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Microbial Communities in Long-Term Heavy Metal Contaminated Ombrotrophic Peats

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Abstract High concentrations of heavy metals are known to be toxic to many soil organisms. The effects of long-term exposure to lower levels of metals on the soil microbial community are, however, less well understood. The southern Pennines of the U.K. are characterised by expanses of ombrotrophic peat soils that have experienced deposition of high levels of heavy metals since the mid to late 1800s. Concentrations of metals in the peat remain high but the effect of the contamination on the *in-situ* microbial communities is unknown. Geochemical and molecular polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and sequencing techniques were used to derive new information on the metal chemistry and microbial populations in peat soils from six locations in the southern Pennines. All sites were highly acidic (pH 3.00–3.14) with high concentrations of potentially toxic heavy metals, particularly porewater Zn and particulate-associated Pb. The results also reveal a split in site characteristics between the most polluted sites with the highest levels of bioavailable metals (Bleaklow, FeatherBed Moss and White Hill) and those with much lower bioavailable metals (Cowms Moor, Holme Moss and Round Hill). There was no difference in the number of dominant bacterial species between the sites but there were significant differences in the species composition. At the three sites with the highest levels of bioavailable metals, bacterial species with a high similarity to acidophilic sulphur- and iron-oxidizing bacteria and those from high metal environments were detected. The transformations carried out by these metal mobilising and acid producing bacteria may make heavy metals more bioavailable and therefore more toxic to higher organisms. Bacteria with similarity to those typically found in forest and grassland soils were documented at the three sites with the lowest levels of bioavailable metals. The data highlight the need for further studies to elucidate the species diversity and functionality of bacteria in heavy metal contaminated peats in order to assess implications for moorland restoration.

Keywords Bacteria · Heavy metals · Microbial communities · Ombrotrophic peat · PCR-DGGE · Polluted upland soils

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1 Introduction

High concentrations of heavy metals are known to be toxic to many soil organisms (see Giller et al. 1998

for a review) but long-term exposure to even modest levels can also be detrimental to soils (McGrath et al. 1988). Metals can adversely affect soil quality by either reducing microbial biomass (Brookes and McGrath 1984; Fliessbach et al. 1994) or by inhibiting specific functional groups of microbes (Frostegard et al. 1996) responsible for processes such as organic matter mineralization and nitrogen fixation (Chaudri et al. 1993). As heavy metals are known to persist in soils (Brookes 1995), the effects on microbial communities and soil functions may be long-lived. The dearth of field-based studies on the microbial populations of metal-affected soils, however, means that the effect of long-term exposure to elevated metals is poorly understood.

Some studies have investigated the impact of heavy metal contamination in soils using culture methods and community-level physiological profiling (e.g. Kelly et al. 1999; Ellis et al. 2001). Traditional culture methods, however, may provide a false impression of the microbial community as many species may not be detected. Biomass and changes in respiration have also been used as indicators of soil pollution (e.g. Chander and Brookes 1991; Baath 1992); however, this will not highlight changes in diversity and therefore functionality of the microbial biomass and may not be a useful indicator of the effects of contamination. Bacterial community shifts may be more helpful in assessing the impact of pollution and molecular analysis based on 16S rDNA genes of soil bacteria has opened up opportunities to investigate total microbial populations in soils. Thus far, however, there are only relatively few studies where the dominant soil microbial community in heavy metal contaminated soils has been identified using molecular analysis (Dell'Amico et al. 2007). Instead, most work has concentrated on effects of metals on numbers of species without actual species identification (Smit et al. 1997; Li et al. 2006) or on community-based microbiological measurements such as phospholipid fatty acid analysis (Sandaa et al. 1999). In peat soils there has been some recent work on methanogen and methanotroph communities, due in part to their important role in biogeochemical cycling of carbon (e.g. Hales et al. 1996; Dedysh et al. 1998; McDonald et al. 1999). Little is known, however, about the overall diversity of bacteria in peat soils; how the indigenous bacterial community structure is affected by heavy metal pollution, or how the bacteria in these soils contribute to the cycling and fate of heavy metals.

The complex relationships between soil micro-organisms and their interactions with each other and their environment may make it difficult to use microbial community composition alone as an indicator of soil quality (Schloter et al. 2003; Dickinson et al. 2005). Species composition, however, affords a relative view of community shifts that may be caused by different levels of pollutants (Sandaa et al. 1999; Avidano et al. 2005) and with a shift in species community composition comes a potential change in the functional diversity and therefore in the biogeochemical processes that are occurring in the environment. The presence or absence of certain indicator organisms which are known to be very important for ecosystem functioning, such as *Rhizobium* or arbuscular mycorrhiza, are also useful in determining soil quality (Schloter et al. 2003).

The role of microorganisms in heavy metal cycling and the effect on microbes of these metals may be particularly relevant in the UK where there is a long history of ore exploitation and industrialisation. Many upland regions have been polluted for centuries at potentially-toxic levels by heavy metal deposition (Tipping et al. 2006). The toxicity of heavy metals to soil organisms depends on many chemical, physical and biological factors including, pH, chelation capacity of the soil substrate, competitive interactions such as plant cover and the presence of water (Sterritt and Lester 1980). The influence of soil chemistry, in particular pH and organic matter, on metal partitioning and bioavailability means that measurement of the total metal concentration is best thought of as a measure of the potential pool of metals available to microorganisms. Current research has shown that inputs of heavy metals into upland reservoirs in the Southern Pennine region of the UK are high as historically contaminated peat soils erode and transport metals into surface waters (Shotbolt et al. 2006; Rothwell et al. 2007). Thus whilst current atmospheric deposition is low in comparison to previous levels (Baker 2001), a legacy of metal contamination and the potential to adversely affect soil micro-organisms remains.

Elucidation of the impact of soil heavy metal pollution on bacterial populations in the field is, however, problematic. It is difficult to isolate the influence of heavy metals on microbial populations as other physical and chemical factors as well as other soil

organisms will influence metal bioavailability. It is particularly difficult to isolate the influence of individual heavy metals on soil microbial populations. Site selection to meet the aims of the study may also be problematic. In particular it may be difficult to find sites with a range of heavy metal concentrations whilst keeping other parameters constant, in particular it is often difficult to find a 'control' site of similar environmental conditions without metal contamination. This is certainly true in the Southern Pennines. A further consideration is that heavy metal concentrations vary considerably over small distances (Rothwell et al. 2005) and it may be difficult to determine the metal concentration relevant to the microenvironment in which the soil bacteria exist. Nevertheless, exploratory field based data are a useful start in investigation of the long term impacts of heavy metal pollution on microbial populations in upland peat soils.

