Antonie van Leeuwenhoek **81:** 509–520, 2002.

Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms

George. A. Kowalchuk[1,*], Douwe S. Buma[1], Wietse de Boer[1], Peter G.L. Klinkhamer[2] & Johannes A. van Veen[1]

[1]*Department of Plant-Microorganism Interactions, Netherlands Institute of Ecology, Centre for Terrestrial Ecology, Boterhoeksestraat 48, Postbox 40, 6666 ZG, Heteren, The Netherlands;* [2] *Department of Biology, Research Group Ecology of Plant-Animal Interactions, University of Leiden, Kaiserstraat 63, PO Box 9516, 2300 RA Leiden, The Netherlands (*Author for correspondence; E-mail. gkowal@cto.nioo.knaw.nl)*

Key words: 16S rDNA, biodiversity, collector's curve, PCR-DGGE

Abstract

A coupling of above-ground plant diversity and below-ground microbial diversity has been implied in studies dedicated to assessing the role of macrophyte diversity on the stability, resilience, and functioning of ecosystems. Indeed, above-ground plant communities have long been assumed to drive below-ground microbial diversity, but to date very little is known as to how plant species composition and diversity influence the community composition of micro-organisms in the soil. We examined this relationship in fields subjected to different above-ground biodiversity treatments and in field experiments designed to examine the influence of plant species on soil-borne microbial communities. Culture-independent strategies were applied to examine the role of wild or native plant species composition on bacterial diversity and community structure in bulk soil and in the rhizosphere. In comparing the influence of *Cynoglossum officinale* (hound's tongue) and *Cirsium vulgare* (spear thistle) on soil-borne bacterial communities, detectable differences in microbial community structure were confined to the rhizosphere. The colonisation of the rhizosphere of both plants was highly reproducible, and maintained throughout the growing season. In a separate experiment, effects of plant diversity on bacterial community profiles were also only observed for the rhizosphere. Rhizosphere soil from experimental plots with lower macrophyte diversity showed lower diversity, and bacterial diversity was generally lower in the rhizosphere than in bulk soil. These results demonstrate that the level of coupling between above-ground macrophyte communities and below-ground microbial communities is related to the tightness of the interactions involved. Although plant species composition and community structure appear to have little discernible effect on microbial communities inhabiting bulk soil, clear and reproducible changes in microbial community structure and diversity are observed in the rhizosphere.

Abbreviations: DGGE – denaturing gradient gel electrophoresis; RFLP – restriction fragment length polymorphism; ARDRA – amplified ribosomal DNA restriction analysis; OTU – operational taxonomic unit

Introduction

The loss of biodiversity has become a major topic of concern in recent decades, and a significant amount of research has been devoted to understanding the consequences of ecosystem simplification. Although the extent of microbial diversity is not yet known, soil microorganisms probably represent the world's greatest reservoir of biological diversity (Torsvik et al. 1990; Dykhuizen 1998). However, little is known as to the forces that drive microbial community structure in soil (Tiedje 1995). As the main input of organic matter in terrestrial ecosystems, plants are thought to have a profound affect in steering soil communities and processes, especially in the rhizosphere (Insam & Domsch 1988; Wardle 1992). However, there exists little data to date to support this assumption.

To date, the majority of studies focussed on examining the role of biodiversity on ecosystem functions and reliability have examined the impact of

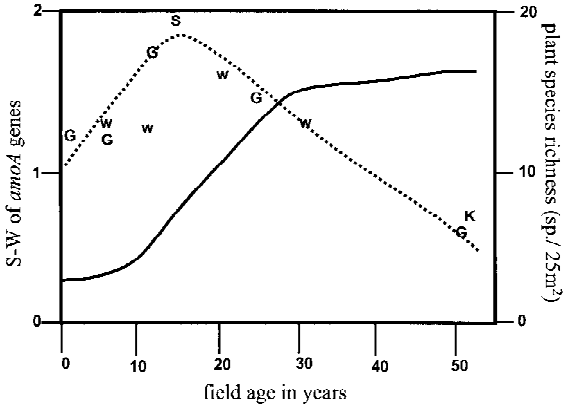

Figure 1. Relationship between plant diversity and diversity of ammonia-oxidising bacteria during secondary succession of chalk grasslands. Field age is given in years since taken out of production. Ammonia oxidiser diversity, shown by the solid curve, is given with respect to the diversity of *amoA* genes (from Kowalchuk et al. 2000a). Plant species richness is given by the broken curve. Letters correspond to the location of the plots examined in the south Limburg province of The Netherlands: G – Gerendal; W – Wrakelberg; S – Stokhem; K – Kunderberg.

changes in plant diversity (Naeem et al. 1994; Tilman & Downing 1994; Tilman et al. 1996; Hooper & Vitousek 1997; Naeem & Li 1997; Hector et al. 1999). Some studies have measured various biological processes of soils in response to plant diversity (Naeem et al. 1994; Tilman et al. 1996 1997; Bardgett et al. 1999; Wardle et al. 1999; Maly et al. 2000; Spehn et al. 2000). However, despite the realisation that microbial communities are key to the functioning of terrestrial ecosystems, their response to changes in macrophyte diversity has rarely been investigated directly (Broughton & Gross 2000; Yin et al. 2000). The lack of information in this area is at least partly due to the difficulties associated with studying microbial diversity, especially in soil. The vast majority of soil microbes (>95%) are not culturable using currently available techniques, morphological characters are insufficient to allow taxonomy, and the definition of a bacterial species is not at all clear. However, recent use of molecular biological techniques has opened up a new window of observation, which now allows us to view microbial diversity and community structure without the need for laboratory cultivation (Amann et al. 1995; Muyzer & Smalla 1998; Tiedje et al. 1999; Stephan & Kowalchuk 2002). Thus, we are now in a position to address more directly this response of soil-borne microbial communities in response to changes in macrophyte diversity and species composition.

In previous studies (Kowalchuk et al. 2000a,b) we observed that the diversity of β-subclass ammonia oxidising bacteria, as judged by restriction analysis of PCR-recovered *amoA* gene fragments was not correlated with the above-ground macrophyte diversity in grasslands at different stages of secondary succession. Surprisingly, ammonia oxidiser diversity appeared to be lowest for fields with the highest plant species richness (Figure 1). Ammonia supply seemed to steer the ammonia oxidiser community structure, selecting for ammonia-oxidising populations that were well adapted to high and low ammonia supplies in early and late successional fields, respectively. Plant diversity and species composition had no, or only an indirect effect on this functional group of microorganisms and the dominant bacterial populations as judged by PCR-DGGE appeared to be more indicative of field location as opposed to successional stage. Thus, the notion that soil-borne microbial communities follow the dynamics of above-ground macrophyte development seemed to be incorrect, or at least a vast oversimplification.

