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Bacterial diversity promotes community stability and functional resilience after perturbation

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Summary

The relationships between bacterial community diversity and stability were investigated by perturbing soils, with naturally differing levels of diversity, to equivalent toxicity using copper sulfate and benzene. Benzene amendment led to large decreases in total bacterial numbers and biomass in both soils. Benzene amendment of an organo-mineral/improved pasture soil altered total soil bacterial community structure but, unlike amendment of the mineral/arable soil, maintained genetic diversity, based on polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis targeting DNA and RNA, until week 9 of the perturbation experiment. Assuming equivalent toxicity, the genetic diversity of the naturally more diverse soil was more resistant to benzene perturbation than the less diverse soil. The broad scale function (mineralization of ^{14}C-labelled wheat shoot) of both benzene- and copper-treated soil communities was unaffected. However, narrow niche function (mineralization of ^{14}C-labelled 2,4-dichlorophenol) was impaired for both benzene-polluted soils. The organo-mineral soil recovered this function by the end of the experiment but the mineral soil did not, suggesting greater resilience in the more diverse soil. Despite a large reduction in bacterial numbers and biomass in the copper-treated soils, only small differences in bacterial community diversity were observed by week 9 in the copper-polluted soils. The overall community structure was little altered and functionality, measured by mineralization rates, remained unchanged. This suggested a non-selective pressure and a degree of genetic and functional resistance to copper perturbation, despite a significant reduction in bacterial numbers and biomass. However, initial shifts in physiological profiles of both copper-polluted soils were observed but rapidly returned to those of the controls. This apparent functional recovery, accompanied by an increase in culturability, possibly reflects adaptation by the surviving communities to perturbation. The findings indicate that, although soil communities may be robust, relationships between diversity and stability need to be considered in developing a predictive understanding of response to environmental perturbations.

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Introduction

Pollution and intensive cultivation of agricultural land may influence soil quality and productivity but little is known of their effects on soil microbial communities, and consequent impacts on soil functioning (Giller, 1996; Hågvar, 1998; Andrén and Balandreau, 1999). Diverse communities are believed to promote ecosystem stability, productivity and sustainability (Giller *et al.*, 1997), as suggested by macro-ecologists (MacArthur, 1955; Elton, 1958), and many (mainly botanically based) studies have investigated the relationship between diversity and stability of community ecosystems. For example, Tilman (1996) observed that diversity stabilized community and ecosystem processes within a grassland ecosystem, but destabilized population processes, leading to a stable community biomass but with changes in individual species abundance. This encompasses May's (1973) proposal that diversity adversely affects population stability, while incorporating the theories of Elton (1958) and MacArthur (1955). The high level of diversity of soil microorganisms may require a functional approach to diversity, as functional redundancy among soil bacteria may be high, as demonstrated in deep-sea sediments (Fredrickson *et al.*, 1991) and tropical forest soils (Heneghan *et al.*, 1999). Atlas and colleagues (1991) noted reduced genetic diversity within a microbial community in a chemically perturbed site, but the surviving community exhibited enhanced physiological tolerances and substrate utilization capabilities. Copper

Table 1. Chemical and biological characteristics of mineral and organo-mineral soils. Values are means of triplicates ± standard error.

	Mineral	Organo-mineral
pH	6.98 ± 0.03	6.90 ± 02
Water holding capacity (%)	38 ± 0.6	143 ± 1.44
Organic matter (%)	1.75 ± 0.23	22.5 ± 0.90
Total carbon (%)	1.02 ± 0.14	13.2 ± 0.88
Total nitrogen (%)	0.10 ± 0.07	0.88 ± 0.03
Biomass (mg 100 g^{-1})	15.9 ± 0.87	63.8 ± 5.6[a]
Diversity of total soil bacteria[b]	3.25 ± 0.02	3.68 ± 0.04
Diversity of culturable soil[b] bacteria	2.95 ± 0.01	2.79 ± 0.12
Extractable DNA (μg g^{-1})	14.0 ± 0.22	37.6 ± 1.51
Total cell concentration (cells g^{-1})	$8.54 \pm 0.62 \times 10^8$	$19.2 \pm 0.74 \times 10^8$
Culturability (%)	0.45 ± 0.07	0.15 ± 0.02

a. As reported by Grayston and colleagues (2001).
b. Shannon diversity index calculated from DGGE bands.

perturbation experiments on soil samples of varying diversities were performed by Griffiths and colleagues (2000), who monitored the ability of the community to mineralize rye grass residue and found a positive correlation between diversity and resilience.

Exposure to metals at high available concentrations has been shown to reduce soil respiration (Hattori, 1992), microbial biomass and bacterial numbers (Brookes and McGrath, 1984; Chander *et al*., 1995) and cause shifts in the composition of soil microbial and faunal populations (McGrath *et al*., 1988; Griffiths *et al*., 1997; Bååth *et al*., 1998; Sandaa *et al*., 1999). Volatile aromatic hydrocarbons such as benzene, toluene, ethyl benzene and xylene (BTEX compounds) are also common environmental pollutants (Zhou *et al*., 1997; Huertas *et al*., 1998), attributed to their use in petroleum fuels and as industrial solvents, although they are amenable to remediation.

The aim of this study was to investigate the relationship between microbial diversity and stability by perturbing two soils, with naturally differing levels of diversity, by the application of contrasting pollutants, copper and benzene, to equivalent levels of toxicity (to achieve an approximately 50% decrease in the culturable community). Genetic diversity was monitored using 16S-rDNA- and 16S-rRNA-DGGE (denaturing gradient gel electrophoresis) profiling. Functional diversity was monitored using BIOLOG community level physiological profiles and the mineralization of ^{14}C-labelled wheat shoot and 2,4-dichlorophenol (2,4-DCP). Plant litter decomposition was estimated by measurement of wheat shoot mineralization over a 24-h period, a process dependent on a wide range of bacteria and fungi, while mineralization by a narrower range of organisms was assessed by decomposition of 2,4-DCP, a widespread pollutant.

Results

Soil chemical and biological analyses

The pH values of pasture (organo-mineral) and arable (mineral) soils were similar but water holding capacity, organic matter and total C and N were all significantly greater in the organo-mineral soil (Table 1). Extractable DNA, biomass, total and culturable cell numbers and bacterial diversity were also greater in the organo-mineral soil, although culturability was lower.

Equivalent toxicity, EC_{50} concentrations

Culturable cell concentrations were reduced to 55% of initial values after 3 days of the application of 25 μg g^{-1} copper to the mineral soil. For the organo-mineral soil, 340 μg g^{-1} copper reduced culturable cells to 65% (Fig. 1A) and an EC_{50} value of 540 μg g^{-1} copper was used for subsequent organo-mineral soil microcosm experiments. Culturable cell concentrations in the mineral and organo-mineral soils were reduced after 3 days by 40 and 80 μl g^{-1} of benzene to approximately 45% and 35% of original values respectively (Fig. 1B).

