

The impact of increasing heavy metal stress on the diversity and structure of the bacterial and actinobacterial communities of metallophytic grassland soil

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Abstract A study was undertaken to investigate the bacterial community found in metallophytic grassland soil contaminated with Zn and Pb. We hypothesised that such communities would be tolerant of additional heavy metal stress due to phylogenetic and functional adaptation. In microcosm experiments, lasting 51 days, denaturing gradient gel electrophoresis (DGGE) analyses was used to compare the total bacterial and actinobacterial communities in non-amended soils and those to which additional Pb and Zn concentrations were added. There was a decrease in total bacterial diversity with each addition of Pb and Zn; in contrast, the actinobacterial community diversity remained unaffected. The community structures were analysed using multivariate analyses of the DGGE profiles. Total bacterial community profiles showed two distinct groups sharing less than 80% similarity, irrespective of Pb and Zn addition. The first contained profiles sampled during the first 7 days of the experiment; the second contained those sampled from day 10 onwards. Actinobacterial profiles from those that were non-amended showed a similar distribution to those of the total bacterial community. However, in soil amended with fivefold additional Pb and Zn, all the profiles shared more than 80% similarity. Raup and Crick analyses suggested that total bacterial soil communities were subject to deterministic selection becoming significantly similar as the experiment progressed, but this was inhibited by the highest concentration of additional Pb and Zn. Actinobacterial communities showed a similar response but were less affected by elevated Pb and Zn concentrations. These data

indicate that the diversity of the actinobacterial community was not negatively affected by additional heavy metal stress in contrast to total bacterial community diversity.

Keyword Soil bacteria · Heavy metal contamination

Introduction

The metallophytic grassland habitats of the northern Pennines were formed as a result of a short period mining activity between the mid-eighteenth and mid-twentieth centuries. They comprise of spoil heaps from abandoned mine workings and alluvial deposits on the flood plains of the local surface waters that receive water from abandoned mine workings. They have high levels of heavy metal contamination, particularly Pb and Zn, and represent a unique habitat, containing a highly diverse flora.

A number of studies have shown that the presence of heavy metals in soils have had a negative impact on the diversity of the microbial community in soil (Joynt et al. 2006; Li et al. 2006) and have also led over time to the adaptation of a community more able to tolerate their presence (Schmidt et al. 2005; Frey et al. 2006). Indeed, some estimations suggest that heavy metal pollution can reduce the diversity of bacterial populations by 99.9% (Gans et al. 2005) affecting rarer taxa, whose activity may be functionally significant, most especially. Two theories have been postulated to explain how heavy metals affect microbial community diversity, the first suggests that stressed systems are more stable than those which are not, as they have acquired physiological adaptations that enable them to maintain function the face of additional stresses (Odum 1981). The second states that non-stressed systems have a greater stability, as they have more resources at their

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disposal that can be deployed to maintain ecosystem function in the face of stress (Tobor-Kaplan et al. 2005). These hypotheses were tested by Griffiths et al. (2001) using grassland, agricultural and industrial soil and Kuan et al. (2006) with grassland soils subject to various disturbances (biocide, sewage sludge application). Both studies challenged the soils with heat and Cu stress and measured the effects on soil functional stability. In each study, the less stressed soils had greater stability supporting the hypothesis that these soils have more resistance to stress than those previously exposed to environmental challenge.

The significance of Actinobacteria within the microbial communities of soils contaminated with heavy metals is unresolved; Gremion et al. (2003), studying the bacterial community of a soil contaminated with Cd, Cu and Zn, showed that Actinobacteria, dominated clone libraries derived from both the bulk soil and the rhizosphere of *Thlaspi caerulescens*, a heavy metal hyperaccumulator. Moreover, 70% of the actinobacterial clones belonged to the genus *Rubrobacter*, a widespread taxa in soils, that has yet few cultured representatives (Holmes et al. 2000). In contrast, other studies on the impact of heavy metal contamination on bacterial community structure have reported that there was a significant decline in the contribution of Actinobacteria to the bacterial community. In a forest soil contaminated with Cd, Cu, Zn and Pb, there was a significant decrease in the actinobacterial marker using a phospholipid fatty acid (PFLA) protocol compared to control soils (Frey et al. 2006). The microbial community also showed more general changes in response to heavy metal amendment, one that was more tolerant of heavy metals but with a compromised functional activity.