In the present study we sought to examine in more detail how plants can influence the structure and diversity of below-ground microbial communities. We hypothesised that the influence of the plant would be greatest on the root surface and rhizosphere as this zone should be most directly involved with the selective forces exerted by the plant. We examined this hypothesis in two field experiments, which were subsequently analysed in the laboratory using culture-independent, PCR-based methods.

In the first experiment, the bacterial communities inhabiting the rhizosphere of individual plants of two different wild plant species, *Cynoglossum officinale* (hound's tongue) and *Cirsium vulgare* (spear thistle), grown in field experiments, were compared. Comparisons were made across three harvest dates, between the two plant species and with bulk soil samples. The second experiment involved a large-scale field experiment designed to investigate the role of plant diversity treatments in accelerating the secondary succession of grasslands after abandonment of agricultural fields. In a block design, plant species diversity was manipulated, thereby providing an excellent opportunity to examine the role of plant diversity and species composition on microbial communities. In addition to comparing the most dominant bacterial populations in the bulk and rhizosphere soils of these experimental plots by PCR-DGGE profiling, microbial diversity was addressed using collector's curves of 16S rDNA types in clone libraries generated by PCR.

Materials and methods

Meyendel experiment

The Meyendel experimental field was established on a former site of flower bulb production at the foot of coastal sand dunes, near the town of Wassenaar, the Netherlands (52°,08′ N; 4°,20′ E). The experimental design included twelve replicate plots (1 m^2) per treatment. The treatments were planted with either seven seedlings of *Cynoglossum officinale* or seven seedlings of *Cirsium vulgare*, with plots where no plants were planted as controls. No weeding was performed throughout the course of the experiment, and weeds were free to colonise all experimental and control plots. Seedlings were planted in the autumn of 1998, and a portion harvested at each of three harvest times in 1999. Harvests occurred in early spring, at the time of flowering, and in autumn, and two, randomly chosen, plants per plot were removed for each harvest. Although weeds eventually covered that majority of available space in these plots, the experimental plants could easily be followed and harvesting only included root material of the desired plant without interference of surrounding weeds. The species composition and density of weeds was indistinguishable for experimental and control plots. Of the harvested plants, three individual plants of each species were chosen randomly for the molecular analysis of bacterial communities in the rhizosphere. Analysis of bulk soil samples corresponded to half of the plant sites used for the rhizosphere analysis, plus an additional four bulk soil samples from outside the experimental grid, as described below.

CLUE experiment

The experimental field was established within the program Changing Land Usage: Enhancing of biodiversity and ecosystem functioning (CLUE). The field site is located in the Veluwe region (52°04′ N, 5°45′ E) of The Netherlands and was taken out of agricultural production after the 1995 autumn harvest. The soil is a loamy sand with the following particle size distribution: < 2 μm 3.4%, 2–53 μm 17.3%, > 53 μm 79.7%. In April 1996, 20 experimental plots (10 $\times$ 10 m) were established according to a randomised block design (five replicate blocks of four treatments). The treatments were continued agricultural cropping (CA), natural colonisation by weeds (NC), and sowing of low (LD) and high diversity (HD) seed mixtures. Continued agricultural practice consisted of a crop rotation of barley (1996), buckwheat (1997) and corn (1998), and was left to natural colonisation after the 1998 growing season. The HD seed mixture contained 15 plant species in total, namely five grasses (*Agrostis capillaris, Anthoxanthum odoratum, Festuca rubra, Phleum pratense, Poa pratensis*), five legumes (*Lotus corniculatus, Trifolium arvense, Trifolium dubium, Trifolium pratense, Vicia cracca*) and five other forbs (*Hypericum perforatum, Hypochaeris radicata, Linaria vulgaris, Plantago lanceolata, Tanacetum vulgare*). These plant species are commonly found in later successional stages of abandoned agricultural fields in this region. The LD seed mixtures contained a sub-set of four species (two grasses, one legume and one other forb) from the HD mixture. Each of the five LD replicates contained a different sub-set of the species used in the HD treatment to compensate for species-specific effects (Hooper & Vitousek 1997; Huston 1997). All fields were mowed once per year in the autumn and above-ground biomass removed. Plots were not fertilised except for the CA plots, which received 300 kg ha^{-1} of fertiliser (20% N NH_4NO_3 and 14% P_2O_5) and 5000 l ha^{-1} dried cow manure pellets, every spring. No weeding was performed for any of the plots, and although the HD plots were more resistant to invasion by weedy plants, large differences were still observed in the plant species composition and plant diversity of the experimental plots at the time sampling (5 years after the experiment's initiation) (Van Der Putten et al. 2000). More details about the experimental site, as well as description of the other European sites used in the CLUE program, are described by Van der Putten et al. (2000) and Korthals et al. (2001). Details of soil properties can be found in Maly et al. (2000). For convenience in sample handling, reaction numbers and DGGE analysis, only four of the five blocks of the CLUE experiment were subjected to the PCR-DGGE analysis described below.

Soil collection and DNA extraction

For the Meyendel experiment, rhizosphere samples consisted of soil both loosely adhering to roots as well as soil that could be brushed or scraped off the root surface. For the Meyendel experiment, root samples, including adhering soil were sampled for individual plants, bagged, and kept at 4 °C in transport to the laboratory. Recovery of soil from roots was performed the following day. Bulk soil for this experiment was defined as soil, free of roots, taken from the area

where plants were removed for sampling, as well as four additional bulk soil samples, which were taken at a distance of >2 m from the nearest target plant. Bulk soil samples were sieved (4-mm mesh) prior to freezing.

For the CLUE experiment, roots, rhizosphere, and bulk soil were collected in September of 1999, using a 3-cm corer, and each sample combined the material obtained from 30 cores (top 10 cm) taken throughout the area of a 15-m^2grid in the middle of each experimental plot. Roots were carefully removed from each sample, and no attempt was made to determine the plant species for root pieces. Soil adhering to roots was removed using a fine brush and defined as rhizosphere soil. The remaining soil was sieved (4-mm mesh) and defined as bulk soil. All soil samples were frozen at −20 °C for between 2 weeks and 6 months prior to DNA extraction, and all DNA extractions per experiment were performed in parallel. DNA was extracted from soil samples, 0.25 g wet weight per extraction, using the MOBIO soil DNA extraction kit according to the manufacturer's specifications (MO BIO Laboratories; Solana Beach, CA), except that 2 × 30 s bead-beating was substituted for vortex mixing. Samples were eluded in 50 μl 10 mM Tris, pH 8.0, and diluted to a final concentration of 50 ng μl^{-1}.