During incubation of control microcosms for several weeks, total bacterial cell concentration increased in both mineral and organo-mineral soils until week 9, and then decreased in the organo-mineral soil only. The influences of copper and benzene on total cell concentrations over the 9-week experiment were similar for both soils, decreasing to 35–37% of the control counts in the copper-amended mineral and organo-mineral soils, respectively, and to 12% and 13% of control counts, respectively, following addition of benzene (Fig. 2A and B) ($P < 0.01$). Cell concentrations remained at these low levels in both soils during further incubation, ranging from 30–60% of the control for copper addition and 10–30% of the control for benzene treated soils (Fig. 2A and B). Pollutant effects on total bacterial concentrations were more pronounced than those on culturable communities, resulting in increased culturability in amended microcosms (Fig. 2C and D). Although copper led to large reductions in bacterial cell concentration, it had a small, but statistically significant effect (reduction to 80–90% of control values) on extract-

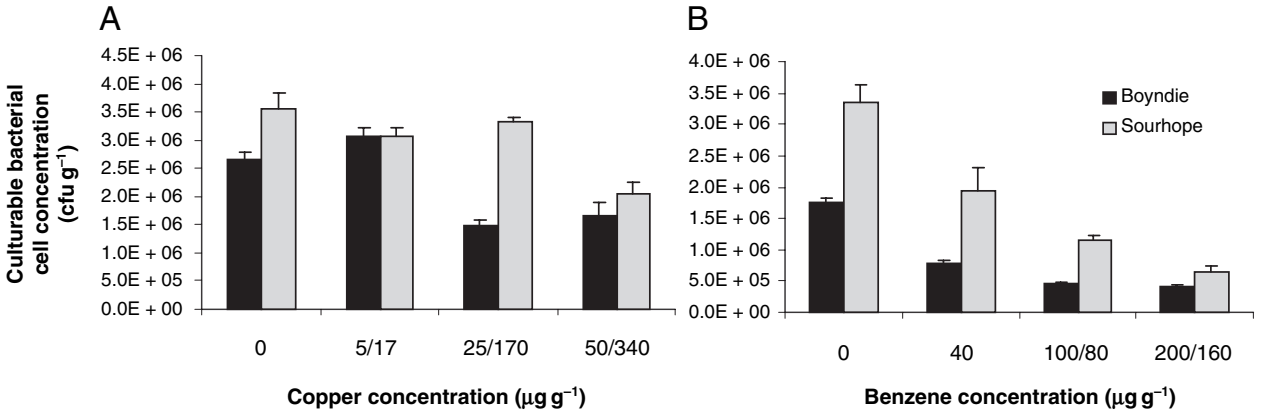

Fig. 1. The influence of concentration of (A) copper and (B) benzene on culturable cell concentration in the mineral (black bars) and the organo-mineral (grey bars) soils measured 3 days after pollutant addition. Concentration ranges used differed for the two soils. Bars represent standard errors of three replicate microcosms.

able DNA concentration in weeks 1 and 4 ($P < 0.05$), although decreases were greater (to 20% of the control) following treatment with benzene ($P < 0.001$) (Fig. 2E and F). Initial DNA concentrations were more than three times greater in the organo-mineral soil while total bacterial numbers were 2.5 times greater than in the mineral soil. DNA levels decreased with time in mineral soil but were relatively constant in organo-mineral soil until week 9, when a large increase occurred.

Mineral soil community diversity and structure

The diversity of bacterial communities was assessed by DGGE analysis of 16S rRNA gene fragments polymerase chain reaction (PCR)-amplified from extracted DNA. Genes were also amplified using reverse transcription (RT)-PCR, targeting 16S rRNA, for the organo-mineral soil. Copper addition did not significantly affect the Shannon diversity index of the total bacterial community in mineral soil (Fig. 3A). Diversity within the benzene-treated mineral soil community fluctuated, but was significantly lower at weeks 4 and 9 ($P < 0.01$) (Fig. 3A). Although all sample replicates were initially analysed individually, direct comparisons for all time points were made by DGGE analysis, on a single gel, of amplification products pooled from triplicate PCRs at each sampling point (Fig. 4A). This also facilitated matching of individual bands. Profiles were compared using principal component analysis (PCA) (Fig. 4B) and Student's *t*-tests were performed to compare ordination scores of each principal axis for treated and control samples, to determine the significance of any shift in community structure. No differentiation in community structure between the copper and control samples was apparent on principal axis 1 but a small differentiation on axis 2 was observed at weeks 4 and 9 ($P < 0.05$). The structure of the benzene-treated mineral soil was different from the control soil at all time points (Fig. 4B) ($P < 0.01$) and communities at weeks 0 and 1 differed significantly from those at weeks 4 and 9. Temporal shifts between weeks 0 and 1 and weeks 4 and 9 were observed in both control and copper-treated soils.

Organo-mineral soil community diversity and structure

Neither copper nor benzene addition significantly affected bacterial diversity in the organo-mineral soil until week 9, when diversity significantly decreased in copper-treated ($P = 0.043$) and, to a greater extent, in benzene-treated soils ($P < 0.01$) (Fig. 3B). PCA analysis of these data (Fig. 4D) indicates no difference in community composition between copper-treated and control soils. Benzene-treated soil communities differed from the other treatments at all time points along principal axis 1 ($P < 0.01$) (Fig. 4D) but were similar to each other until week 9, after which a large difference was observed along axis 2. Temporal shifts in diversity in control and copper-treated soils were observed, as in the mineral soils. The RNA- and DNA-derived DGGE profiles of the organo-mineral soil samples differed (Fig. 4C and E), with no significant decrease in diversity for any treatment until week 9, when diversity decreased in both treatments ($P < 0.01$) (Fig. 3C). PCA analysis of the pooled data showed no significant difference between control and copper-treated communities, except at week 9 (Fig. 4F), and temporal shifts were again observed in copper-treated and control soil communities. The benzene-treated community differed from copper-treated and control communities ($P < 0.05$) but the temporal change within the benzene-treated community was more gradual than for DNA profiles.