This paper presents new molecular information on the dominant microbial populations of heavy metal contaminated peat soils from the Peak District, U.K. The objectives are to:

1. Determine the degree of heavy metal contamination of peat soils and potential bioavailability at six locations across the Peak District.
2. Use molecular analysis based on 16S rDNA genes to determine and identify the dominant microbial populations at each of the sites.
3. Determine whether the degree of metal toxicity has had an effect on the dominant microbial population.

2 Materials and Methods

2.1 Site Description and Sample Collection

The Southern Pennines are characterised by wide expanses of ombrotrophic or blanket peat bogs (Fig. 1). Since the early 19th century the area has experienced high levels of atmospheric heavy metal pollution and acid deposition (Evans and Jenkins 2000; Tipping and Smith 2000; Shotbolt et al. 2006) originating from the urban-industrial conurbations of Greater Manchester and Merseyside to the west; Sheffield, Bradford and Leeds to the north and east. Localised pollution associated with historical lead mining and smelting has also contributed to metal deposition onto the peat bogs (Livett et al. 1979). Recent research has demonstrated that high concentrations of heavy metals persist in the peat soils (Rothwell et al. 2005) and that erosion continues to transfer peat contaminated with metals to Pennine reservoirs (Shotbolt et al. 2006).

In August 2005 peat samples were collected from six ombrotrophic bog sites located between 450 and 610 m above sea level in the Southern Pennines of the UK (Table 1). At all sites the water and nutrient supply to the soils is solely from rainfall with no mineralogical input from the underlying bedrock. Variations in mean annual rainfall and proximity to historical and contemporary pollution sources meant we expected to find a range of heavy metal contamination. Three samples of peat were collected from each site at a depth of 8–10 cm below the surface. This depth was chosen as Pb contamination is greatest in the upper layers of the peat profile (Jones and Hao 1993) and peaks at around 8–10 cm in peats in the Southern Pennines (Rothwell et al. 2007). It is also above the anoxic layer. Sterilised equipment and sealable clean polythene bags were used to collect samples which were kept dark and stored at 4°C prior to analysis.

Vascular plant species diversity is low at all sites and dominated by *Eriophorum vaginatum*. Vegetation assemblages are closest to the National Vegetation Classification M19 and M20 habitats (*E. vaginatum* blanket mire (M20) or *Calluna vulgaris* – *E. vaginatum* blanket mire (M19)) although *Erica tetralix* is found in equal or greater numbers than *Calluna vulgaris* at all sites. *Vaccinium myrtillis* was also observed at four sites (Featherbed Moss, Bleaklow, Cowms Moor and Round Hill).

2.2 Geochemical Analyses

The pH, water and organic matter content of the soils were determined on three samples from each of the six sites. pH was determined using a glass probe in a one part soil, one part deionised water slurry immediately on return to the laboratory. Water content was determined after drying at 35°C until a constant weight was obtained. Organic matter was determined by loss-on-ignition after heating 2 g of oven-dry peat to 550°C for 4 h (Heiri et al. 2001). The three sub-samples were then amalgamated to provide sufficient material for the remaining analysis.

Porewater was extracted from the combined samples for dissolved organic carbon (DOC), major anions

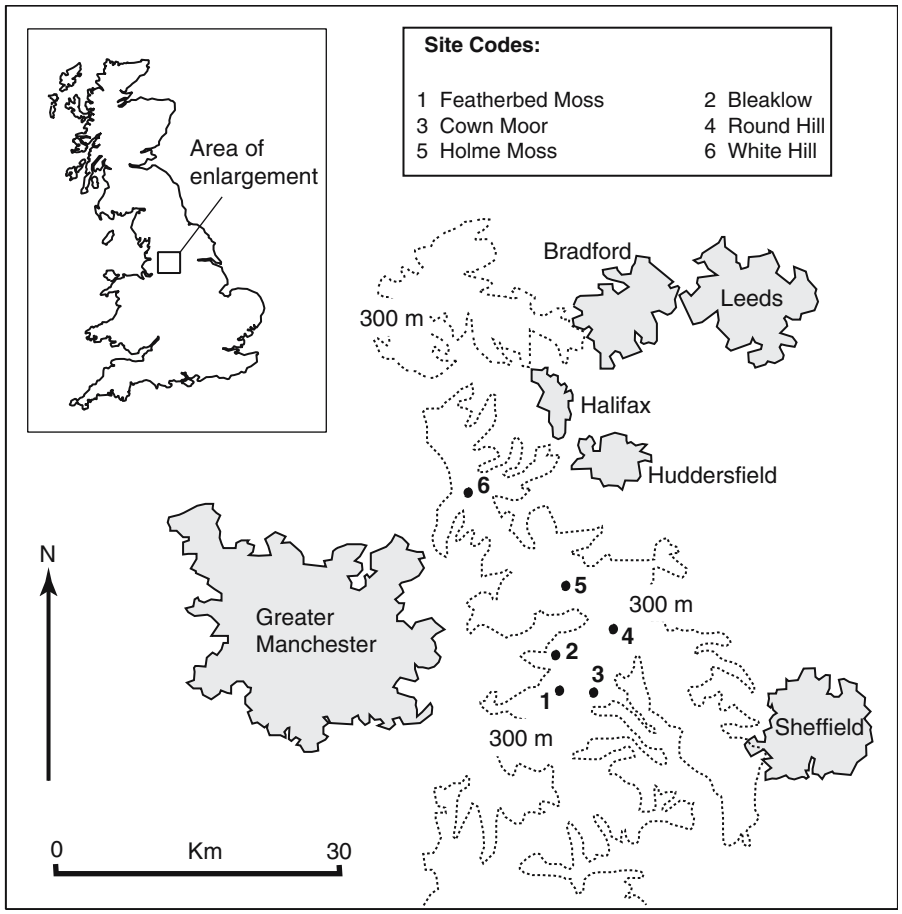

Fig. 1 Southern Pennines and study sites showing their proximity to major conurbations in the area

and cations and heavy metal analysis. 20 ml deionised water was added to 20 g sample and the resulting slurry centrifuged at 5000 g and filtered to 0.45 μm. Dissolved organic carbon (defined as organic carbon remaining in solution after centrifugation) was determined by combustion using an Elementar LiquiTOC. Major anions fluoride (Fl^-), chloride (Cl^-), nitrate (NO_3^-), phosphate (PO_4^{3-}) and sulphate (SO_4^{2-}), were analysed by ion chromatography (Dionex ICS-2500). The base cations sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) were analysed by Inductively Coupled Plasma Optical Emission Spectrometry (Varian Vista-Pro).