PCR-DGGE analyses

The V6–V8 region of the 16S rRNA gene was amplified from soil DNA with the primers 968f-GC and 1378r (Heuer et al. 1997, 1999), and the following thermocycling program: (120 s 94 °C) × 1 cycle; (30 s 92 °C, 60 s 55 °C, 45 s 72 °C +1 s / cycle) × 35 cycles; (300 s 68 °C) × 1 cycle. Each amplification reaction consisted of 30 nM of each primer, 1 μl template DNA (50 pg), 1 U Expand High Fidelity DNA polymerase (Boehringer, Mannheim, Germany) and the manufacture's recommended buffer conditions. PCR products were examined by standard 1.5% (w/v) agarose 0.5 × TBE gel electrophoresis with ethidium bromide staining, to confirm product integrity and estimate yield. Approximately 0.5 μg of PCR product was used for DGGE analysis, using the method of Muyzer et al. (1993) as modified below. Gels contained 6% (w/v) polyacrylamide (37:1 acrylamide:bis-acrylamide) 0.5 × TAE, and were 1.5 mm thick. (20 × 20 cm) The linear gradient used was from 25–35% denaturant, where 100% denaturing acrylamide is defined as containing 7 M urea and 40% (v/v) formamide (Muyzer et al. 1993). To ensure well-polymerised slots, a 10-ml top gel containing no denaturants was added before polymerisation was complete. All DGGE analyses were run using a D-Gene system (Bio-Rad Laboratories, Hercules, CA) at a constant temperature of 60 °C. Electrophoresis was for 10 min at 200 V, after which the voltage was lowered to 80 V for an additional 16 h. Gels were stained in MilliQ (Millipore BV, Etten-Leur, The Netherlands) water containing 0.5 mg l^{-1} ethidium bromide and de-stained twice in MilliQ water prior to UV transillumination. Gel images were digitally captured using the ImaGo system (B&L; Maarssen, The Netherlands). Comparisons of DGGE profiles used a Pearson's index, taking both band number and intensity into account after signal normalisation, and dendrogram construction was performed using the ImageMaster Elite Database program (version 2.0) (Amersham Pharmacia Biotech) as described by Duineveld et al. (2001).

16S rRNA gene library construction, ARDRA and rarefaction analysis

One series of plots from the CLUE experiment was chosen for examination of 16S rDNA diversity by cloning and ARDRA analysis. The LD plot of this series was sown with the following plant species: *Poa pratensis*, *Festuca rubra, Vicia cracca,* and *Hypochaeris radicata*. Nearly complete 16S rRNA genes were amplified from soil DNA extracts with the primers pA and 1492r (Edwards et al. 1989), using the conditions and thermocycling program described above, except that extension was for 75 s +1 s / cycle. Products were analysed on 1.2% (w/v) agarose 0.5 × TBE gels with ethidium bromide staining, and the resulting 1.5-kbp fragment excised. DNA was recovered from the gel matrix using the QiaQuick Gel extraction kit (Qiagen; Hilden, Germany), in a final volume of 30 μl. PCR fragments were ligated into the pGEM-Teasy vector (Promega, Madison, WI) for transformation of Epicurian Coli XL2-Blue supercompetent cells (Stratagene, La Jolla, CA) according to the manufacturer's recommended protocol. The presence of inserts of the expected size was confirmed by colony PCR using the vector-targeting primers SP6 and T7 (Promega). Reactions (25 μl) were performed using 1 unit of Tbr polymerase according to the manufacturer's recommended buffer conditions (Finnzymes, Ipoo, Finland) and the following thermocycling program: 2 min 94°C followed by 30 cycles of 94 °C (30 s), 55 °C (60 s) and 72 °C (120 s) and a final extension step at 72 °C (300 s). Five