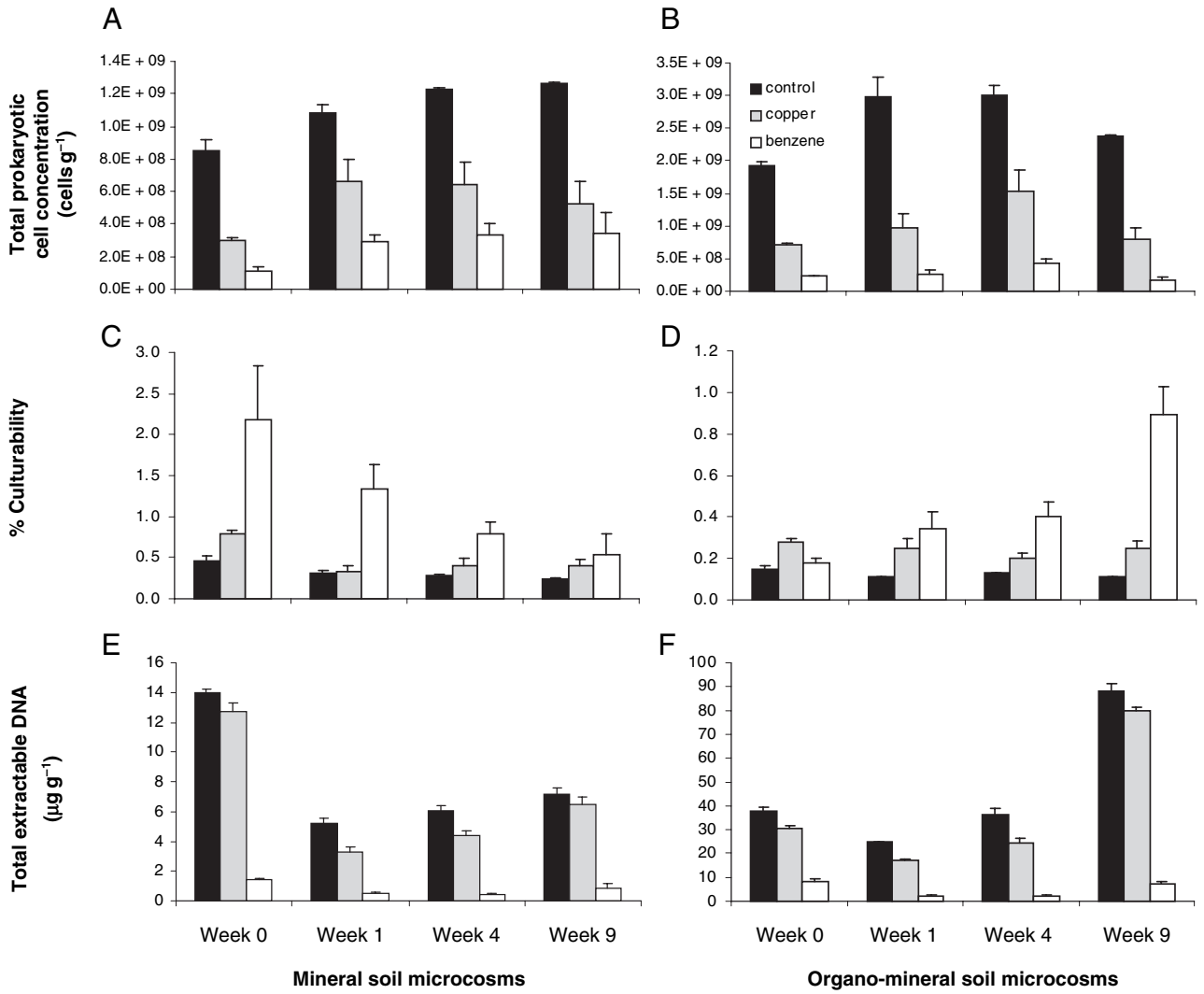

Fig. 2. Changes in total prokaryotic cell concentration (A, B), culturability (C, D) and extractable DNA (E, F) during incubation for 0 (72 h), 1, 4 and 9 weeks of mineral (A, C, E) and organo-mineral (B, D, F) control soils (black bars) and after addition of copper (grey bars) and benzene (white bars). Copper was added at final concentrations of 25 and 540 μg g^{-1} to mineral and organo-mineral soils, respectively, and benzene was added at final concentrations of 40 and 80 μl g^{-1} to mineral and organo-mineral soils, respectively. Culturability was estimated as the concentration of cells growing on 1/10 strength TSA as a percentage of total cell concentration. Bars represent standard errors for three replicate microcosms.

Phylogenetic analysis of 16S rRNA gene sequences

DGGE bands that discriminated treatments were excised and 16S rRNA gene fragments were re-amplified and sequenced. All of the sequences identified in mineral soil control microcosms were present in the copper-treated soils. Two bands associated with the temporal shift in mineral soil community structure by week 4 (2 and 3, Fig. 4A, Table 2) were most closely related to *Bacillus* (>96%). Bands that disappeared or became less dominant in benzene-treated soil were most similar to Gram-negative bacteria while those that increased in dominance were most similar to Gram-positive bacterial clones (Table 2). Fewer sequences were successfully sequenced from the organo-mineral soil (Table 2, Fig. 4C and E) because of the high diversity and subsequent mixed sequences from the gel. The most dominant band in the DNA-derived profiles of the benzene-treated soil (band 2, Fig. 4C, Table 2) was again most closely related to a *Bacillus* species, while a band showing greater dominance in benzene-treated soil (band 1, Fig. 4C, Table 2) than in control copper-treated soils was most closely related to a Gram-negative *Hyphomicrobium* clone. The sequence of a band from the RNA-derived profiles (band 5, Fig. 4C, Table 2) that was associated with the temporal change in copper-treated and control soils (weeks 1 and 4), and present in all samples except week 9 in the benzene-treated soil, was closely related to *Pedomicrobium*

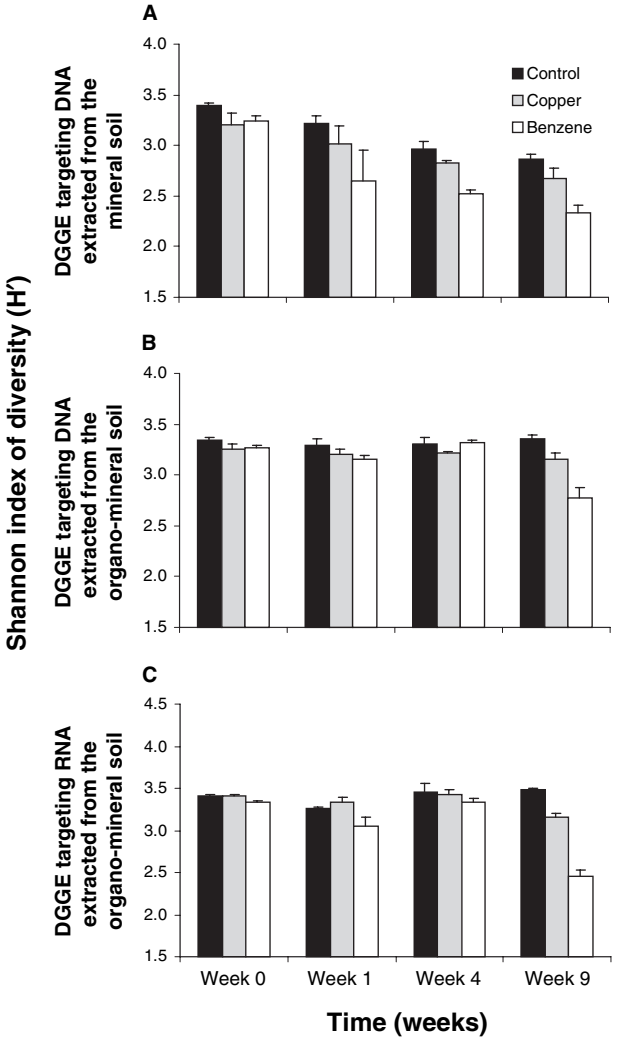

Fig. 3. The effects of copper and benzene on the Shannon index of diversity (H′) calculated from DGGE profiles of 16S rRNA genes PCR-amplified from DNA from mineral (A) and organo-mineral (B) soil microcosms and RT-PCR-amplified from RNA extracted from organo-mineral microcosms (C). Profiles reflect bacterial community structure and were determined at weeks 0 (72 h after perturbation), 1, 4 and 9. Copper was added at final concentrations of 25 and 540 μg g^{-1} to mineral and organo-mineral soils, respectively, and benzene was added at final concentrations of 40 and 80 μl g^{-1} to mineral and organo-mineral soils respectively. Bars represent standard errors for three replicate microcosms.

australacium (97%). None of the sequences from the last sample point from the RNA-derived, benzene-treated soil community was representative of *Bacillus*; all were closely related to pseudomonads and sphingomonads and to an isolate from metal contaminated soil (Fig. 4E, Table 2).