In this study, we wished to test the hypothesis that microbial diversity and structure would be resistant to additional heavy metal input in to the soil due to adaptation and functional changes within the community predisposing it to cope with the increased stress (Odum 1981). Although previous studies have identified that short-term addition of heavy metals do not model long-term effects on microbial activity (Renella et al. 2002), our aim was to explore the effects of sudden acute exposure on communities subjected to long-term heavy metal concentrations in order to understand how bacterial and actinobacterial communities responded.

Materials and methods

Field sites and sampling strategy

The site at Bardon Mill, grid reference 779 644 (OS Landranger 87) was a former spoil heap. The site was predominantly bare earth, but there were patches of sparse

vegetation, comprised of grass (*Festuca ovina*). Soil was collected from non-vegetated areas; triplicate samples (1 kg) were excavated from the top 10 cm of the soil.

Immediately after collection, soil was transported to the laboratory and stored at 4°C until required. Prior to being used in microcosms, soil was sieved (2 mm) and air-dried overnight (Baxter et al. 2006).

Analyses of soil samples

Total metal content was determined in duplicate. Samples (0.5 g) were air-dried and digested in 10 ml of boiling aqua regia (HNO₃/HCl, 1:3, v/v). Once cool, solutions were filtered (Whatman no. 1 filter paper) to remove any insoluble sediment or organic material. The solution was then made up to 100 ml with distilled water. Bioavailable concentrations of Pb and Zn were determined using ethylenediaminetetraacetic acid (EDTA) and acetic acid extraction (Quevauviller 1998). Samples were analysed for total metal content using a flame atomic absorption spectroscope (Perkin Elmer/AAAnalyst 100), with “Calib” software. Samples were calibrated against Pb and Zn solutions. Bioavailable metal analyses were performed using inductively coupled plasma mass spectrometry (ICP mass spectrometer XSeries II Thermo Electron) according to the protocol of Intawongse and Dean (2008).

Carbon and nitrogen analyses were done with duplicate samples of soil (1 g) using a Carlo Erba 1108 Elemental Analyser controlled with CE Eager 200 software, run in accordance with the manufacturer’s instructions and weighed using a Mettler MT 5 Microbalance by the Advanced Chemical and Material Analysis, University of Newcastle-upon-Tyne, Newcastle, UK.

Soil microcosms

For each treatment, three microcosms, consisting of 500 g of soil in each replicate, were established in 600-ml glass beakers. The soil was loamy sand (84.2% sand, 10.5% silt and 5.3% clay) of pH 6.0. Elemental analyses showed that the soil contained 0.57% organic carbon and 0.12% nitrogen. The concentration of extractable Pb and Zn in Bardon Mill soil was 3 g kg⁻¹ and 1 g kg⁻¹, respectively. In experimental microcosms, these levels were supplemented with PbNO₃ and Zn ZnSO₄ to give a twofold and fivefold increase over those concentrations. A carrier system was used to ensure consistent metal application. Appropriate concentrations of PbNO₃ and ZnSO₄ dissolved in dH₂O were applied to 1-g aliquots of sterile soil; these were incubated overnight to allow evaporation of the water. The aliquots were cut into the rewetted soil by thorough mixing. The bioavailable concentrations of Pb and Zn were then determined. In the non-supplemented soil, these were found

to be 60 ± 3 and 57 ± 2 mg kg⁻¹ of Pb and Zn, respectively. After the two- and fivefold supplementation with PbNO₃ and ZnSO₄, the bioavailable concentrations were 147 ± 20 and 369 ± 24 mg kg⁻¹ for Pb and 110 ± 9 and 203 ± 15 mg kg⁻¹ for Zn, respectively.

Soil water content was measured and found to be constant during the experiment at 20% of the water-holding capacity of the soil. Initial samples (day 0) were taken immediately after the microcosms were set up. Between samplings microcosms were incubated at 18°C in a water-saturated atmosphere (Rasmussen and Sorensen 2001). Samples of soil (1 g) were collected immediately after setting up the microcosms (day 0) and then on days 2, 7, 10, 16, 23, 30, 37, 44, and 51.