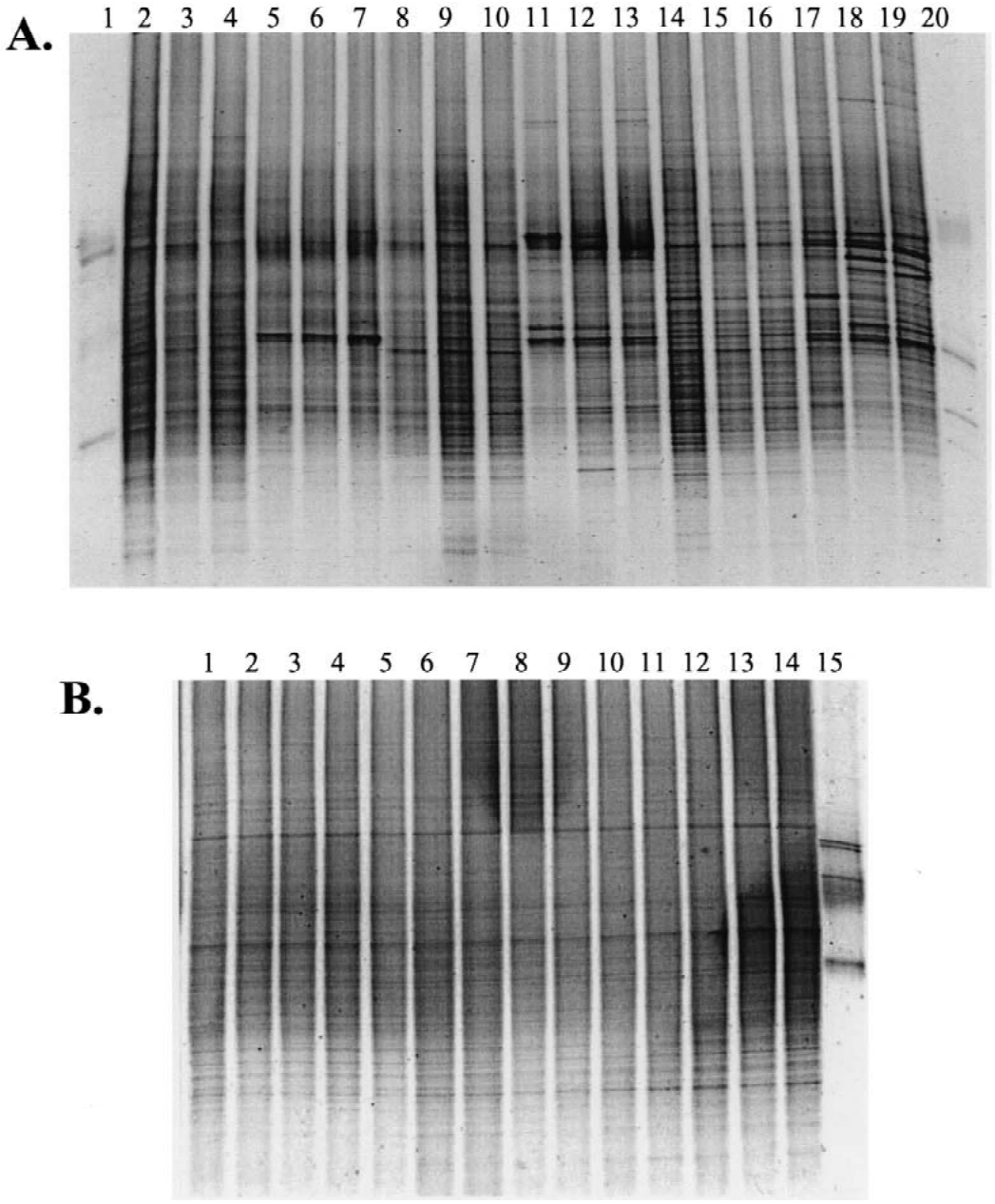

Figure 2. Bacterial PCR-DGGE analysis of (A) rhizosphere and (B) bulk soil from the Meyendel field experiment. Rhizosphere samples were as follows: (A) 1 & 20, bacterial marker; 2–4, *Circium vulgare* first harvest; 5–7, *Cynoglossum officinale* first harvest; 8–10, *Circium vulgare* second harvest; 11–13, *Cynoglossum officinale* second harvest; 14–16, *Circium vulgare* third harvest; 15–17, *Cynoglossum officinale* third harvest. (B) The bulk samples were taken from the study areas of every other rhizosphere sample (lanes 1–10) as well as four samples randomly chosen outside the experimental plots (lane 11–14). Lane 15 – bacterial marker.

μl of each reaction were inspected by 1.2% (w/v) agarose gel electrophoresis as above, and positive reactions further subjected to restriction analysis with the enzyme *Hae*III according to the manufacturer's recommended protocol (Promega). RFLP patterns were analysed by 3% (w/v) agarose gel electrophoresis with ethidium bromide staining, and digitised as described above. Band calling and RFLP pattern comparison was performed within the ImageMaster Elite Database program (version 2.0; Pharmacia). Bands below 100

bp were excluded from the analysis. Coverage of the 16S rDNA clone libraries was determined using the formula, $C = \{$(total # clones examined per sample – # of different RFLP patterns detected in sample) / total # clones examined per sample$\} \times 100\%$. Rarefaction analysis was performed using Analytic Rarefaction software (www.uga.edu/ strata/Softwarte.html) and version 1.2 of the rarefaction calculator (Krebs 1989; http://www.biology.ualberta.ca/jbrzusto/rarefact.php#Calculator).

Results and discussion

Bacterial community analysis of bulk and rhizosphere soil from Meyendel experiment

PCR-DGGE banding patterns of the rhizosphere samples within each plant species of a given harvest were highly consistent (Figures 2a and 3). In contrast, the band patterns from rhizosphere soil from the two plant species examined were highly different from each other. Analysis of DNA extracted from bulk soil samples however, produced a highly consistent result across all samples examined, whether from the vicinity of *Cynoglossum officinale, Cirsium vulgare* or neither (Figure 2b). Similar results were obtained using a separate bacterial primer set targeting the V-3 region of the 16S rRNA gene (not shown; Muyzer et al. 1993). These results suggest that the selection of the most dominant bacterial populations is a highly plant-driven process and that this process is highly specific for the two plant species tested. This effect is restricted to the rhizosphere, defined here as the root surface and adhering soil. Previous studies have shown various degrees of a 'rhizosphere effect' using either culture-dependent (Miller et al. 1989; Germida et al. 1998; Grayston et al. 1998) or culture-independent strategies (Marilley & Aragno 1999; Miethling et al. 2000; Duineveld et al. 2001; Smalla et al. 2001). The variable nature of previous results may be due to different sampling strategies for retrieving rhizosphere soil, different plant species, and differences in the methods used (Broughton & Gross 2000). Sampling strategies that include a greater amount of soil not under the direct influence of the plant, may serve to dilute out any 'rhizosphere' effects. Different plant species may differ in the degree and manner in which they influence microbial community structure in the rhizopshere. For instance, Smalla et al. (2001) observed that the changes induced in the rhizosphere of potato and oilseed rape were more similar than those induced by strawberry.

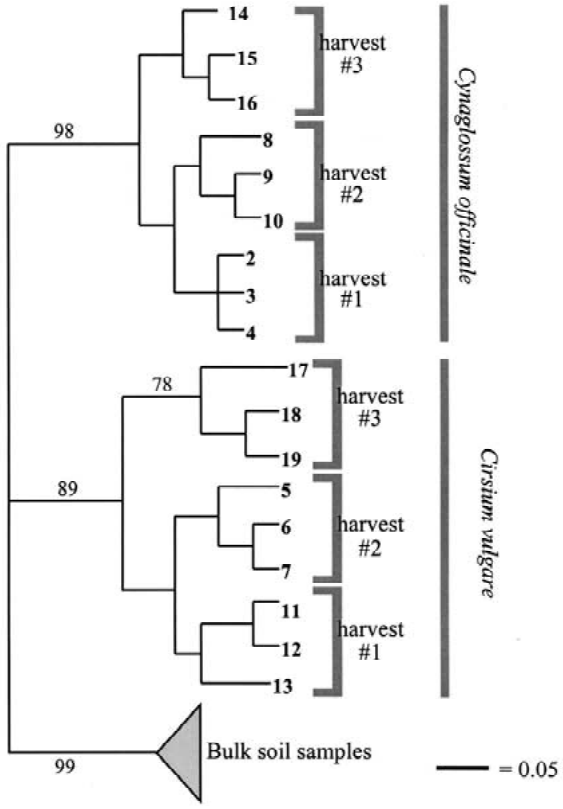

Figure 3. Dendrogram analysis of bacterial DGGE profiles from Meyendel experiment. The dendrogram is based upon the DGGE banding patterns presented in Figure 2, and numbers refer to lane numbers used in Figure 2. Details of the dendrogram analysis can be found in the text and Duineveld et al. (2001). The width of the triangle used to depict the bulk samples is proportional to the total variation found within the bulk samples, but the height does not correspond to the number of samples analysed.

Some succession was observed in the dominant microbial populations present in the rhizosphere of a given plant species over the course of the growing season (Figure 2A). However, the main characteristics of community profiles of a given plant species were maintained throughout the growing season (Figure 3).