The influence of copper and benzene on potential activity

BIOLOG data for benzene-treated soils were not comparable with those for copper-treated or control soils because of very low cell concentration. However, PCA analysis of the BIOLOG data indicated differences between copper-treated and control mineral soil communities (Fig. 5A), with a large separation at week 0 on both axes ($P < 0.001$) and a smaller separation on both axes at week 1 ($P \leq 0.01$). All subsequent copper and control samples clustered together. Temporal changes were again observed during incubation. Analysis was performed on the results for both MT and GN plates, but separation of treatments was chiefly determined by the MT plate substrates. Separation between the copper-treated and control soils was attributed to a large decrease in tartaric acid and lysine utilization for the former, and small increases in ribose, hydroxybenzoic acid and tryptophan utilization. BIOLOG data also discriminated copper-treated and control organo-mineral soils (Fig. 5B) ($P < 0.001$ on both axes), largely because of a decrease in oxalic acid utilization for the copper-treated community and a small increase in methionine, glycine and tryptophan utilization. Control and copper-treated soils were tightly grouped after week 1 and temporal shifts were again observed.

Mineralization of wheat shoot carbon increased in copper-treated mineral and organo-mineral soils at week 1 (Fig. 6A and B) ($P < 0.05$), but thereafter remained constant. There was a small but statistically significant decrease in the ability of both copper-treated soils to mineralize 2,4-DCP at week 1 ($P < 0.05$), although these soils subsequently recovered. Mineralization of 2,4-DCP (Fig. 6A and B) decreased for both benzene-treated soils at all time points ($P \leq 0.001$), except in the organo-mineral soil community, which recovered by week 9.

Discussion

The maintenance of healthy soil requires an understanding of the response of microbial communities to stress and perturbation. This study investigated the potential resilience of soil bacterial communities following severe stress, determined the level of genetic diversity associated with maintenance of function and assessed the relationship between genetic diversity and stability. Two soils with differing organic matter were chosen in the expectation that diversity would be greater in the more organic soil (Øvreås and Torsvik, 1998; Tiedje *et al.*, 2000). As predicted, organic matter content, extractable DNA, cell concentration, biomass and diversity were greater in the organo-mineral soil (Table 1), but culturability was lower, possibly because of lower levels of inorganic fertilizer application.

Equivalent toxicity, EC_{50} concentrations

The organo-mineral soil required much greater pollutant concentrations, particularly of copper, than the mineral soil to achieve similar EC_{50} values. This is probably