Three heavy metal fractions were determined: total metals (which include all particulate-associated and dissolved metals and represents the potentially available metal pool), metals in porewaters (which represents the available metal pool to soil water dwelling

Table 1 Sample site location and altitude

Site	Latitude/Longitude	Altitude(m asl)
Featherbed Moss	53°25′32″N, 01°51′57″W	520
Bleaklow	53°27′25″N, 01°51′36″W	610
Cowms Moor	53°25′15″N, 01°49′04″W	500
Round Hill	53°29′02″N, 01°46′42″W	490
Holme Moss	53°31′38″N, 01°51′13″W	520
White Hill	53°36′52″N, 02°00′43″W	450

organisms) and free metal ions. Free-ions represent the fraction of porewater metals that are not associated with any substance other than water; i.e. not sorbed to dissolved organic material or inorganic ligands and are therefore the most bioavailable fraction.

To determine total metal concentrations, 0.5 g of sample was digested in 10 ml HNO_3 for 10 min following European Protection Agency method 3051 using a CEM Mars X Microwave. Porewater metals were determined on samples extracted as above by centrifugation and filtration then acidified to 2% HNO_3. Copper (Cu), zinc (Zn), cadmium (Cd), lead (Pb), aluminium (Al), iron (Fe) and manganese (Mn) in the digests and porewater were determined by Inductively Coupled Plasma Mass Spectrometry (Perkin Elmer Elan 6100).

Free-ion concentration was estimated using a chemical speciation model (WHAM/Model VI) that describes sorptive interactions in soils (Tipping 1998; Tipping et al. 2003). The model allows for proton buffering by weak organic acid groups and the competitive sorptive interactions of protons and major and minor metallic cations (Tipping et al. 2006). Input parameters were porewater heavy metals (including Al, Fe and Mn), base cations, DOC and pH.

2.3 Extraction of DNA, PCR-DGGE and Sequence Analysis

DNA was extracted from the three replicate samples from each site and pooled using a method described by Cresswell et al. (1992). Extraction buffer (containing 0.1 M Tris–HCl 0.1 M sodium-EDTA, 0.1 M sodium phosphate, 1.5 M sodium chloride, 0.3 mM hexadecyltrimethylammonium bromide) was added to 0.5 g of each sample which was then incubated in a sonic water bath for 10 min to detach bacteria from the particles. The samples were then centrifuged for 5 min at 5,000 g and the supernatants recovered.

Cell lysis was achieved by adding 5 µl of proteinase K (10 mg ml^{-1}) and incubating at 37°C and then adding 75 µl of SDS solution (20% *w*/*v*) and incubating for a further 2 h at 65°C. Organics in the samples were complexed using chloroform/isoamyl alcohol (24:1 *v*/*v*) and the aqueous layer recovered. The DNA was precipitated by adding 80% isopropanol and washing in 70% ethanol. The DNA pellet was then air-dried and re-suspended in 50 µl sterile water.

Polymerase Chain Reaction (PCR) was carried out to amplify the 16S rDNA regions using eubacterial, universal primers with GC-clamps attached to the forward primer. The primers were 101F-GC clamp 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GTG GCG GAC GGG TGA GTA A-3′ and 518R 5′-GGT ATT ACC GCG GCT GCT GG-3′ (Liu et al. 1997). PCR was carried out in a PTC-200 Thermocycler (GRI) in a 50 µl reaction mixture containing 10 × NH_4 reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM total dNTPs, 0.5 U Taq (BIOLINE), 1 µM of each primer (MWG-Biotech) and 0.5 µl template DNA. The temperature cycling conditions began with an initial denaturation step at 92°C for 2 min, and then five cycles of 94°C for 30 s and 40°C for 1 min and then 30 cycles of 94°C for 30 s, 50°C for 1 min and 72°C for 3 min.

PCR products were separated using the Ingeny phor-U system (GRI), on a 2 mm thick vertical gel containing 7% (*w*/*v*) polyacrylamide (SIGMA) (acrylamide/bisacyrlamide ratio of 37.5:1) with a gradient of urea and formamide denaturants from 30% to 60%. Electrophoresis was performed in 0.5 × TAE buffer for 3.5 h at 250 V and 60°C. The gel was stained for 45 min in 1× SYBR gold (Invitrogen) in 0.5 × TAE buffer. It was then rinsed with sterile milli-Q water and visualised and recorded using Gene Genius Genesnap software (Syngene). The DGGE step was repeated three times to ensure reproducibility of band formation.

Individual bands were excised from the gel using sterile 10 µl pipette tips and incubated in 30 µl of sterile Milli-Q water for 24 h at 4°C. The eluent was used in a re-amplification PCR using the original primers and the product purified using a wizard spin-column kit (Promega). These products were then used as templates in sequencing reactions with a DTCS Quick Start Kit (Beckman-Coulter United Kingdom Ltd). Sequencing reaction products were analysed with a CEQ 8000 genetic analysis system (Beckman-Coulter United Kingdom Ltd) using both forward and reverse primers (101F and 518R).

2.4 Phylogenetic Analysis

The sequences were analysed using CEQ software and forward and reverse sequences were aligned using CAP EST ASSEMBLER software (http://bio.ifom-ieo-campus.it/cap). Similarity with sequences

deposited in the Genbank EMBL and DDBJ databases was checked using BLAST (Altschul et al. 1997). Nearest relatives were determined and percentage identities with the determined sequences were recorded. 16S rDNA sequence identities between genera have been found to range from 86% to 91%, within genera from 92% to 96%, while the same species within each genus usually share similarity of 97% and above (Benlloch et al. 1995). Sequence data from this study are listed within the Genbank database under accession numbers EF367149 to EF367168.

3 Results and Discussion

3.1 Soil Acidity, Organic Matter, DOC, Anions and Base Cations

The ombrotrophic peat soils in this study were characterised by high acidity (pH 3.00–3.14) (Table 2). Soil pH was slightly lower than reported for other peat soils in the North of England and substantially lower (up to 1.9 pH units lower) than peat soils remote from urban-industrial sources of acidic precipitation. (Table 3). Concentrations of chloride, sulphate and nitrate (11.3 mg l^{-1} Cl^-, 24.1 mg l^{-1} SO_4^{2-} and 4.05 mg l^{-1} NO_3^-) are high in comparison to levels at less acidic sites. For example Proctor (2006) found an average 3.6 mg l^{-1} Cl^-, 4.7 mg l^{-1} SO_4^{2-} and 0.02 mg l^{-1} NO_3^{-1} from 55 samples collected at Plym Head, Dartmoor between 1992 and 1997.