DNA extraction and PCR amplification of 16S rRNA genes from soil microcosms

DNA was extracted from 1 g of soil with an UltraClean Soil DNA kit (MoBio) and stored at -20°C. Total bacterial diversity was monitored by polymerase chain reaction amplification of V3 region of the bacterial 16S rRNA gene performed using primers 1 and 2 as described by Muyzer et al. (1993). Selective amplification of Actinobacteria were performed using Primer F243 (5'-GGAT-GAGCCCGCGGCCTA-3') and primer R513 (5'-CGGCCGCGGCTGCTGGCACGTA-3') (Heuer et al. 1997). Amplification of DNA and the thermocycling conditions used were as described by Baxter and Cummings (2006). All reactions were carried out using the Eppendorf Mastercycler Gradient PCR machine. PCR products were analysed by agarose (1.5% w/v) gel electrophoresis and ethidium bromide staining.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) analysis was performed using the D-Code DGGE system (Bio-Rad) as described by Baxter and Cummings (2006). For analysis of bacterial populations, PCR products were loaded on to polyacrylamide gels (12%) with a denaturant gradient ranging from 35% to 55% denaturant (with 100% denaturant corresponding to 7 M urea plus 40% v/v formamide). Gels were run at 60°C for 10.5 h at 80 V. Gels were stained in SYBR Green I (1:10,000 dilution in 1× Tris-acetate-EDTA buffer; Molecular Probes) for 30 min and viewed with UV transillumination and photographed using the Gel Doc 2000 gel documentation system (Bio-Rad).

Statistical analysis of DGGE profiles

For each treatment, PCR products, derived from DNA extracted from three independently derived communities,

were analysed by DGGE. The patterns obtained from these replicates were highly reproducible. To facilitate sample processing, PCR products were pooled for each treatment. The pooled PCR products were then subjected to triplicate DGGE analyses to confirm that the band patterns were reproducible with those derived from the independently derived community replicates (Sigler and Turco 2002; Brodie et al. 2003).

DGGE profiles were analysed using Quantity One 4.1.1 software (Bio-Rad). The lane background was subtracted by using a rolling disc algorithm, and the lanes were 'normalised' to ensure that all lanes contained the same amount of total signal. Lane profiles were corrected for differences in migration rate by manually assigning R_f lines to marker lane bands (Griffiths et al. 2003). To facilitate the efficient handling of samples and data, each sample from each replicate was screened with PCR-DGGE to assess replicate variability in DGGE profiles. When the reproducibility of PCR profiles within a treatment had been ensured, all PCR products derived from a common treatment were then pooled for the final analysis in order to accommodate the electrophoresis of samples on a single gel.

Band patterns were analysed using three methods. Correspondence analysis (CA) was performed to visualise the relationships among band patterns using Statistica 6.0 software (StatSoft). Cluster analyses of band patterns were also performed with the unweighted pair group method using averages based on the Sorensen index of similarity. Band pattern data were analysed using Raup and Crick's index of similarity. The Raup and Crick index of similarity was used to test if similarities observed between soil cultures profile patterns were significantly higher or lower than would be observed by chance (Rowan et al. 2003). Raup and Crick and cluster analyses were calculated using the PAST statistical analysis software. Student's *t* test was used for statistical difference between means ($P < 0.05$).

Results and discussion

DGGE analyses of microcosm soil

Visual comparison of the total bacterial DGGE profiles and Shannon–Weaver diversity index (H') calculated for each treatment indicated that, in non-amended microcosms soil, there were 70 individual bands ($H' = 3.8\pm 0.06$), significantly more ($P < 0.05$) than the 46 ($H' = 3.3\pm 0.1$) and 30 ($H' = 2.8\pm 0.13$) bands in soils amended with two- and fivefold additional Pb and Zn, respectively. The actinobacterial profiles showed no significant difference with 36 bands in non-amended soils ($H' = 2.9\pm 0.16$) and 33 bands ($H' = 3.0\pm 0.06$) in soils amended with fivefold more Pb and Zn. However, straightforward measures of diversity do not

resolve community structure and diversity, due to primer bias, resulting in preferential amplification of more abundant sequences and multiple taxa being represented by a single band or a single taxa by several bands (Duarte et al. 2001; Joynt et al. 2006). Therefore, the use of multivariate analysis provides a more sensitive means of evaluating the overall changes in microbial community profiles, enabling a higher resolution by exploring both pattern and intensity of bands within profiles (Ogino et al. 2001).