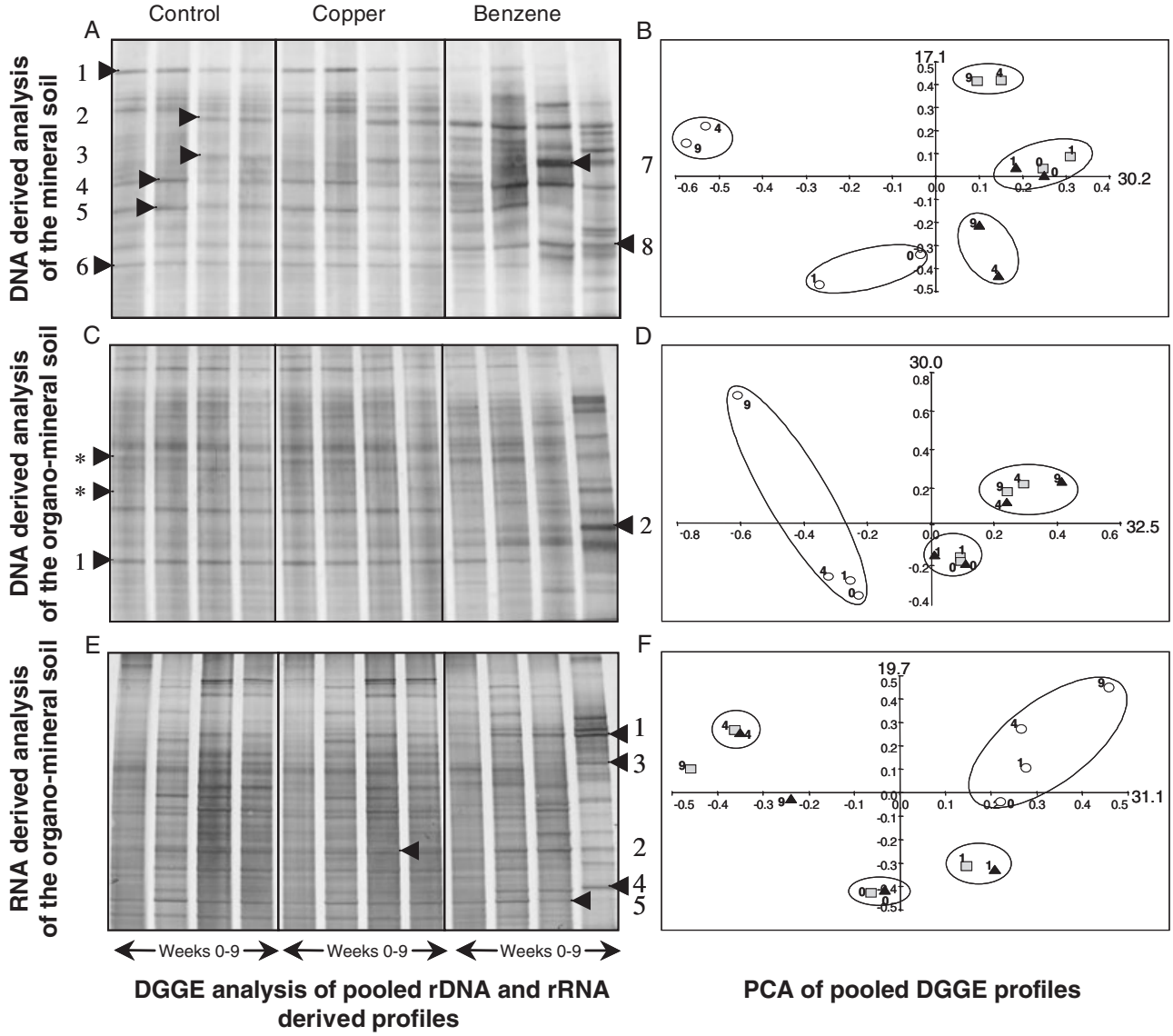

Fig. 4. The effects of copper and benzene on bacterial community structure in microcosms incubated for 9 weeks. Silver nitrate stained DGGE profiles of 16S rRNA genes derived from DNA from mineral soil (A) and organo-mineral soil (C) and from RNA extracted from organo-mineral soil (E). DNA or RNA was pooled from triplicate microcosms (samples) prior to amplification using primers Bf (McCaig *et al*., 1994) and 1390r (Embley, 1991) followed by Muyzer primers (Muyzer *et al*., 1993). Copper was added at final concentrations of 25 and 540 μg g^{-1} to mineral and organo-mineral soils, respectively, and benzene was added at final concentrations of 40 and 80 μl g^{-1} to mineral and organo-mineral soils respectively. Bars represent standard errors for three replicate microcosms. Arrows indicate bands that were subsequently excised and bands marked * are those that changed with time. B, D and F are the PCA ordinations of the first two axes generated from the corresponding DGGE profiles (A, C and E); control (▲), copper (□) and benzene (○); numbers indicate sampling times of 0 (72 h after perturbation), 1, 4 and 9 weeks.

because of the higher organic matter levels, greater complexation of the copper and lower availability than in the mineral soil (Saha *et al*., 1999), with similar effects observed by others (Bååth *et al*., 1998; Giller *et al*., 1998). A reduction in total bacterial numbers in the mineral soil (Fig. 2A) was achieved at copper concentrations lower than observed in other studies (Sandaa *et al*., 1999). Organic content may also lead to sequestration of organic pollutants (Luthy *et al*., 1997), a process known as ageing, and this may explain the differences in the dose response to benzene in the two soils observed in our study. The influence of benzene and copper on culturable cell concentrations was reflected in equivalent decreases in total bacterial numbers and biomass for both soils, particularly for benzene.

Copper-treated bacterial community diversity, structure and function

Despite its impact on bacterial numbers and biomass,

Table 2. 16S rRNA partial gene sequence homologies for bands excised from gels presented in Fig. 4A, C and E obtained using BLAST searches in GenBank.

Source of excised bands	Microcosm	Organism	% Similarity	Accession No.
Fig. 4A	1	Uncultured soil bacterium	95.6	AF013530
Fig. 4A	2	Unidentified bacterium	98.0	UEU232784
Fig. 4A	3	Unidentified bacterium	96.9	UEU232784
Fig. 4A	4	*Pseudomonas saccharophilia*	100	AB021407
Fig. 4A	5	Denitrifying Fe <II>-oxidizing	99.4	DFU51102
Fig. 4A	7	Low GC Gram-positive bacterium	95.6	LGRR16SR
Fig. 4A	6	Unidentified bacterium	98.5	UEU232882
Fig. 4A	8	*Bacillus macquariensis*	91.2	BM16SRNA
Fig. 4C	1	*Hyphomicrobium W1-1B*	91.7	U59505
Fig. 4C	2	*Bacillus* sp.	97.4	BI20385
Fig. 4E	1	*Unidentified gamma*	98.0	AB010853
Fig. 4E	2	*Unidentified alpha*	97.4	AB015565
Fig. 4E	3	*Pseudomonas* sp. *PsI*	96.8	AF105387
Fig. 4E	4	*Uncultured bacterium*	91.2	AF125204
Fig. 4E	5	*Pedomicrobium americanum*	97.0	PSRNA

copper treatment had little effect on bacterial diversity or community structure in either soil, as measured by analysis of amplified 16S rRNA gene fragments. That DGGE did not distinguish communities with greatly differing culturable and total numbers may be attributed to a lack of selectivity in the acute toxicity of copper and detection of community changes resulting from adaptation may require a longer period of incubation. High concentrations of copper in the environment have led to the evolution of chromosomally regulated and plasmid-borne copper resistance mechanisms, involving a combination of efflux, complexation, compartmentalization or reduction of copper ions (Cervantes and Gutierrez-Corona, 1994). Many resistant bacteria are enteric and survive by efflux of ions regulated by plasmid-borne *pco* genes (Cooksey, 1993; Williams *et al*., 1993) but many other soil bacteria exhibit resistance mechanisms, e.g. *Pseudomonas syringae*, through involving accumulation and compartmentalization regulated by the *cop* operon (Cooksey, 1994; Lin and Olson, 1995). Changes in more specific components of the community may have been masked by analysis of community structure using universal bacterial primers.

Culturable cell counts determined using 1/10 strength TSA were greater in the copper-treated soil than in control soils, suggesting an adaptive response to stress by the surviving culturable community (Atlas *et al*., 1991). A shift in physiological (BIOLOG) profile was also observed immediately after copper addition to both soils but the control recovered rapidly. Community analysis by BIOLOG is based on the growth of primarily fast growing bacteria (Smalla *et al*., 1998). As this shift was not associated with changes in bacterial diversity measured by DGGE, it may

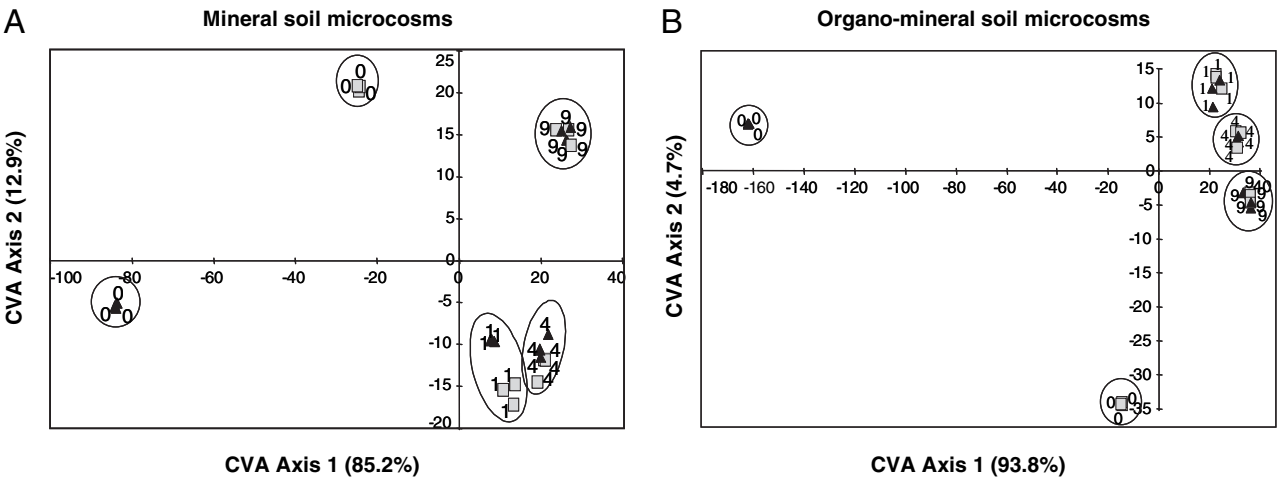

Fig. 5. Canonical variate analysis of carbon source utilization patterns, measured by BIOLOG, in mineral (A) and organo-mineral (B) soil microcosms after incubation for 0 (72 h), 1, 4 and 9 weeks after addition of copper (□) or benzene (○) or in control soils (▲). Copper was added at final concentrations of 25 and 540 μg g^{-1} to mineral and organo-mineral soils, respectively, and benzene was added at final concentrations of 40 and 80 μl g^{-1} to mineral and organo-mineral soils respectively. Numbers indicate sampling times.