The combination of low pH and high levels of sulphate may be indicative of long-term atmospheric deposition of sulphur in the region (Tipping and Smith 2000) primarily from emissions of sulphur dioxide from the Greater Manchester conurbation. This is despite the decline in sulphur emissions from an estimated 6400 thousand tonnes in 1970 to 700 thousand tonnes in 2005 (DEFRA 2006). The region is also downwind of two of Britain's major farming areas; Cheshire and the England–Wales border, leading to high nitrogen deposition from agricultural livestock in addition to nitrogen from fossil fuel combustion and road traffic. (Moors for the Future 2006).

pH is often the most significant factor in determining the partitioning of metals between particulate-associated and dissolved forms. In acid soils a relatively high proportion will be found as dissolved forms in the porewater and therefore more easily available to soil organisms, although the effect will vary between metals and the impact will be counteracted, to a certain extent, by competition from other cations in solution (Lofts et al. 2004).

Table 2 Water content, organic matter, pH, dissolved organic carbon (DOC) and major anions and cations found in samples from six sites across the Southern Pennines

		Bleaklow	Cowms Moor	Featherbed Moss	Holme Moss	Round Hill	White Hill
Water content	g g^{-1}	5.12	4.68	5.22	9.01	6.61	7.75
Organic matter	%	93.5	92.4	96.8	72.0	91.7	95.0
pH		3.14	3.09	3.14	3.10	3.00	3.05
DOC	mg l^{-1}	171	348	95.3	199	269	88.5
Anions							
Fluoride	mg l^{-1}	0.34	0.58	0.26	0.36	0.32	0.18
Chloride	mg l^{-1}	8.81	12.9	9.04	14.5	13.8	8.52
Nitrate	mg l^{-1}	6.51	5.15	2.49	5.04	3.07	2.13
Sulphate	mg l^{-1}	7.60	26.2	21.7	27.1	28.4	33.7
Phosphate	mg l^{-1}	0.15	Bdl	bdl	bdl	bdl	0.14
Cations							
Ca	mg g^{-1}	0.77	0.49	1.05	0.27	0.51	0.34
Mg	mg g^{-1}	0.34	0.21	0.35	0.16	0.23	0.25
Na	mg g^{-1}	0.12	0.10	0.19	0.10	0.20	0.19
K	mg g^{-1}	0.12	0.13	0.10	0.23	0.33	0.33
Al	mg g^{-1}	2.21	1.68	1.76	3.17	1.87	2.79
Fe	mg g^{-1}	8.02	2.59	2.76	2.18	3.27	4.12
Mn	μg g^{-1}	10.5	5.02	7.52	4.05	6.19	5.10

Table 3 pH in UK deep peats from studies using pH analysis methods similar to this study

Site	Location	pH
Moss Moor, Southern Pennines[a]	53°37′14″N, 02°01′32″W	3.13
North York Moors (average of two sites)[a]	54°24′08″N, 00°55′06″W 54°24′05″N, 00°49′00″W	3.29
Oxenhope Moor, West Yorkshire[a]	53°47′26″N, 01°58′54″W	3.49
North Yorkshire (average of three sites)[a]	54°02′32″N, 02°25′12″W54°05′23″N, 02°11′00″W 54°14′55″N, 02°12′42″W	3.50
Peak District (average of three sites)[a]	53°20′44″N, 01°35′18″W 53°26′01″N, 01°51′47″W 52°38′20″N, 01°51′29″W	3.62
Berwyn, N. Wales[a]	52°51′30″N, 03°26′27″W	3.91
Exmoor, S. England[a]	50°09′40″N, 03°48′28″W	3.92
Dyfed, Wales (average of three sites)[a]	52°14′31″N, 03°55′44″W 52°20′21″N, 03°00′41″W52°21′39″N, 03°40′43″W	4.11
Plym Head, Dartmoor, S. England[b]	50°22′08″N, 03°56′51″W	4.30
Sutherland, N. Scotland[c]	58°22′N, 5°00′W	4.80
Foula, Shetland Islands[c]	60°09′N, 2°06′W	4.89

[a] Proctor and Maltby (1998); [b] Proctor (2006); [c] Shotyk (1997).

In peat soils, partitioning will also be influenced by the availability of solid and dissolved organic matter (DOC) that provide the majority of sites for metal complexation. Organic matter (72–97%) and DOC (89–348 mg l^{-1}) are high at all sites (Table 2). DOC is particularly high when compared to concentrations measured in zero-tension soil lysimeter studies from other UK peatlands (13–153 mg l^{-1}) (Shotbolt and

Table 4 Total, porewater and modelled free-ion heavy metal concentrations from six sites in the Southern Pennines

		Bleaklow	Cowms Moor	Featherbed Moss	Holme Moss	Round Hill	White Hill
Total metals							
Cu	μg g^{-1}	89.1	49.0	82.5	84.1	80.3	101.2
Zn	μg g^{-1}	61.9	36.4	66.2	28.2	27.2	30.0
Cd	μg g^{-1}	0.82	0.57	2.27	0.77	1.20	0.80
Pb	μg g^{-1}	1,066	354	465	492	425	448
Porewater metals							
Cu	μg l^{-1}	28.6	18.6	25.5	17.3	19.6	17.8
Zn	μg l^{-1}	1,632	1,341	841	1,006	1,274	1,167
Cd	μg l^{-1}	0.29	0.31	0.76	0.43	2.45	1.35
Pb	μg l^{-1}	204	51.0	29.6	49.0	34.7	32.7
Porewater as % of total metals							
Cu	%	0.16	0.18	0.16	0.19	0.16	0.14
Zn	%	13.5	17.3	6.6	32.1	30.9	30.3
Cd	%	0.19	0.24	0.18	0.45	1.32	1.27
Pb	%	0.10	0.07	0.03	0.09	0.05	0.06
Free ion metals							
Cu	μg l^{-1}	6.02	0.38	3.15	0.82	0.67	4.17
Zn	μg l^{-1}	1274	615	666	638	804	983
Cd	μg l^{-1}	0.22	0.13	0.57	0.25	1.44	1.10
Pb	μg l^{-1}	65.3	6.2	11.1	10.9	7.5	16.8
Free ions as % of porewater							
Cu	%	21.1	2.05	12.3	4.75	3.40	23.5
Zn	%	78.0	45.8	79.2	63.4	63.1	84.2
Cd	%	75.8	42.3	75.9	59.4	58.9	81.3
Pb	%	32.0	12.2	37.7	22.3	21.5	51.4

Ashmore 2004). It is, however, within the range of those found by Smith et al. (2005) along a 400 km transect of metal contaminated ombrotrophic peat soils in Scotland and England. Although high DOC was expected, the very high values may also reflect the extraction method adopted in this study. Centrifugation will extract soil water held at relatively high matric potentials compared to zero-tension lysimeters which sample only free-flowing soil water. As microbes exist in all fractions of the soil water, centrifugation was considered the most appropriate method for this study. It should be noted that DOC is not truly dissolved but is operationally defined as carbon molecules smaller than 0.45 μm.