Minimal variation was observed for all bulk soil samples analysed from the Meyendel experimental field, regardless of sampling date, or sampling location (Figures 2B and 3). These results are similar to those observed by Duineveld et al. (1998, 2001) and Smalla et al. (2001). The present study targeted 16S rDNA, and therefore only addressed detection of numerically dominant bacterial populations. Thus, although it can be argued that the bacterial populations that become dominant in the rhizosphere must actively grow to

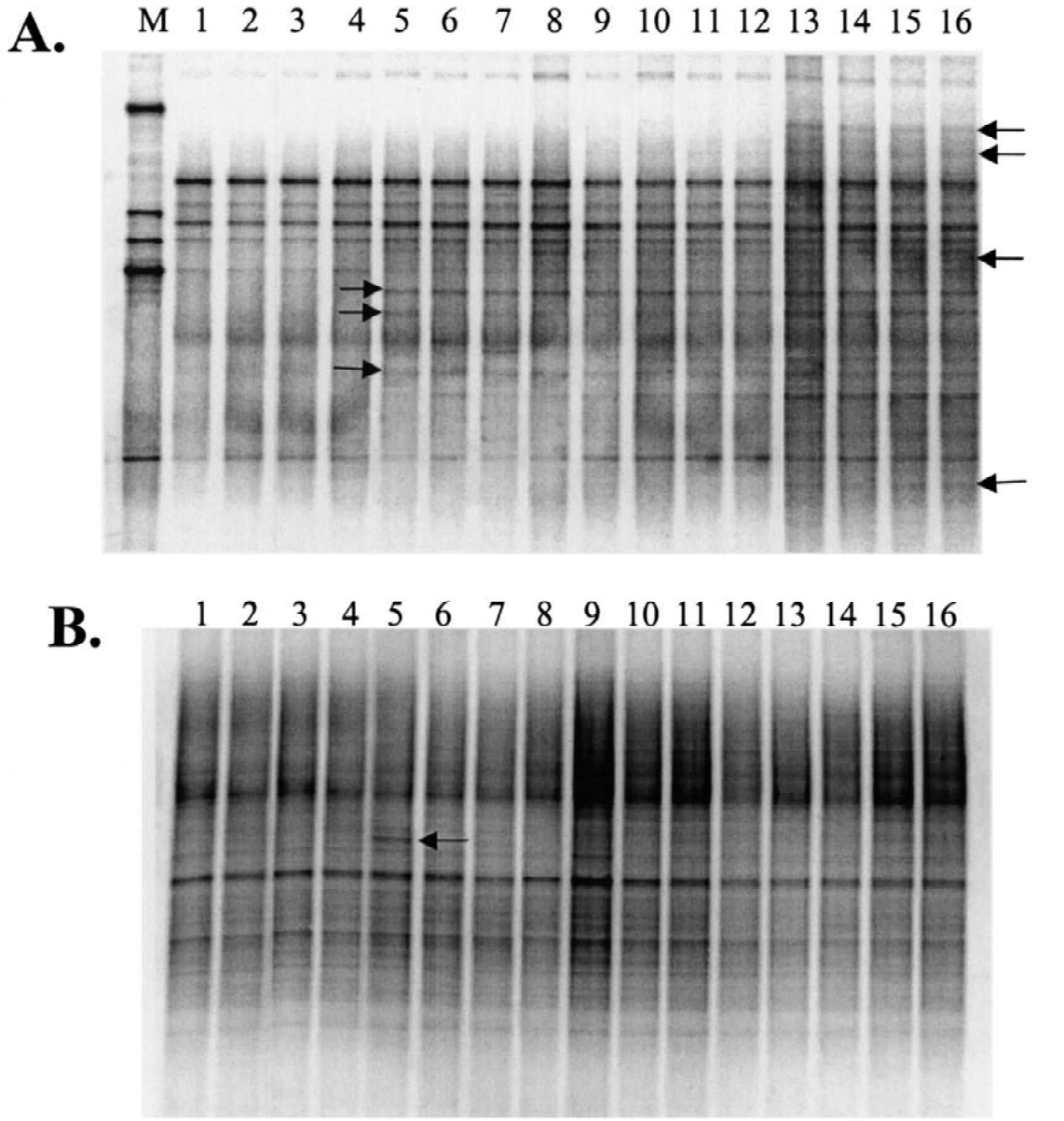

Figure 4. Bacterial PCR-DGGE analysis of (A) rhizosphere and (B) bulk soil from CLUE fields. PCR-DGGE across the V-6/V-8 region of the 16S rRNA gene from DNA extracted from CLUE field (A) rhizosphere and (B) bulk soil samples. Lane designations are as follows: (M) bacterial marker; (1–4) CA samples (5–8) LD samples (9–12) NC samples; (13–16) HD samples. Each sample within a treatment was taken from a different plot of the grid set up in the field experiment. For panel (A), arrows pointing to the right, designate bands that are absent from the CA samples, yet present in all other samples, and arrows pointing to the left designate bands unique to the HD samples. The arrow in panel (B) points to a unique band detected in one of the LD samples.

achieve this status, future studies targeting 16S rRNA, as performed by Duineveld et al. (2001), would address this issue more directly. Also, sequence analysis of those bands distinguishing between the rhizosphere samples of the different plant species would provide insight into the identity of bacterial populations stimulated by the two plant species examined, and such analysis is currently underway.

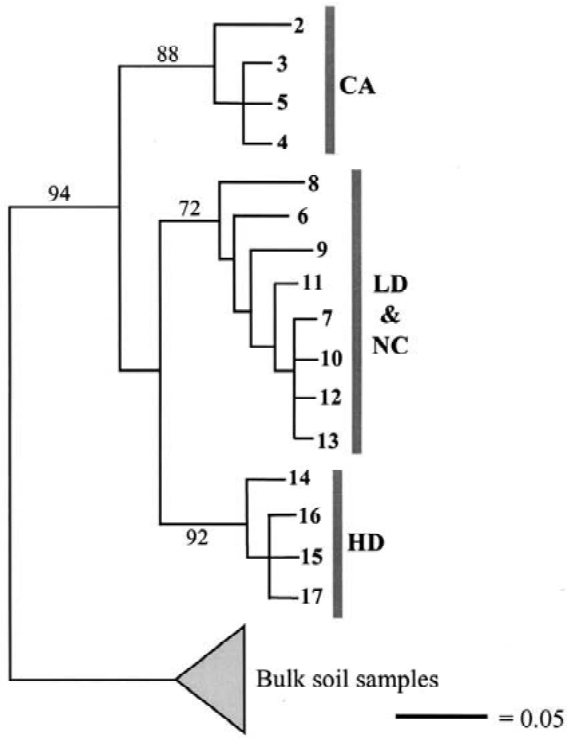

Figure 5. Dendrogram analysis of bacterial DGGE profiles from the CLUE experiment. The dendrogram is based upon the DGGE banding patterns presented in Figure 4, and numbers refer to lane numbers used in Figure 4. The width of the triangle used to depict the bulk samples is proportional to the total variation found within the bulk samples, but the height does not correspond to the number of samples analysed.