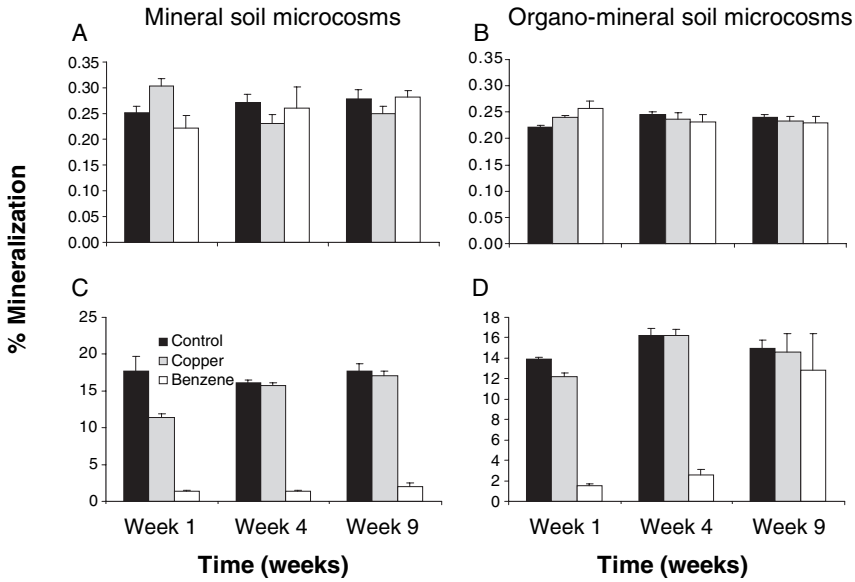

Fig. 6. Carbon mineralization in mineral soils (A, C) and organo-mineral (B, D) control soils (black bars) and soils amended with copper (grey bars) or benzene (black bars). Mineralization was determined by decomposition of ^{14}C-labelled wheat shoot after 24 h (A, B) or of ^{14}C-labelled 2,4-DCP after 120 h (C, D) at weeks 1, 4 and 9. Copper was added at final concentrations of 25 and 540 μg g^{-1} to mineral and organo-mineral soils, respectively, and benzene was added at final concentrations of 40 and 80 μl g^{-1} to mineral and organo-mineral soils respectively. Bars represent standard errors for three replicate microcosms.

be attributed to physiological changes in the community or indirect effects on other members of the community, such as fungi, that are sensitive to copper (Bååth *et al*., 1998). However, community analysis by BIOLOG reflects potential rather than *in situ* functional ability of the community (Garland and Mills, 1991). More compelling evidence of changes in function was provided by the mineralization tests. Stimulation of wheat shoot mineralization in the copper-treated soil at week 1 was correlated with recovery in the BIOLOG profiles but coincided with a decrease, in week 2, of 4-DCP mineralization. This, combined with the increased culturability of the copper-treated soil bacterial community, may suggest adaptation of surviving organisms. Atlas and colleagues (1991) showed that communities had lower genetic diversity following addition of chemical pollutants, but that the dominant populations within the community possessed enhanced physiological tolerances and substrate utilization capacities. Our results suggest that an adaptive tolerance response may have occurred in survivors (Díaz-Raviña and Bååth, 1996; Bååth *et al*., 1998), which is consistent with a lack of influence of copper on bacterial diversity.

Benzene-treated bacterial community diversity, structure and function

Benzene addition also resulted in large decreases in bacterial and DNA concentrations but, in contrast to copper treatment, led to larger shifts in community structure. Benzene can be selectively toxic as its main effect is on cell membrane integrity, which may exert less effect on spore forming Gram-positive bacteria and Gram-negative species with cell membrane adaptations (Heipieper *et al*., 1992; Gutierrez *et al*., 1999). The toxicity of benzene, and other aromatic hydrocarbons, is attributed to the partitioning of lipophilic compounds into the lipid bilayer of cell membranes, causing loss of integrity and dissipation of the proton motive force. Bacteria may develop resistance by increasing the amount of saturated fatty acids present in their membrane (Keweloh *et al*., 1991; Gutierrez *et al*., 1999) and conversion from the *cis*- to the *trans*-configuration of unsaturated fatty acids has been observed for strains of *Pseudomonas putida* exposed to organic solvents (Heipieper *et al*., 1992; Weber *et al*., 1994). Saturated and *trans* fatty acids show a higher degree of ordering and therefore increased surface density, which is believed to oppose the partitioning of lipophilic solutes into the lipid bilayer (Marqusee and Dill, 1986).

For both soils, the dominant sequences in benzene-polluted soil DNA-derived profiles were related to bacilli, while those decreasing in relative abundance were related to Gram-negative bacteria. Thompson and colleagues (1999) observed an increase in the *Bacillus* population, measured by fatty acid methyl ester (FAME) analysis, after repeated treatment with 1,2-dichlorobenzene. Similarly, selective mineralization of benzene by organisms adapted to benzene degradation, including *Pseudomonas* sp. (Shim and Yang, 2002) and *Rhodococcus* sp. (Kim *et al*., 2002) might also have occurred, explaining the community shift. In contrast to the mineral soil communities, in which the benzene-treated community diversity fluctuated, diversity of the organo-mineral community was constant until week 9, suggesting a possible link between resistance and diversity. Although the structure of organo-mineral DNA- and RNA-derived DGGE profiles differed, similar trends were observed with maintenance of diversity until the final sampling point, and similar PCA ordination plots. However, sequences of the dominant bands from RNA-

derived profiles did not contain representatives of bacilli but were dominated by proteobacteria, including two pseudomonads. Pseudomonads are commonly associated with BTEX degradation (Wunsche *et al*., 1995; Hamann *et al*., 1999).

This prevalence of bacilli in the DNA-derived profiles from the benzene-treated soil, and their failure to dominate the active community, are not surprising. Although bacilli survive unfavourable conditions by spore formation (Zelles *et al*., 1997), and have been shown to increase after addition of 1,2-DCB (Thompson *et al*., 1999), they have limited ability to mineralize BTEX compounds (Sikkema *et al*., 1995). Conversely, the Gram-negative xenobiotic degrading organisms (Green *et al*., 2000; Kim *et al*., 2002; Shim and Yang, 2002) may not be present in sufficient numbers to dominate the total bacterial community profiles, identified by DNA-derived DGGE analysis but, due to higher activities, were dominant in the RNA-derived DGGE profiles for organo-mineral soil.