3.2 Total Heavy Metal Concentrations

Total Pb concentrations were very high at all sites (Table 4), particularly at Bleaklow where 1,065.9 μg g^{-1} was recorded. Levels of Pb at three of the sites are, in fact, above the 450 μg g^{-1} U.K. Contaminated Land Exposure Assessment (CLEA) guideline value for protecting human health (DEFRA 2002). Concentrations of total Cu and Zn ranged between 49.0 and 101.2 μg g^{-1} and 27.2–66.2 μg g^{-1}, respectively. Bleaklow also had the highest total Zn and second highest total Cu concentrations. There are no guideline values for Cu and Zn. Cd levels ranged from 0.6–2.3 μg g^{-1} and also exceed CLEA guideline of 1 μg g^{-1} at two sites.

Cu and Pb concentrations at the study sites are an order of magnitude higher than those reported from remote peat soils (Tables 4 and 5). This is both the result of centuries of heavy metal deposition from surrounding urban-industrial conurbations and the behaviour of Cu and Pb in organic soils. Cu and Pb are strongly complexed to organic matter and can potentially be retained in peat soils for centuries (Tipping et al. 2006). It is likely that Zn and Cd will also have been deposited onto the peat soils of the southern Pennines in higher quantities than at remote sites; however, Zn and Cd are more soluble in organic acidic soils and are less effectively retained. Despite the proximity of industrial sources, levels of Zn are similar to levels measured in Shetland (Sugden 1993) and lower than measured at Lochnagar, NE Scotland

Table 5 Maximum levels of heavy metals in cores taken from deep peats in the U.K. Values in parenthesis are lowest reported value in core

Site	Cd μg g^{-1}	Cu μg g^{-1}	Zn μg g^{-1}	Pb μg g^{-1}
Remote sites				
Glenshieldaig, NW Scotland[1]		12 (6)		40 (3)
Lochnagar, NE Scotland[6]	1.8 (0.2)	10 (0.1)	150 (60)	100 (20)
N. Uist, Shetland[2]		18 (2)	27 (4)	37 (4)
Easter Dean, E Scotland[2]				95 (40)
Loch Laxford, W Scotland[3]				36 (6)
Sites close to industrial sources				
Flanders Moss, C Scotland[2]		14 (3)	105 (60)	388 (20)
S.Drumboy Hill, C Scotland[4]		9 (1)	64 (05)	
Grassington Moor, N. England[1]		50 (5)	200 (30)	800 (100)
Dartmoor, SW England[5]		220 (50)	250 (25)	400 (10)
Cornwall, SW England[5]		400 (50)	200 (50)	75 (10)
Gordano Valley, W England[7]		34 (05)	500 (20)	230 (<10)
Lower Don Valley, N. England[10]		472 (50)	613 (50)	827 (100)
Snake Pass, Peak District, N. England[11]				570 (05)
Upper North Grain, N. England[9]				1,084 (<50)
Alston Moor, N England[1]		30 (5)	500 (75)	620 (75)
Moor House, N England[1]			325 (100)	725 (65)
Ringinglow Bog, N England[8]		240 (10)	1,300 (100)	1,230 (100)

[1] Livett et al. (1979); [2] Sugden (1993); [3] Weiss et al. (2002); [4] MacKenzie et al. (1998); [5] West et al. (1997); [6] Yang et al. (2001); [7] Martin et al. (1979); [8] Jones and Hao (1993); [9] Rothwell et al. (2005); [10] Gilbertson et al. (1997); [11] Lee and Tallis (1973).

(Yang et al. 2001; Table 5). Cd levels are similar to those measured at Lochnagar.

3.3 Porewater Metals

Metals in soil porewater better reflect toxicity to many soil organisms than total metals because soluble metals are more readily available (Chaudri et al. 1999). This fraction does, however, include some less available metals complexed to dissolved organic matter and associated with inorganic ligands. In contrast to the total metals, porewater concentrations of Zn were higher than other metals at all sites (Table 4), ranging from 840.8–1632.7 μg l^{-1}. This reflects the greater solubility of Zn under acidic conditions and its relatively low affinity to organic matter. A mean of 21.8±0.43% (95% Confidence Interval) of the total Zn pool is found in the porewater. Porewater Pb ranged from 29.6–204.1 μg l^{-1}, only a tiny fraction (0.07±0.02%) of the total Pb pool. Cu ranged from 17.3–28.6 μg l^{-1}, 0.16±0.01%, of the total pool. Perhaps surprisingly, the percentage of Cd found in the porewater was also relatively small, from 0.3 to 2.4 μg l^{-1}, an average of 0.61±0.43% of the total pool. Bleaklow had the highest porewater concentrations of Pb, Zn and Cu.

3.4 Free Metal Ions

Many studies have shown that metal toxicity in soils can be best related to the free ion fraction of the total soil metal pool (e.g. Sauvé et al. 1998; Lofts et al. 2004). Free ion metals were determined using the WHAM chemical speciation model (Tipping 1994). As with the porewater metals, Zn dominates the free-ion metal pool (Table 4). An average of 69.0±11.4 % and 65.5±11.8% of porewater Zn and Cd respectively are estimated to be in free ion form, whereas only 11.2±7.5% of porewater Cu and 29.5±11.1 % of Pb are in this fraction, reflecting the greater importance of DOC as a sorbant for Cu and Pb. The high DOC at Cowms Moor results in low free ion concentrations for all metals at this site.

3.5 Metal Toxicity

Recent research has calculated the free-ion concentrations that may lead to detrimental impacts on soil organisms (Ashmore et al. 2004; Lofts et al. 2004). These concentrations are referred to as 'critical limits' and have been derived through statistical analysis of exotoxicological studies on a wide range of plants and soil invertebrates, although not soil microbial communities (Lofts et al. 2004). The critical limit defines an acceptable maximum concentration of metal below which deleterious effects to organisms should not occur. Free-ion based critical limits are not constant, but vary according to the availability of other free cations in the soil solution. This is because other free-ions (e.g. H^+, Na^+ and Ca^{2+}) compete with the heavy metals for uptake (Campbell 1995; Lofts et al. 2004; Hall et al. 2006). Thus, under acid conditions while there may be relatively high concentrations of free metal ions, they may be less available due to competition from other cations.