Table 1. Diversity within 16S rDNA clone libraries from CLUE field soil samples

Sample site	Number of clones examined	Number of ARDRA patterns observed	Coverage (%)
CA – rhiz	94	71	24
CA – bulk	96	86	10
LD – rhiz	88	64	27
LD – bulk	92	82	11
NC – rhiz	95	76	20
NC – bulk	96	87	9
HD – rhiz	92	77	16
HD – bulk	93	86	8
Total rhiz	369	239	35
Total bulk	377	289	23

Bacterial community analysis of CLUE field rhizosphere and bulk soil samples

The complex DGGE patterns recovered from rhizosphere DNA from the CLUE fields was highly consistent within a given treatment (Figures 4a and 5). The greatest variability within a treatment was observed for the LD plots (Figure 5). These plots were sown with different subsets of the plant species used in the HD treatment and showed a wide disparity with respect to the dominant plant species present (Van Der Putten et al. 2000). Clear differences were observed between the different diversity treatments, with a number of bands absent from the CA treatment and a number of bands only present in the HD treatment, which contained the most complex DGGE banding patterns. All rhizosphere profiles were clearly distinct from the community profiles observed for bulk soil. In contrast to the results from rhizosphere samples, bulk soil samples were nearly indistinguishable from each other, except for a single unique band in one of the LD samples (Figure 4b). Thus, as observed in the Meyendel experiment, the detectable effect of plant species composition seemed to be limited for the most part to the rhizosphere. The differences observed between the different plant diversity treatments was less than that observed for the two plant species tested in the Meyendel experiment. This may be due to the fact that CLUE rhizosphere samples were taken from all plant roots present in a sample, and not traced back to individual plants or plant species. Thus, these samples sum up the potential rhizosphere effects of all the plants growing in a particular plot.

Although the sowing of different plant species combinations resulted in clear differences in the plant diversity and species composition of the different plots, some naturally colonising plant species were present in all plots (Van Der Putten et al. 2000), causing some redundancy of plant species between the different CLUE plots. As with the Meyendel experiment, future studies directed at 16S rRNA and sequence analysis of DGGE bands should provide further insight into differences in the dominantly active bacterial populations in the rhizosphere and bulk soils subjected to different plant diversity treatments.

Collector's curves based upon 16S rDNA clones

Although community profiling techniques such as PCR-DGGE can be highly informative for detecting shifts between the most dominant populations in microbial communities, such methods are not suitable for assessments of diversity. Although one can extrapolate band number and intensity of a community profile to produce a biodiversity index (Hendrick et al. 2000; Kowalchuk et al. 2000a; Yin et al. 2000; McCaig et

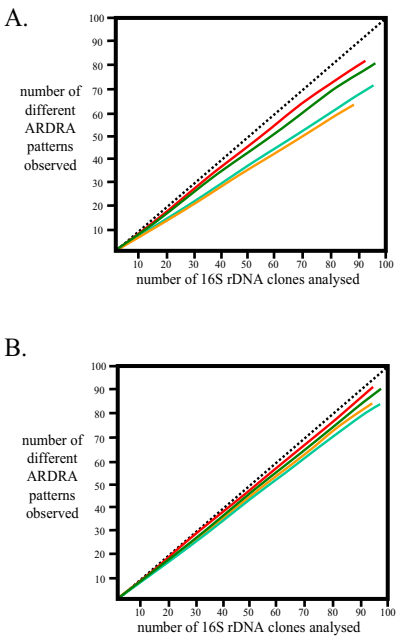

Figure 6. Collector's curves of 16S rDNA bacterial OTUs from CLUE fields: (A) rhizosphere and (B) bulk soils. The dotted line gives a slope of one, depicting the case if every ARDRA pattern sampled were unique. Colour codes are as follows: orange – CA; blue – LD; green – NC; and red – HD.

al. 2001), band number and intensity are not valid indicators of species richness and evenness. We therefore also adopted an approach of PCR, cloning, and clone screening by ARDRA to address the issue of the diversity within the rRNA gene pool of bulk and rhizosphere samples taken from the CLUE experimental fields. In agreement with previous estimates (Torsvik et al. 1990), analysis of 16S rDNA clone libraries revealed an extremely high level of bacterial diversity, as evidenced by the low coverage observed for all of the clone libraries examined (Table 1). This low level of coverage is in agreement with other PCR-cloning based studies targeting soil-borne bacterial communities (Borneman et al. 1996; Zhou et al. 1997; McCaig et al. 1999; Marilley & Aragno 1999; Nogales et al. 2001). Coverage was very low for all bulk samples and somewhat higher for rhizosphere samples, suggesting a lower bacterial diversity in the rhizosphere. This result is consistent with the suggestions made by Yin et al. (2000), who claimed a greater functional redundancy in soils supporting a more varied plant community. These results also agree with those based upon culture-dependent analysis of microbial responses to increased plant diversity (Stephan et al. 2000). Collector's curves and rarefaction analysis (not shown) showed no differences in the diversity of the clone libraries recovered from the bulk soil samples (Figure 6a). Some differences were however found in the rhizosphere samples, with a trend toward lower diversity in the LD and CA plots (Figure 6a).

Since all bulk samples appeared highly similar both in terms of community structure (Figure 4) and diversity (Figure 6b), we pooled the clone libraries of the four bulk samples analysed, revealing a total coverage of 23%, which was more than twice as high as any individual library (Table 1). Even though a combination of the rhizosphere data may not be valid due to clear differences in community structures of these samples, we combined these data as well for comparison. The total coverage of rhizosphere clones was estimated at 35%, which is only marginally higher than the coverage observed for the LD rhizosphere libraries (Table 1). Thus, combining the four rhizosphere samples, had a less additive effect on coverage than pooling of bulk soil data, suggesting that the individual rhizosphere samples were more distinct from each other than the individual bulk soil samples were from each other. Even with the pooling of data, sample size was not large enough to circumscribe the bacterial diversity in the samples analysed. Nonetheless, clear trends could be observed in the rate of collection of new OTUs in the rhizosphere versus bulk soil and in the different rhizosphere samples.