No difference in the mineralization of wheat shoot carbon was observed in benzene-treated soils, despite large reductions in biomass and dramatic changes in community structure. This maintenance of broad-spectrum function may be attributed to high functional redundancy in soil systems (McGrady-Steed *et al*., 1997; Andrén and Balandreau, 1999; Heneghan *et al*., 1999) or to increased substrate utilization capacity (Atlas *et al*., 1991). A similar result was observed when mercury contamination led to a reduction in soil bacterial community diversity with no subsequent reduction in substrate utilization diversity (Muller *et al*., 2002). In contrast, the ability to mineralize 2,4-DCP was significantly reduced in both benzene-treated soils in comparison with the untreated control. Degradation of 2,4-DCP is carried out by a limited number of xenobiotic proteobacterial degraders, such as *Pseudomonas* sp. and *Burkholderia* sp. (Bengtsson and Carlsson, 2001; Shaw *et al*., 2002), and by eukaryotes, such as the white-rot fungus *Phanerochaete chrysosporium* (Nanny *et al*., 1996). Fewer organisms can mineralize 2,4-DCP and this process may be more sensitive to a decrease in biomass. The microbial community within the organo-mineral soil, however, recovered the ability to mineralize 2,4-DCP by week 9, perhaps because of the increase in numbers and activity of the xenobiotic community. This apparent greater resistance and resilience of the microbial community in the organo-mineral soil, compared with the mineral soil, at equivalent toxicity again suggests a link between diversity, resistance and resilience.

The relationship between bacterial community diversity, stability and function

Other studies have examined the relationships between diversity, function and stability. McGrady-Steed and colleagues (1997) manipulated biodiversity in an aquatic ecosystem and, using respiration as a measure for stability, observed that ecosystem predictability became more stable with increasing diversity. Griffiths and colleagues (2000) observed a correlation between diversity and resilience following copper addition to soil, although no attempt was made to obtain equivalent toxicity in different soils. A similar correlation was found following heat shock stress, although this may have been attributed to pretreatment of soil, rather than naturally occurring and functionally stable communities.

Our study suggests that the soil microbial community is resilient to acute perturbations and can support community function despite loss of biomass and genetic diversity. This supports the theory that there are a limited number of functions to be performed within an ecosystem (Andrén and Balandreau, 1999) and that soil communities exhibit a high degree of functional redundancy (Fredrickson *et al*., 1991; Staddon *et al*., 1997; Muller *et al*., 2002). However, narrow niche function was severely reduced in the benzene-treated soils, and reduced genetic diversity may compromise capacity for adaptive responses to further perturbation (Hågvar, 1998). Consequently functional redundancy should not be used to suggest that soils are infinitely capable of dealing with toxic insults. Although this study has shown that soil is highly robust when subjected to short-term perturbations, the mineral soil appeared to be less stable than the organo-mineral soil. The more diverse organo-mineral soil exhibited greater resistance to genetic perturbation and greater functional resilience and so may be more capable of enduring perturbation stresses; the complex soil structure and more diverse community may provide some protection from toxic shock. Organic matter content has been shown to be fundamental to the development of diversity in soil (Øvreås and Torsvik, 1998; Tiedje *et al*., 2000). Mineral soils tend by the nature of their predominant land use to be exposed to higher levels of physical disturbance, shown to impact adversely upon the fungal community (Boddington and Dodd, 2000), and biocide additions that may also reduce overall diversity. Therefore, in general, these soils may be more vulnerable to damage. The findings of this study support the view that decreased diversity could lead to decreased stability following environmental fluctuations (Tilman, 1996). Microbial community analysis reveals a complex relationship between genetic diversity, function and stability within microbial systems. Understanding how essential ecosystem functioning relates to soil biodiversity will therefore contribute to maintenance of ecosystem productivity.

Experimental procedures

Sample sites

Soils studied were an improved sandy loam with a low

organic matter content (mineral soil) taken from an arable site in north-east Scotland, Boyndie, near Elgin (Grid Ref NJ 224658) and an agriculturally improved pasture soil with higher organic matter content (organo-mineral soil), from the Sourhope Research Station in the Borders Region of Scotland (Grid Ref NT850205). At each site, random soil samples were taken, within a heterogeneous plot as determined by surface vegetation, from the top 20 cm with a 2.5-cm diameter augur. These samples were pooled to reduce spatial heterogeneity, sieved (2-mm mesh), removing plant roots by hand, and stored for less than 1 month at 4°C. Organic matter content was measured using a muffle furnace by loss on ignition, and total C and N concentrations by CHN analyses (Perkin Elmer, Beaconsfield, UK). Initial biomass was determined by chloroform fumigation/recovery (Vance *et al*., 1987). Water holding capacity was determined using saturation followed by free draining.

Trial experiments

EC_{50} values for copper and benzene were determined in triplicate with 5 g of each soil placed within a 250-ml Erlenmeyer flask, adjusted to approximately 40% of water holding capacity with sterile deionized H_2O and amended with benzene and copper sulfate in aqueous form in the concentration ranges 5–340 μg Cu per gram and 40–200 μl benzene per gram. Pollutants were added to the soil surface and mixed and the flasks sealed with silicone subaseals. After incubation at 21°C for 24 h, destructive sampling was performed for determination of culturable cell numbers and calculation of the concentration resulting in 50% reduction in culturable cell numbers.

Microcosm construction

Microcosms consisted of 20 g soil, equilibrated for 24 h at room temperature, placed in a 1-l Kilner jar with either copper or benzene at concentrations equivalent to EC_{50} in culturable numbers. Sterile distilled water was added to achieve a moisture content of approximately 40% of the WHC and the soil was mixed thoroughly. The jar was sealed, incubated at 21°C and opened daily in a laminar flow cabinet to effect gas transfer. Moisture content was maintained by addition of autoclaved dH_2O to replace that lost through evaporation. Soil pH was measured in water for samples at the beginning and end of the experiment. Samples were taken 0, 1, 4 and 9 weeks after pollutant addition (0 weeks being 72 h after pollutant application) for determination of total prokaryotic cell concentration, using acridine orange staining (Hobbie *et al*., 1977), culturable cell concentration, by dilution plate counting on 1/10 strength TSA (Tryptone Soya Agar, Oxoid Ltd, Basingstoke, UK), and community structure.

Nucleic acid extraction and quantification

Total DNA and RNA were coextracted by vortexing (Fisons Scientific) 0.5 g of soil, 0.5 ml of phosphate buffer (100 mM, pH 7.5), 0.5 ml of Tris-equilibrated phenol and 0.5 g acid-washed UV-treated glass beads (150- and 212-μm diameter, Sigma/Aldrich) for 30 s. The mixture was placed on ice for 20 s, and the process repeated, before centrifuging for 10 min at 12 000 *g*. Nucleic acids were purified by mixing phenol, chloroform : isoamyl (24:1) and supernatant in equal volumes for 1 min and centrifugation for 5 min at 12 000 *g*. DNA was quantified by agarose gel electrophoresis of 13 μl of this lysate with 3 μl loading buffer and of DNA molecular weight markers ranging from 5 to 150 ng (constructed from λ bacteriophage DNA) (Gibco BRL Life Technologies) run on a 1% agarose gel. DNA concentrations were estimated following calibration of gels using markers and the Phoretix 1D Advanced Analysis package (Non-linear Dynamics, Newcastle, UK). Nucleic acids were further purified for PCR amplification by agarose gel electrophoresis and using the Hybaid Recovery™ DNA Purification Kit II (Hybaid, Middlesex, UK) and the Bio 101 RNaid® Kit (Anachem, Bedfordshire, UK), following the manufacturer's instructions. Nucleic acids were eluted in nuclease-free water.