Free ion critical limits can be described by the following function:

$$\mathrm{Log}[\mathrm{M}]_{\mathrm{free,CRIT}}(\mathrm{mol}/L) = \alpha\mathrm{pH} + \gamma, \quad (1)$$

where, for Cu, Zn, Cd and Pb respectively, α is −1.23, −0.31, −0.32 and −0.91 and γ is −2.05, −4.63, −6.34 and −3.80 (Ashmore et al. 2004). A term for pH rather than the whole range of competing cations is used as these have been found to co-vary (Lofts et al. 2004).

We cannot use the critical limit approach to determine whether metals are likely to have ecotoxicological effects for two reasons. Firstly, the approach is primarily designed for large-scale (country-wide) assessment of heavy metal critical loads. Secondly, critical limits are not based on exotoxicological work on microbial populations. The critical limit functions, however, also account for the competing effect of cations and thus provide a means of making a relative assessment of the potential toxicity of metals at the six sites. Table 6 shows critical limits calculated from Eq. 1 and the concentration above or below the critical limit at each site. Sites were ranked such that a value of 1 indicates the site the furthest below the critical limit (or least above) and 6 indicates the site with the highest exceedance of critical limit or nearest to the critical limit for each metal. As we cannot be certain which metals are having the most significant effect on the microbial population, rankings have been combined to provide an initial indication of the relative potential

Table 6 Free-ion critical limits, amount above or below at each site and ranking where 1 is the least metal contaminated site in relation to critical limits and 6 is highest

		Bleaklow	Cowms Moor	Featherbed Moss	Holme Moss	Round Hill	White Hill
Critical Limits							
Cu	$\mu g\ l^{-1}$	77.3	88.5	76.8	87.9	114.2	99.7
Zn	$\mu g\ l^{-1}$	163	168	163	168	180	174
Cd	$\mu g\ l^{-1}$	5.07	5.26	5.07	5.26	5.62	5.42
Pb	$\mu g\ l^{-1}$	45.4	50.2	45.2	49.9	60.6	54.8
Level above/below critical limits							
Cu	$\mu g\ l^{-1}$	−71.3	−88.1	−73.7	−87.1	−114	−95.5
Zn	$\mu g\ l^{-1}$	1111	447	503	470	624	809
Cd	$\mu g\ l^{-1}$	−4.85	−5.13	−4.50	−5.01	−4.18	−4.32
Pb	$\mu g\ l^{-1}$	19.9	−44.0	−34.1	−39.0	−53.1	−38.0
Ranking							
Cu		6	4	5	3	1	2
Zn		6	1	3	2	4	5
Cd		3	1	4	2	6	5
Pb		6	2	5	3	1	4
Combined Rank		21	8	17	10	12	16

The data in this table were used to split sites into two categories (more and less contaminated):

More contaminated sites: Bleaklow, Featherbed Moss and White Hill.

Less contaminated sites: Round Hill, Holme Moss and Cowms Moor.

toxicity at each site (Table 6). Combined rankings indicate the greatest ecotoxicological effects may occur at Bleaklow and that toxicological effects on the microbial population are likely to decrease in the order: Bleaklow > Featherbed Moss > White Hill > Round Hill > Holme Moss > Cowms Moor. The data also reveal a split in site characteristics between the most polluted sites with the highest levels of bioavailable metals (Bleaklow, FeatherBed Moss and White Hill) and those with much lower bioavailable metals (Cowms Moor, Holme Moss and Round Hill).

3.6 Bacterial Populations

Figure 2 shows the 16S rDNA fragments from the samples from the six sites as resolved by DGGE. The bands were labelled A-T and sequenced, confirming that each band corresponds to a different species. The discernable bands were assumed to represent the dominant members of the mixed eubacterial community present in each sample; i.e. those that are present in highest numbers in the environment. After comparison with those sequences contained on the Genbank database many of the nearest relative sequences are from uncultured bacteria and thus their physiology and functionality have not been characterised. Therefore, we must rely on information about the characteristics of the environment from which the sequences originated and the bacterial members that dominate these environments to give us an idea of the types of dominant bacteria that are present at the sites. Percentage identities can usually be used to determine the main phylogenetic group to which an unidentified bacterium might belong (Stackebrandt and Rainey 1995). There is currently no concensus, however, regarding the classification of prokaryotes at species level based on phylogenetic data (Forney et al. 2004). Therefore this classification of species and genus can only be used as a tentative measure of species or genus level identity.

There were no significant differences in the number of dominant species identified at each site with between 8 and 11 bands identified in each sample (Fig. 2). There are, however, marked differences in species composition. Shifts in microbial communities without change in species richness have also been found by Mench et al. (2006) and Herrera et al. (2007) in metal contaminated soils. With a shift in species community composition comes a potential

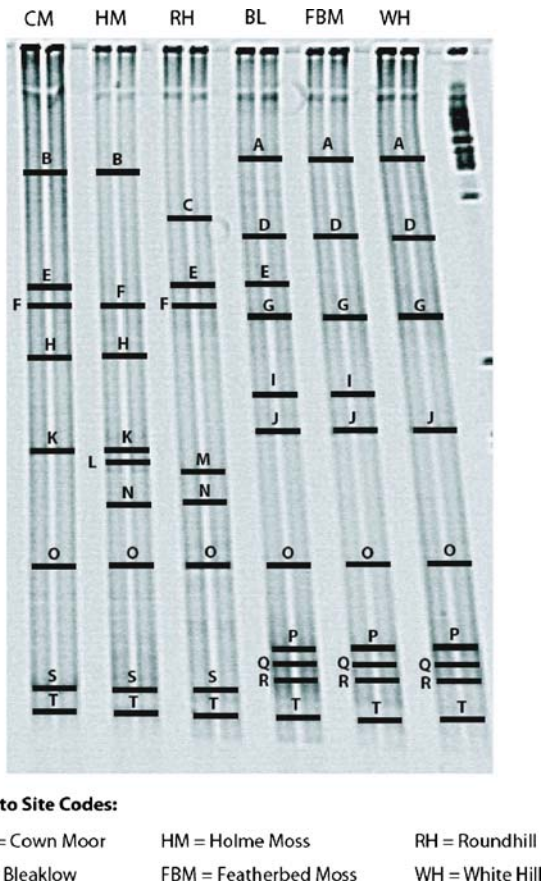

Fig. 2 Denaturing gradient gel electrophoresis (DGGE) line-up showing bacterial diversity at the study sites. DGGE separates DNA fragments with different sequences based on their base composition. *Labels* highlight the dominant bands that were then sequenced. Bands *A–T* represent different types of bacteria as confirmed by sequence data

change in the functional diversity and therefore in the biogeochemical processes that are occurring in the soil environment. Analysing species diversity alone would fail to detect variations between microbial populations in these samples, demonstrating the importance of using molecular techniques such as sequencing in studies on the diversity of soil microorganisms.