Discussion and conclusions

This report represents the first culture-independent demonstration of a clear plant-induced effect on bacterial community structure in the rhizosphere of non-agricultural plant species. This effect appears to be limited to the direct rhizosphere, and the level of coupling between above-ground macrophyte communities and below-ground microbial communities therefore appears to be related to the tightness of the interactions involved. Nevertheless the clear and reproducible changes in microbial community structure and diversity observed in the rhizosphere may have important implications for plant growth, vegetational succession and other critical functions (Bever 1994). We would hypothesise that the diversity within mi-

crobial functional groups that interact more directly and necessarily with plant roots (e.g. mycorrhiza and obligate plant pathogens) should be more affected by plant diversity and species composition than microbial groups with less direct root associations (e.g. nitrifying bacteria; Kowalchuk et al. 2000a).

The results presented here suggest that the rhizosphere selects for specific soil-borne microbial populations, and that this effect is highly plant-specific and reproducible for a given plant species. Thus, although general soil characteristics may be most important in determining the most dominant bacterial populations in bulk soil (Duineveld et al. 1998; Felske & Akkermans 1998; Kowalchuk et al. 2000a) microbial communities in the rhizosphere are plant-driven to a far greater extent. It is, however, not yet clear to what extent soil characteristics predetermine the pool of bacterial populations from which plants can select, and future studies will need to incorporate both plant species composition and bulk soil properties. Also, plant species diversity and species composition can have indirect and long-term effects on general soil properties, for instance by the uptake of nutrients by plants and the deposition of various quantities and qualities of litter (Berendse 1990, 1999). Thus, while the dominant microbial populations of bulk soil appear to be more a reflection of the general soil properties than plant species composition, these general soil properties can be steered by long-term vegetation effects.

The selective effect of plants appears to lower the diversity of bacterial communities in the rhizosphere. Intuitively, a greater level of plant diversity should lead to a higher level of heterogeneity in structure and exudate patterns, capable of supporting a higher degree of microbial diversity (Korona et al. 1994). This indeed appears to be the case. The differential selection imposed by a greater variety of plant species in fields of high macrophyte diversity maintains a higher level of bacterial diversity within the collective rhizosphere as compared to low diversity fields. This diversity effect is however not translated to the bulk soil, at least not within the timeframe of the experiments presented here.

The results presented demonstrate that one cannot assume that plant diversity and species composition will act as a driver of microbial diversity. Rather, the level of interaction with the plant is critical in determining the degree of coupling between macrophyte and microbial communities. Thus, it remains an open question whether loss of macrophyte diversity will eventually lead to losses in microbial diversity. On the basis of recent RFLP data of bacterial clones recovered from soil, it has been speculated that high bacterial diversity is fostered by spatial isolation of bacteria in low carbon topsoil environments (Zhou et al. 2002). Our data from bulk soil microbial communities support this idea and suggest that competition for specific resources becomes more important in the rhizosphere, especially when plant diversity is low. Thus, although we are starting to recognise patterns of microbial diversity in soil and the role of plants in shaping the distribution of microbial populations, we still do not know the exact mechanisms that serve to maintain extremely high levels of microbial diversity in soil ecosystems (Dykhuizen 1998).

Acknowledgements

The authors thank Saskia Gerards and Karin van der Veen for assistance with the Meyendel experiment, and Wim van der Putten for assistance with the CLUE experiments and for critical reading of the manuscript. This represents publication #2933 of Centre for Terrestrial Ecology of the Netherlands Institute of Ecology Netherlands, which is an institute of the Dutch Royal Academy of Arts and Sciences (NIOO-KNAW).

References

Amann R, Ludwig W & Schleifer K-H (1995) Phylogenetic identification and *in situ* detection of individual cells without cultivation. Microbiol. Rev. 59: 143–169.

Bardgett RD, Mawdsley JL, Edwards S, Hobbs PJ, Rodwell JS & Davies WJ (1999) Plant species and nitrogen effects on soil biological properties of temperate upland grasslands. Funct. Ecol. 13: 650–660.

Berendse F (1999) Implications of increased litter production for plant biodiversity. Trends Ecol. Evol. 14: 4–5.

Berendse F (1990) Organic-matter accumulation and nitrogen mineralization during secondary succession in healthland ecosystems. J. Ecol. 78: 413–427.

Bever JD (1994) Feedback between plants and their soil communities in an old field community. Ecology 75: 1965–1977.

Borneman J, Skroch PW, O'Sullivan KM, Palus JA, Rumjanek NG, Jansen JL, Nienhuis J & Triplett EW (1996) Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. 62: 1935–1943.

Broughton LC, Gross KL (2000) Patterns of diversity in plant and soil microbial communities along a productivity gradient in a Michigan old-field. Oecologia 125: 420–427.

Duineveld BM, Rosado AS, Van Elsas JD & Van Veen JA (1998) Analysis of the dynamics of bacterial communities in the rhizosphere of the chrysanthemum *via* denaturing gradient gel electrophoresis and substrate utilization patterns. Appl. Environ. Microbiol. 64: 4950–4957.

Duineveld BM, Kowalchuk GA, Keizer A, Van Elsas JD & Van Veen JA (2001) Analysis of the bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR amplified 16S ribosomal RNA and DNA fragments. Appl. Environ. Microbiol. 67: 172–178.

Dykhuizen DE (1998) Santa Rosalia revisited: Why are there so many species of bacteria? Antonie van Leeuwenhoek 73: 25–33.

Edwards U, Rogall T, Blöcker H, Emde M & Böttger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. 17: 7843–7853.

Felske A & Akkermans ADL (1998) Spatial homogeneity of abundant bacterial 16S RRNA molecules in grassland soils. Microb. Ecol. 36: 31–36.

Germida JJ, Siciliano SD, De Freitas RJ & Seib AM (1998) Diversity of root-associated bacteria associated with filed-grown canola *Brassica napus* L.) and wheat (*Triticum aestivum* L.). FEMS Microbiol. Ecol. 26: 43–50.

Grayston SJ, Wang S, Campbell CD & Edwards AC (1998) Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biol. Biochem. 30: 369–378.

Hector A, Schmid B, Beierkuhnlein C, Caldeira MC, et al. (1999) Plant diversity and productivity experiments in European grasslands. Science 286: 1123–1127.

Hedrick DB, Peacock A, Stephen JR, Macnaughton SJ, Bruggemann J & White DC (2000) Measuring soil microbial community diversity using polar lipid fatty acid and denaturing gradient gel electrophoresis data. J. Microbiol. Methods 41: 235–248.

Heuer H, Krsek M, Baker P, Smalla K & Wellington EMH (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl. Environ. Microbiol. 63: 3233–3241.

Heuer H, Hartung K,Wieland G, Kramer I & Smalla K (1999) Polynucleotide probes that target a hypervariable region of 16S rRNA genes to identify bacterial isolates corresponding to bands of community profiles. Appl. Environ. Microbiol. 65: 1045–1049.