Cluster analyses were combined with ordination using CA to aid in visualising the relationship between different community profiles generated by DGGE analyses (Ramette 2007). The cluster analysis of the total bacterial community in non-amended soil demonstrated that samples taken between days 0 and 16 and those derived from the later sampling dates (day 23 onwards) formed two clusters (Fig. 1a), and indicated that the similarity between the two clusters was less than 80%. Correspondence analysis indicated that 46.2% of the variance in the total bacterial community patterns could be explained by the first two dimensions (Fig. 1a). The first axis that explained 27.8% of the inertia separated the earlier from the later profiles and reflected the groupings observed in the cluster analysis (Fig. 1b). In microcosms amended with an extra two- or fivefold concentration of Pb and Zn, CA showed that there

was again a differentiation of the total bacterial community profiles into those derived from samples from days 0, 2 to 7 and those from the later time points (Fig. 1b,c). Cluster analyses indicated that the differentiation of sample profiles from the early sample points (days 0, 2 and 7) compared to the later samples was robust in soils amended with both two- and fivefold additional heavy metal and that the profiles from these early samplings shared less than 75% similarity with the later profiles (Fig. 1b,c).

The actinobacterial profiles derived from microcosms without supplementation and with an additional fivefold Pb and Zn were also analysed. In non-amended microcosm soil, three distinct clusters were seen in the CA; the profiles derived from days 0 to 2 were separated from the other profiles along the first axis that explained 40.3% of the inertia. The profiles from days 23 and 30, were then separated from the remaining patterns (days 7, 10, 16, 34–51) along the second axis (Fig. 1d), explaining 16.6% of the variation. Analyses using the Sorensen coefficient demonstrated that the similarity between the three clusters was less than 80% (Fig. 1d). In contrast to the total bacterial community profiles, it appeared that adding fivefold more Pb and Zn to the soil increased the similarity between profiles. Although CA indicated that the initial two samples, days 0 and 2, were separated from the remaining

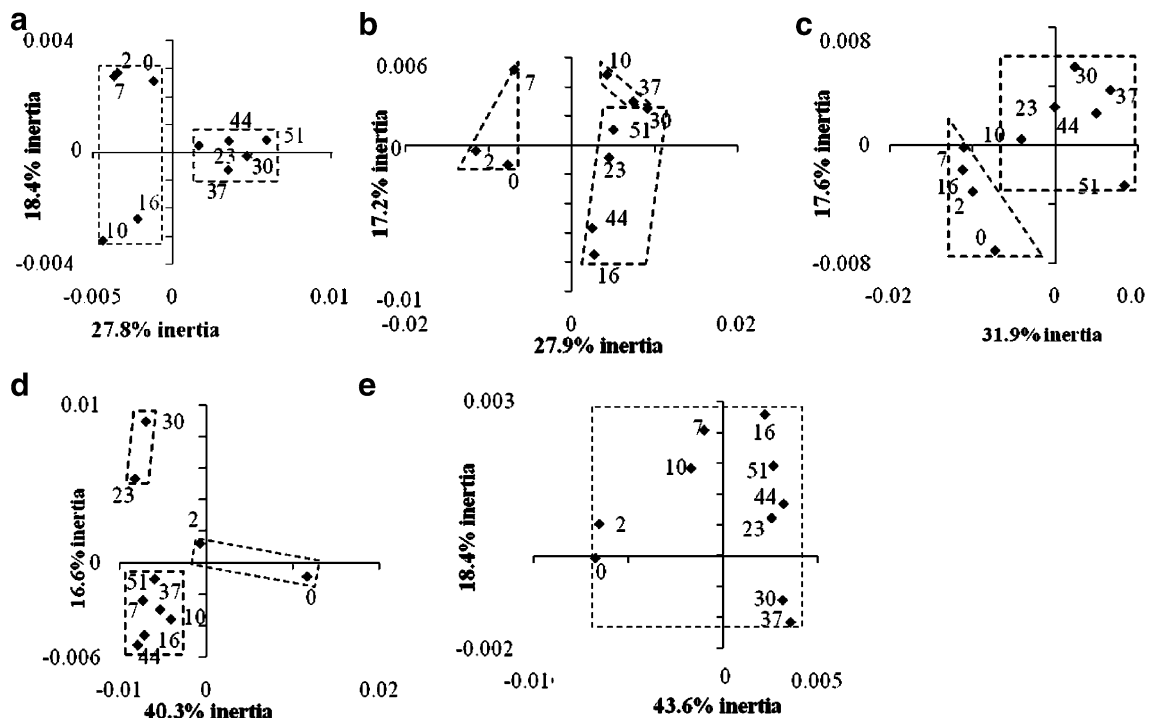


Fig. 1 Cluster and ordination analyses of bacterial and actinobacterial DGGE community profiles identifying the relationships between the band patterns. Correspondence analyses of the total bacterial community profiles of microcosm soils subjected to **a** no amendment or with **b** twofold **c** fivefold additional extractable Pb and Zn. Similarly, **d**