PCR amplification and DGGE analysis

For initial characterization of soil diversity, bacterial 16S rRNA gene fragments were PCR-amplified from DNA using Muyzer 16S rRNA gene primers 2 and 3 (Muyzer *et al*., 1993). Culturable diversity was determined by DGGE analysis of DNA washed from the 1/10 TSA plates, purified by ethanol precipitation and amplified with Muyzer primers (Muyzer *et al*., 1993). For subsequent analyses, PCR amplification was performed on RNA and DNA using a nested approach. First round amplification was carried out using primers Bf (McCaig *et al*., 1994) and 1390r (Embley, 1991) (synthesized by Custom Primers, Paisley, UK), designed to amplify almost full-length 16S rRNA bacterial genes. The second round of amplification was carried out on a 10^{-2} dilution of this PCR product using Muyzer primers p2 and p3 (Muyzer *et al*., 1993). PCR mixtures for the full-length primer set consisted of 5 μl of PCR buffer (Bioline, London, UK), 3 mM $MgCl_2$, 250 μM of each deoxynucleoside triphosphate, 400 nM of each primer, 1 unit of Biopro DNA polymerase (Bioline), 10 ng of template DNA and sterile nuclease free water to a final volume of 50 μl. PCR conditions were: 5 min at 95°C followed by 10 cycles of 30 s at 94°C, 30 s at 50°C and 2 min at 72°C and a final extension for 10 min at 72°C. PCR mixtures for the Muyzer primer set were as above, except that 2 mM $MgCl_2$ was used and PCR conditions were: 5 min at 95°C followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C and a final extension for 10 min at 72°C. Amplification of 16S rRNA gene fragments from RNA was achieved by RT-PCR following treatment of purified nucleic acid extract (10 μl) with DNase RQ1 (Promega, Southampton, UK), according to the manufacturer's instructions. Reverse transcription was performed using SUPERSCRIPT II reverse transcriptase (Gibco BRL Paisley, UK) according to the manufacturer's instructions, followed by PCR reactions as for DNA.

PCR products were analysed using DGGE (Muyzer *et al*., 1993) using 40–60% denaturant (100% denaturant was 7 M urea and 9 M formamide). Gels were poured using the gradient delivery system supplied with the Bio-Rad DCode system (Bio-Rad), run for 5 h at 200 V at 60°C and then fixed overnight with 10% ethanol and 0.5% glacial acetic acid (v/v). Gels were stained using silver nitrate solution (0.1% w/v), developed using a solution of 0.01% (w/v) sodium borohy-

dride and 0.4% (v/v) formaldehyde, fixed with 0.75% sodium carbonate (w/v) and preserved using a solution containing 25% ethanol and 10% glycerol (v/v). Gels were scanned using an Epson GT9600 scanner and analysed using the Phoretix 1D Advanced Analysis package (Non-linear Dynamics, Newcastle, UK). Bands of interest were excised with a sterile scalpel from ethidium bromide-stained DGGE gels, homogenized (using a sterile toothpick) with 30 μl of sterile double-deionized H_2O and placed at 4°C overnight. After brief centrifugation, 1 μl of a 10^{-1} dilution of this eluent was used for PCR amplification using bacterial 16S rRNA primers p2 and p3 (Muyzer *et al*., 1993). Sequencing reactions were performed and analysed by NCIMB (Aberdeen, UK) ABI310 automated genetic analyser (Applied Biosystems California, USA). Sequences were submitted in FastA format and analysed using 'BLAST' similarity searches (Pearson and Lipman, 1988) found within the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov).

Mineralization

Mineralization was measured in 5 g soil samples in 250-ml Erlenmeyer flasks amended with either 100 μg g^{-1} (final concentration) 2,4-DCP (specific activity, 344.1 KBq $mmol^{-1}$; radiochemical purity 98%, Sigma; 250.8 Bq g^{-1}) or 20 μg g^{-1} wheat shoot material, pulse labelled by $^{14}CO_2$ assimilation (31 160 Bq g^{-1}). Mineralization rates, expressed as percentage substrate mineralized per day, were calculated from evolution of $^{14}CO_2$ collected in traps containing 10 ml 0.1 M KOH following incubation for 24 h and 120 h for 2,4-DCP and wheat shoot carbon mineralization respectively. The radioactivity in samples of KOH was measured using a Minaxi Tri-Carb™ 500 liquid scintillation counter 2500TR (Packard, Pangbourne, UK) with Ultima Gold™ scintillation cocktail (Packard) at a sample : scintillant ratio of 1.5:1.

BIOLOG analysis

The metabolic diversity of the culturable community was determined using BIOLOG™ GN plates (BIOLOG, Hayward, USA), containing 95 carbon substrates, and in BIOLOG MT plates containing buffered nutrient medium supplemented with 31 sugars, phenolic acids, amino acids, carboxylic acids and aliphatic acids representative of root exudates found within the soil environment (Campbell *et al*., 1997). Each well was inoculated with 150 μl of a 10^{-3} dilution of a 10% (w/v) soil suspension in 1/4 strength Ringer's solution, centrifuged at low speed (750 *g* for 10 min) to remove soil particles. Changes in the concentration of tetrazolium dye were measured during incubation for 7 days using a Dias microplate reader (Dynex Technologies, Ashford, UK) at a wavelength of 600 nm. Control values (tetrazolium dye only) and initial readings were subtracted to eliminate background colour generated from the substrates and bacterial suspension. BIOLOG data were analysed as described by ^{q23}Garland (1996). The average well colour development (AWCD) was calculated for each sample at each time point by dividing total optical density by the number of substrates. Further analysis was then performed on sample data when AWCD values were approximately equivalent to 1. The data were normalized by dividing absorbance values by AWCD to reduce bias between samples of differing inoculum concentration. Principal component analysis was performed and the first 10 components were used to perform canonical variate analysis (CVA). Data were analysed using Genstat 5.3 (NAG, Oxford, UK).

Statistical analysis

The Shannon diversity index, $H' = -\Sigma p_i \ln p_i$, and equitability, *J*, were calculated from the DGGE bacterial community profiles, where p_i is the relative abundance of species *i* within the community. DGGE gels were examined using the Phoretix 1D Advanced Package (Non Linear Dynamics, Newcastle, UK). Relative abundance of each phylotype (band) was estimated as relative intensity of individual bands on each community (lane) and was expressed as a proportion of the total community volume, thus normalizing the data and standardizing any loading differences. Principal component analysis was then performed on these data using Genstat 5.3 (NAG, Oxford, UK). Other data were analysed by analysis of variance (ANOVA) and the Tukey test (Microsoft Excel 7a) with a *P*-value of <0.05 to establish a significant difference between samples.

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