Hooper DU & Vitousek PM (1997) The effects of plant composition and diversity on ecosystem processes. Science 277: 1302–1305.

Huston MA (1997) Hidden treatments in ecological experiments: re-evaluating the ecosystem function of biodiversity. Oecologia 110: 449–460.

Insam H & Domsch KH (1988) Relationship between soil organic-carbon and microbial biomass on chronosequences of reclamation sites. Microb. Ecol. 15: 177–188.

Korona R, Nakatsu CH, Forney LJ & Lenski RE (1994) Evidence for multiple adaptive peaks from populations of bacteria evolving in a structured habitat. Proc. Natl. Acad. Sci. USA 91: 9037–9041.

Korthals GW, Smilauer P, Van Dijk C & Van Der Putten WH (2001) Linking above- and below-ground biodiversity: abundance and trophic complexity in soil as a response to experimental plant communities on abandoned arable land. Funct. Ecol. 15: 506–514.

Kowalchuk GA, Stienstra AW, Heilig GHJ, Stephen JR, & Woldendorp JW (2000b) Composition of communities of ammonium-oxidising bacteria in wet, slightly acid grassland soils using 16S rDNA-analysis. FEMS Microbiol. Ecol. 31: 207–215.

Kowalchuk GA, Stienstra AW, Heilig GHJ, Stephen JR & Woldendorp JW (2000a) Changes in the community structure of ammonia-oxidizing bacteria during secondary succession of calcareous grasslands. Environ. Microbiol. 2: 99–110.

Krebs CJ (1989) Ecological Methodology. Harper & Row, New York.

Maly S, Korthals GW, Van Dijk C, Van der Putten WH & De Boer W (2000) Effect of vegetation manipulation of abandoned arable land on soil microbial properties. Biol. Fertil. Soils 31: 121–127.

Marilley L & Aragno M (1999) Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. Appl. Soil Ecol. 13: 127–136.

McCaig AE, Glover LA & Prosser JI (1999) Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl. Environ. Microbiol. 65: 1721–1730.

McCaig AE, Glover LA & Prosser JI (2001) Numerical analysis of grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. Appl. Environ. Microbiol. 67: 4554–4559.

Miethling R, Wieland G, Backhaus H & Tebbe CC (2000) Variation of microbial rhizosphere communities in response to crop species, soil origin, and inoculation with *Sinorhizobium meliloti* L33. Microb. Ecol. 41: 43–56.

Miller HJ, Henken G & Van Veen JA (1989) Variation and composition of bacterial populations in the rhizosphere of maize, wheat, and grass cultivars. Can J. Microbiol. 35: 656–660.

Muyzer G & Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73: 127–141.

Muyzer G, De Waal EC & Uitterlinden AC (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes for 16S rRNA. Appl. Environ. Microbiol. 55: 695–700.

Naeem S & Li SB (1997) Biodiversity enhances ecosystem reliability. Nature 390: 507–509.

Naeem S, Thompson LJ, Lawler SP, Lawton JH & Woodfin RM (1994) Declining biodiversity can alter the performance of ecosystems. Nature 368: 734–737.

Nogales B, Moore ERB, Llobet-Brossa E, Rossello-Mora R, Amann R & Timmis KN (2001) Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. Appl. Environ. Microbiol. 67: 1174–1184.

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H & Berg G (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl. Environ. Microbiol. 67: 4742–4751.

Spehn EM, Joshi J, Schmid B, Alphei J & Körner C (2000) Plant diversity effects on soil heterotrophic activity in experimental grassland ecosystems. Plant Soil 224: 217–230.

Stephan A, Meyer AH & Schmid B (2000) Plant diversity affects culturable soil bacteria in experimental grassland communities. J. Ecol. 88: 988–998.

Stephen JR & Kowalchuk GA (2002) Ribotyping methods for assessment of *in situ* microbial community structure. In: Gabrielle Bitton (ed.) Encyclopedia of Environmental Microbiology, Volume 5. John Wiley & Sons, New York, USA. pp. 2728–2741.

Tiedje JM (1995) Approaches to the comprehensive evaluation of prokaryote diversity of a habitat. In: Allsopp D, Hawksworth DL, Colwell RR (Eds) Microbial Diversity and Ecosystem Function (pp 73–97). CAB International, Wallingford, UK.

Tiedje JM, Asuming-Brempong, Nüsslein K, Marsh TL & Flynn SJ (1999) Opening the black box of soil microbial diversity. Appl. Soil Ecol. 13: 109–122.

Tilman D & Downing JA (1994) Biodiversity and stability in grasslands. Nature 367: 363–365.

Tilman D, Wedin D & Knops J (1996) Productivity and sustainability influenced by biodiversity in grassland ecosystems. Nature 379: 718–720.

Tilman D, Knops J, Wedin D, Reich P, Ritchie M & Siemann E (1997) The influence of functional diversity and composition on ecosystem processes. Science 277: 1300–1302.

Torsvik V, Goksoyr J & Daae FL (1990) High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. 56: 782–787.

Van der Putten WH, Mortimer SR, Hedlund K, Van Dijk C, Brown VK, Leps J, Rodriguez-Barrueco C, Roy J, Len TAD, Gormsen D, Korthals GW, Lavorel S, Regina IS & Smilauer P (2000) Plant species diversity as a driver of early succession in abandoned fields: a multi-site approach. Oecologia 124: 91–99.

Wardle DA (1992) A comparative assessment of factors which influence microbial biomass carbon and nitrogen levels in soil. Biol. Rev. Cambridge Phil. Soc. 67: 321–358.

Wardle DA, Bonner KI, Barker GM, Yeates GW, Nicholson KS, Bardgett RD, Watson RN & Ghani A (1999) Plant removals in perennial grassland: vegetation dynamics, decomposers, soil biodiversity, and ecosystem properties. Ecol. Monogr. 69: 535–568.

Yin B, Crowley D, Sparovek G, De Melo WJ & Borneman J (2000) Bacterial functional redundancy along a soil reclamation gradient. Appl. Environ. Microbiol. 66: 4361–4365.

Zhou JZ, Davey ME, Figueas JB, Rivkina E, Gilichinsky D & Tiedje JM (1997) Phylogenetic diversity of a bacterial community determined from Siberian tundra soil DNA. Microbiology 143: 3913–3919.

Zhou JZ, Xia B, Treves DS, Wu L-Y, Marsh TL, O'Neill RV, Palumbo AV & Tiedje JM (2002) Spatial and resource factors influencing high microbial diversity in soil. Appl. Environ. Microbiol. 68: 326–334.