. BMC Bioinformatics 10, 171. doi:10.1186/1471-2105-10-171

El Azhari, N., Lainé, S., Sappin-Didier, V., Beguet, J., Rouard, N., Philippot, L., Martin-Laurent, F., 2012. Long-term impact of 19 years' farmyard manure or sewage sludge application on the structure, diversity and density of the protocatechuate-degrading bacterial community. Agric. Ecosyst. Environ. 158, 72–82. doi:10.1016/j.agee.2012.05.015

Garcia-Sánchez, M., Garcia-Romera, I., Cajthaml, T., Tlustoš, P., Száková, J., 2015. Changes in soil microbial community functionality and structure in a metal-polluted site: The effect of digestate and fly ash applications. J. Environ. Manage. 162, 63–73. doi:10.1016/j.jenvman.2015.07.042

Hong, J., Hong, J., Otaki, M., Jolliet, O., 2009. Environmental and economic life cycle assessment for sewage sludge treatment processes in Japan. Waste Manag. 29, 696–703. doi:10.1016/j.wasman.2008.03.026

Joshua, W.D., Michalk, D.L., Curtis, I.H., Salt, M., Osborne, G.J., 1998. The potential for contamination of soil and surface waters from sewage sludge (biosolids) in a sheep grazing study, Australia. Geoderma 84, 135–156. doi:10.1016/S0016-7061(97)00125-0

Kandeler, E., Tscherko, D., Bruce, K.D., Stemmer, M., Hobbs, P.J., Bardgett, R.D., Amelung, W., 2000. Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil. Biol. Fertil. Soils 32, 390–400. doi:10.1007/s003740000268

Kelly, J.J., Häggblom, M., Tate, R.L., 1999. Effects of the land application of sewage sludge on soil heavy metal concentrations and soil microbial communities. Soil Biol. Biochem. 31, 1467–1470.

Leckie, S.E., 2005. Methods of microbial community profiling and their application to forest soils. For. Ecol. Manag., Forest Soils Research: Theory, Reality and its Role in TechnologYSelected and Edited Papers from the 10th North American Forest Soils Conference held in Saulte Ste. Marie, Ontario, Canada, 20-24 July 2003 220, 88–106. doi:10.1016/j.foreco.2005.08.007

Legendre, P., Gallagher, E., 2001. Ecologically meaningful transformations for ordination of species data. Oecologia 129, 271–280. doi:10.1007/s004420100716

Macdonald, C.A., Clark, I.M., Zhao, F.-J., Hirsch, P.R., Singh, B.K., McGrath, S.P., 2011. Long-term impacts of zinc and copper enriched sewage sludge additions on bacterial, archaeal and fungal communities in arable and grassland soils. Soil Biol. Biochem. 43, 932–941. doi:10.1016/j.soilbio.2011.01.004

Mao, L., Bailey, E.H., Chester, J., Dean, J., Ander, E.L., Chenery, S.R., Young, S.D., 2014. Lability of Pb in soil: effects of soil properties and contaminant source. Environ. Chem. 11, 690. doi:10.1071/EN14100

Martin, S.L., Mooney, S.J., Dickinson, M.J., West, H.M., 2012. Soil structural responses to alterations in soil microbiota induced by the dilution method and mycorrhizal fungal inoculation. Pedobiologia 55, 271–281. doi:10.1016/j.pedobi.2012.06.001

Marzouk, E.R., Chenery, S.R., Young, S.D., 2013a. Measuring reactive metal in soil: a comparison of multi-element isotopic dilution and chemical extraction. Eur. J. Soil Sci. 64, 526–536. doi:10.1111/ejss.12043

Marzouk, E.R., Chenery, S.R., Young, S.D., 2013b. Predicting the solubility and lability of Zn, Cd, and Pb in soils from a minespoil-contaminated catchment by stable isotopic exchange. Geochim. Cosmochim. Acta 123, 1–16. doi:10.1016/j.gca.2013.09.004

Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G., 2003. Microbial diversity and soil functions. Eur. J. Soil Sci. 54, 655–670. doi:10.1046/j.1351-0754.2003.0556.x

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2016. vegan: Community Ecology Package.

Pennanen, T., Frostegard, A., Fritze, H., Baath, E., 1996. Phospholipid Fatty Acid Composition and Heavy Metal Tolerance of Soil Microbial Communities along Two Heavy Metal-Polluted Gradients in Coniferous Forests. Appl. Environ. Microbiol. 62, 420–428.

R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Sánchez-Monedero, M.A., Mondini, C., de Nobili, M., Leita, L., Roig, A., 2004. Land application of biosolids. Soil response to different stabilization degree of the treated organic matter. Waste Manag. 24, 325–332. doi:10.1016/j.wasman.2003.08.006

Scheckel, K.G., Chaney, R.L., Basta, N.T., Ryan, J.A., 2009. Chapter 1 Advances in Assessing Bioavailability of Metal(Loid)s in Contaminated Soils, in: Advances in Agronomy. Elsevier, pp. 1–52.

Schütte, U.M.E., Abdo, Z., Bent, S.J., Shyu, C., Williams, C.J., Pierson, J.D., Forney, L.J., 2008. Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. Appl. Microbiol. Biotechnol. 80, 365–380. doi:10.1007/s00253-008-1565-4

Singh, R.P., Agrawal, M., 2008. Potential benefits and risks of land application of sewage sludge. Waste Manag. 28, 347–358. doi:10.1016/j.wasman.2006.12.010

Stefanowicz, A.M., Niklińska, M., Laskowski, R., 2008. Metals affect soil bacterial and fungal functional diversity differently. Environ. Toxicol. Chem. 27, 591–598. doi:10.1897/07-288.1

Tipayno, S., Kim, C.-G., Sa, T., 2012. T-RFLP analysis of structural changes in soil bacterial communities in response to metal and metalloid contamination and initial phytoremediation. Appl. Soil Ecol., Microorganisms and the Sustainable Management of Soil 61, 137–146. doi:10.1016/j.apsoil.2012.06.001

Tipping, E., 1994. WHAMC—A chemical equilibrium model and computer code for waters, sediments, and soils incorporating a discrete site/electrostatic model of ion-binding by humic substances. Comput. Geosci. 20, 973–1023. doi:10.1016/0098-3004(94)90038-8