actinobacterial community profiles for soils subjected to no additional Pb and Zn or with **e** fivefold additional Pb and Zn added. *Dashed lines* enclose those profiles that share >80% similarity in using cluster analyses with Sorensen coefficient. *Numbers* identify the sample day

community profiles (Fig. 1e), cluster analyses demonstrated that all the profiles shared a greater than 80% similarity, and indeed, all but the initial day 0 sample had population patterns with similarities of 88% or more (Fig. 1e).

The use of the Raup and Crick (S_{RC}) index to quantify the relationships between the total bacterial community profiles indicated that, from day 23 in non-amended soils and day 16 in soils of twofold additional Pb and Zn, profiles showed a similarity to each other, greater than could be explained by the chance matching of bands. This suggests that deterministic selection was acting on the community (Table 1, non-amended soil and soil amended with a twofold increase in Pb concentration; Rowan et al. 2003). However, the trend towards significantly similar population profiles was much less pronounced in microcosms amended with an additional

fivefold Pb and Zn, occurring only from day 37 onwards (Table 1, soil amended with a fivefold increase in Zn concentration). The Raup and Crick analyses showed that actinobacterial communities under both treatments showed a drift towards significantly similar community profiles, such that the comparison of profiles from day 10 onwards under both treatments showed high similarity between the community profiles (Table 2).

The total bacterial community diversity decreased in response to additional heavy metal application in a dose-dependent manner. This response does not support the hypothesis that pre-exposed communities would be more tolerant of additional heavy metal stresses but rather supports the observation by Tobor-Kaplan et al. (2005) that ecosystem stability to heavy metals was not enhanced by long-term

Table 1 A comparison of similarity values (S_{RC}) for the bacterial community profiles from Bardon Mill soil microcosms over a 51-day incubation

	Day 0	Day 2	Day 7	Day 10	Day 16	Day 23	Day 30	Day 37	Day 44	Day 51
Non-amended soil										
Day 0	1	1	0.98	0.74	0.56	0.84	0.77	0.94	0.94	0.43
Day 2		1	1	0.99	0.95	0.96	0.89	0.96	0.81	0.79
Day 7			1	0.79	0.92	0.97	0.92	0.53	0.56	0.33
Day 10				1	0.97	0.67	0.49	0.88	0.74	0.34
Day 16					1	0.98	0.93	0.90	0.57	0.76
Day 23						1	1	1	0.99	0.99
Day 30							1	1	1	1
Day 37								1	1	1
Day 44									1	1
Day 51										1
Soil amended with a twofold increase in Pb concentration										
Day 0	1	1	0.97	0.28	0.62	0.60	0.28	0.40	0.64	0.83
Day 2		1	0.99	0.41	0.39	0.17	0.01	0.26	0.25	0.51
Day 7			1	0.77	0.21	0.74	0.57	0.97	0.61	0.70
Day 10				1	0.94	0.94	0.90	0.99	0.60	0.88
Day 16					1	0.98	0.98	0.95	0.96	0.99
Day 23						1	0.99	1	0.99	0.99
Day 30							1	0.99	0.96	0.95
Day 37								1	0.95	0.98
Day 44									1	0.99
Day 51										1
Soil amended with a fivefold increase in Zn concentration										
Day 0	1	0.97	0.76	0.34	0.84	0.89	0.92	0.90	0.84	0.84
Day 2		1	0.99	0.32	0.99	0.98	0.63	0.98	0.99	0.8
Day 7			1	0.50	0.98	0.82	0.80	0.80	0.92	0.34
Day 10				1	0.82	0.28	0.28	0.002	0.24	0.33
Day 16					1	0.98	0.86	0.64	0.84	0.14
Day 23						1	0.99	0.99	0.88	0.51
Day 30							1	0.93	0.89	0.54
Day 37								1	0.99	0.97
Day 44									1	0.99
Day 51										1

A $0.95 > S_{RC} > 0.05$ similarity no greater than expected by chance (null hypothesis upheld), $S_{RC} < 0.05$ significant dissimilarity, $S_{RC} > 0.95$ significant similarity (null hypothesis fails)