Table 7 shows details of all the sequences along with their nearest relative, the environment from which the nearest relative was detected and their percentage identity with the sequences determined during this study. Little sequence overlap occurred between the sites with the highest and lowest level of potential toxicity. 16S rDNA sequences with genus level similarity to an uncultured methanotroph from the upper 10 cm of soil covering a landfill site (Wise et al. 1999) and a gamma proteobacterium from an acidic sphagnum peat soil under a *Sphagnum* plant community in Western Siberia (Dedysh et al. 2006) were detected across all sites. Methanotrophs are a group of methane consuming bacteria which have been shown to be active in acidic ombrotrophic peats (Dedysh et al. 1998) and therefore it is not unexpected that this type of bacteria was detected. Sequences with genus level similarity to *Bradyrhizobium* sp. were found at Cowms Moor, Round Hill and Bleaklow which is unsurprising since this species is a ubiquitous symbiotic nitrogen-fixer which is involved in nodule formation in leguminous plants (Kaneko et al. 2002).

The sites with the lowest level of potential toxicity (Cowms Moor, Holme Moss and Round Hill) were all found to have bacteria present which had similarity to those from natural forest soils (Hackl et al. 2004) and acidic sphagnum peat bogs (Dedysh et al. 2006). However these bacteria may represent new genera as their percentage identities with their closest relatives on Genbank were below 92%. Sequences with genus level similarity to those from low nutrient soil, disturbed forest soils (Axelrood et al. 2002), pine rhizosphere soils (Chow et al. 2002) were found at two out of the three lower metal level sites.

A sequence with species level identity with that from a hydrocarbon-contaminated soil was detected in two of the less polluted sites (Holme Moss and Round Hill). Polyaromatic hydrocarbons occur in peats naturally through decay processes and fires and due to anthropogenic factors such as atmospheric deposition and surface run-off from roads (Malawska et al. 2006). Samples from Holme Moss also contained a sequence with genus level identity to that from a polychlorinated, dioxin-dechlorinating microbial community. Polychlorinated dibenzo-*p*-dioxins sometimes occur naturally in soils subjected to fire (Hoekstra et al. 1999) or atmospheric deposition (Green et al. 2001). The presence of these sequences suggests that there may be bacteria present in the peats that could be responsible for transformations of hydrocarbons and polychlorinated dibenzo-*p*-dioxins or may be tolerant to their presence. To date there are no reports in the literature of the effect of these chemicals on microbial communities in the Southern Pennines.

Two sequences were unique to Roundhill. One had genus level similarity to a methanotroph from landfill soil (Wise et al. 1999), the other had genus level

Table 7 Phylogenetic relationships and occurrence of partial 16S rRNA genes of dominant bacteria detected at each site

DGGE band identification	Occurrence at different sites determined by occurrence on DGGE gel						Nearest Relative and Genbank accession number (a)	% identity (b)	Description of environment of nearest relative (a)	Reference
	CM	HM	RH	BL	FBM	WH				
B	×	×					Uncultured soil bacterium clone RFS-C168 (DQ154488)	0.92	Low nutrient soil	unpublished
C			×				Uncultured methanotroph T2-07 (AF177320)	0.95	Methanotroph community in the top 10cm of soil covering a landfill site.	Wise *et al.*, 1999
F	×	×	×				Uncultured clone D30ST (AY395146)	0.9	Natural forest soils; Austrain pine forest with natural vegetation	Hackl *et al.*, 2004
H	×	×					Uncultured bacterium clone SMS9.95WL (AF432697)	0.95	Forest soils in British Columbia subjected to disturbance. Dominated by *Proteobacteria, Actinobacteria*, *Acidobacterium.*	Axelrood *et al.*, 2002
K	×	×					Uncultured gamma proteobacterium clone C46.84PG (AF431376)	0.93	Lodgepole pine rhizosphere soils from British Columbia forest soils; deep, medium textured loam soils	Chow *et al.*, 2002
L		×					Uncultured bacterium TSBW01 (AB186861)	0.95	Polychlorinated, dioxin-dechlorinating microbial community in sediment samples from a polychlorinated dibenzofurans	Yoshida *et al.*, 2005
M			×				Grassland soil clone saf3_102 (AF078291)	0.96	Community structure in the rhizosphere soil from a grassland previously grazed by sheep, with a pH between 5.5 to 6.9.	McCaig *et al.*, 1999
N		×	×				Uncultured bacterium isolate DGGE gel band 28 (AY673805)	0.97	Hydrocarbon contaminated soils in South Africa	Unpublished
S	×	×	×				Uncultured bacterium clone (AM162419)	0.87	Acidic sphagnum peat bog. Sample was taken from under a Sphagnum-Carex plant community	Dedysh *et al.*, (2006)
A				×	×	×	Bacterium Ellin645 (DQ075309)	0.97	Acidophilic *Acidobacterium* communities in a rotationally grazed perennial ryegrassa and white clover pasture.	Sait *et al.*, 2006
D				×	×	×	Uncultured epsilon proteobacterium clone LKC3-102B.15 (AY510220)	0.9	Filamentous microbial mats from acidic cave sulphidic springs dominated by chemolithotrophic *Epsilonproteobacteria* and sulfur-oxidising bacteria such as *Thiothrix* sp. and *Acidithiobacillus* sp.	Engel *et al.*, 2004
G				×	×	×	Iron-oxidsing acidophile M-1 (AF387301)	0.95	Culture of *Acidithiobacillus ferrooxidans* DSM 2392 – an acidophilic chemoautotrophic bacterium capable of iron and sulphur-oxidation.	Unpublished
I				×	×		Uncultured clone ML-NA-1 DQ206416	0.95	Microbial community in a soda lake carrying out sulphide-oxidation coupled to arsenate reduction	Hollibaugh *et al.*, 2006
J				×	×	×	Uncultured clone DSJB30 (DQ499213)	0.94	Extremely acidic (pH 0-1) biofilm from a cave system in Italy. Dominated by *Acidithiobacillus*, *Sulfobacillus* and *Acidimicrobium.*	Macalady *et al.*, 2007
P				×	×	×	Uncultured bacterium clone FW130 (AF523962)	0.94	Forested wetland impacted by reject coal, dominated by iron & sulphur-oxidising acidophiles (e.g. *Leptospirillium*, *Ferromicrobium*)	Brofft *et al.*, 2002
Q				×	×	×	Uncultured bacterium clone Molly90Delta (AY775534)	0.94	New England Sphagnum Bogs, mean pH = 4.97, with an active Sphagnum layer of 40 cm and high numbers of Deltaproteobacteria	Morales *et al.*, (2006)
R				×	×	×	Uncultured bacterium clone JCL9-73 (AF499344)	0.92	Bacterial diversity in mining impacted lake sediments	Unpublished
E	×			×	×		*Bradyrhizobium japonicum* USDA 110 (AP005935-AP005965)	0.92	An agriculturally important, symbiotic, nitrogen-fixing species, important in forming root nodules with leguminous plants.	Kaneko *et al.*, 2002
O	×	×	×	×	×	×	Uncultured gamma proteobacterium clone WCB21 (AY217474)	0.92	Acidic sphagnum peat bog. Sample was taken from under a Sphagnum-Carex plant community	Dedysh *et al.*, 2006
T	×	×	×	×	×	×	Uncultured type II methanotroph T2-17 (AF177324)	0.94	Methanotroph community in the top 10cm of soil covering a landfill site.	Wise *et al.*, 1999

(a) nearest relative based on Genbank BLAST searches, (b) % identity as determined by neighbour-joining alogorithm; > 0.97 = same species >0.92 = same genus as nearest relative (Benlloch *et al.*, 1995). See Materials and Methods for a fuller description of criteria involved in phylogeneic relatedness. **CM** – Cowms Moor, **HM** – Holme Moss, **RH** – Round Hill, **BL** – Bleaklow, **FBM** – Featherbed Moss, **WH** WhiteHill. Sequences detected only at Cowms Moor, Holme Moss, Round Hill (lowest level of potential toxicity). Sequences detected only at Bleaklow, FBM – Featherbed Moss, WH - White Hill. (highest level of potential toxicity). Sequences detected across the two sets of sites.

similarity to bacteria from the rhizosphere of a sheep-grazed grassland (McCaig et al. 1999). A sequence with genus level identity to another uncultured methanotroph from the same environment was detected across all sites.

In samples from sites with higher level of potential metal toxicity (Bleaklow, Featherbed Moss and White Hill) there was a markedly different community composition from those with lower potential toxicity. From these sites one sequence was detected only at Bleaklow and Featherbed Moss and the rest were detected across all three sites. The sequence found at Bleaklow and Featherbed Moss showed genus level identity with that from a microbial community carrying out sulphide-oxidation coupled to arsenate reduction (Hollibaugh et al. 2006). The other sequences detected across all three sites includes one that has only 90% identity with its nearest neighbour (from acidic cave sulfidic springs dominated by iron-oxidising bacteria such as *Acidithiobacillus* sp. and *Thiothrix* sp. (Engel et al. 2004)) and so may belong to an uncharacterised genus.

Other sequences from the more polluted sites share genus level similarity with; acidophilic *Acidobacterium* communities from a grassland (Sait et al. 2006), *Acidithiobacullus ferroxidans* (a common iron-oxidising acidophile), bacteria from an acidic cave biofilm of pH 0–1 dominated by *Acidithiobacillus* sp. and *Sulfobacillus* sp. which are iron and sulphur-oxidising acidophiles (Macalady et al. 2007). There were sequences with similarity to those from wetlands impacted with reject coal again dominated by iron and sulphur oxidising acidophiles such as *Leptospirillium* and *Ferromicrobium* (Brofft et al. 2002), those from mining impacted lake sediments and those from sphagnum bogs (Morales et al. 2006).

3.7 The Impact of Heavy Metals on Bacterial Populations

Bleaklow, Featherbed Moss and White Hill were identified as sites with the highest bioavailable metals and therefore the most likely to be affected by the ecotoxicological effects of heavy metals. Conversely, Round Hill, Holme Moss and Cowms Moor were identified as being less metal contaminated. The significant difference between the bacterial community composition and the dominance of 'extreme' bacteria at the most contaminated sites suggests heavy metals are influencing bacterial populations at these sites.

Bleaklow, Featherbed Moss and White Hill have bacterial populations dominated by acidophilic, sulphur- and iron-utilising bacteria characteristic of extreme environments with high levels of metals and low acidity. These types of bacteria are known to be metal tolerant and have been previously isolated from areas with high levels of metal contamination and low acidity such as mine spoil heaps, bioleaching heaps and in metal contaminated rivers (Johnson 1998). This indicates that many of the bacteria found in sites with high potential toxicity may play an important role in biogeochemical transformation and mobility of available metal species. Biochemical transformations by acidophilic chemolithotrophic bacteria may result in the dissolution of insoluble metal compounds and minerals (Gadd 2004). The transformation of soluble ferrous iron to insoluble ferric iron during growth of iron- and sulphur-oxidising bacteria (Leduc and Ferroni 1994) may also decrease the mobilisation of soluble iron in acidic peats. The occurrence and speed of these processes, however, will depend on the mineralogical and organic composition of the soil, the presence of plant and root material and the presence of other bacteria and higher organisms (Pennanen 2001).

The presence of a high number of bacteria with similarity to acidophiles is of interest, as this type of bacteria produce inorganic acids as a by-product of their metabolism (Alexander et al. 1987) and may be responsible for further acidifying the already acidic soils. The result of this acidification or activity of these acidophilic bacteria may be the solubilisation of heavy metals, making them more bioavailable and increasing their toxicity to microorganisms, higher plants and animals (Speir et al. 2003). It is already established that heavy metals are released from peats in the Southern Pennines as a result of erosion processes (Rothwell et al. 2005, 2006). The potential of microorganisms to mobilise heavy metals in these areas is less well understood and further work on the role of acidophiles in maintaining low pH and releasing heavy metals in contaminated peat soils is required.

Not all sites were dominated by 'extreme' bacteria and soils at the three sites with the lowest levels of potential toxicity contained bacteria characteristic of forest, grassland and low nutrient soils with no history

of metal contamination. The degree to which this deviates from normal peat bacterial populations is, however, unclear at this stage.

4 Conclusions

This study provides the first picture of bacterial population diversity and species characteristics in metal contaminated upland peat soils. Sites with highest bioavailable metals were found to contain a greater proportion of 'extreme' bacteria (i.e. acidophilic, sulphur and iron utilising bacteria) indicating heavy metal pollution is likely to be a key factor in influencing bacterial species composition. High metal concentrations, however, only affects species composition and not diversity, a change that may be missed unless DNA sequencing techniques are employed.

Relationships between metal concentration and bacterial populations are complex and more work is needed to quantify the effects on different microbial populations, especially in ombrotrophic peats where few data exist. In particular, further work focusing on the role of acidophiles in maintaining low pH and releasing heavy metals in contaminated peat soils is required.

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