Table 2 A comparison of similarity values (S_{RC}) for the actinobacterial community profiles from Bardon Mill soil microcosms over a 51-day incubation

	Day 0	Day 2	Day 7	Day 10	Day 16	Day 23	Day 30	Day 37	Day 44	Day 51
Non-amended soil										
Day 0	1	0.59	0.06	0.27	0.05	0.28	0.16	0.34	0.02	0.22
Day 2		1	0.79	0.63	0.46	0.89	0.87	0.94	0.65	0.95
Day 7			1	0.99	0.8	0.77	0.80	0.97	0.99	0.96
Day 10				1	0.98	0.88	0.88	0.99	0.99	0.99
Day 16					1	0.99	0.9	1	1	0.97
Day 23						1	1	1	0.98	0.99
Day 30							1	0.99	0.95	0.98
Day 37								1	1	1
Day 44									1	0.99
Day 51										1
Soil amended with a fivefold increase in Pb and Zn concentrations										
Day 0	1	0.92	0.69	0.88	0.56	0.61	0.50	0.28	0.40	0.60
Day 2		1	0.96	0.99	0.66	0.97	0.64	0.45	0.83	0.93
Day 7			1	0.99	0.99	0.99	0.91	0.70	0.96	0.99
Day 10				1	0.98	0.99	0.99	0.91	0.95	0.99
Day 16					1	0.97	0.89	0.84	0.88	0.97
Day 23						1	0.99	0.98	1	1
Day 30							1	1	0.97	0.99
Day 37								1	0.95	0.91
Day 44									1	1
Day 51										1

A $0.95 > S_{RC} > 0.05$ similarity no greater than expected by chance (null hypothesis upheld), $S_{RC} < 0.05$ significant dissimilarity, $S_{RC} > 0.95$ significant similarity (null hypothesis fails)

exposure to this stress. Other studies have identified negative impacts on bacterial community diversity exposed to elevated heavy metal concentrations that agree with these observations (Sandaa et al. 1999; Joynt et al. 2006; Li et al. 2006; Wu et al. 2006). Frey et al. (2006) analysed the impact on community structure of heavy metal contamination and observed a shift in the community structure measured using both PLFA and terminal restriction fragment length polymorphism suggesting that the resulting community had a reduced functional diversity compared to control soils. They speculated that the changes caused by the heavy metal application were to shift to more metal tolerant species. However, in studies of hyporheic (sediment) communities from heavy metal contaminated watercourses, no correlation between microbial diversity and metal concentration was apparent nor that the presence of heavy metals led to a more resistant bacterial community (Feris et al. 2003).

In contrast to the total bacterial community, the actinobacterial diversity was not significantly affected by the addition of fivefold more extractable heavy metal, a response that would appear to support the original hypothesis. There are conflicting data on the specific changes of soil microbial communities subjected to heavy metal stress (Giller et al. 1998). For example, PFLA and DGGE data have indicated that fungi, Proteobacteria and

Gram-negative bacteria increase under such conditions, whereas Actinobacteria, rhizobia and Firmicutes decrease (Kelly et al. 1999; Hinojosa et al. 2005; Lorenz et al. 2006; Renella et al. 2007). However, a study of forest soil indicated an increase in Gram-positive bacteria and a decrease in Gram-negative organism (Åkerblom et al. 2008). In contrast, in some heavily contaminated soils, Actinobacteria have been shown to dominate clone libraries, high numbers have also been identified in serpentine soils (Gremion et al. 2003; DeGroot et al. 2005). The observations of the effects of additional Pb and Zn addition to actinobacterial community structure may be an artefact of the analyses; for example, it has been demonstrated that Actinobacteria are dominant with micro-aggregates of soil (Mummey and Stahl 2004), where they may be protected from additional heavy metal addition. However, in metallophytic soils, it seems that Actinobacteria are a diverse taxa that are resistant to exposure to additional Pb and Zn. Whether this reflects a community that has become functionally adapted to this soil environment (Almås et al. 2004; Schmidt et al. 2005) or simply an artefact of their dominance within protected micro-environments requires further study.

The findings that the most common bacterial taxa present in the metallophytic soils are adversely affected by increasing

Pb and Zn levels while actinobacterial community diversity is not suggest that studies on the impact of heavy metals in soil need to account for these differences between different functional and phylogenetic groupings in order to draw robust conclusions.

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