

DGGE-fingerprinting of arable soils shows differences in microbial community structure of conventional and organic farming systems

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Abstract

Microbial diversity and community structure in soil samples from conventional (CF) and organic farming sites (OF) were analysed using denaturing gradient gel electrophoresis (DGGE) and shotgun cloning. DNA extracted directly from soil was amplified with different primer combinations to allow DGGE fingerprinting of the total eubacterial community, as well as of *Actinomycetes*, ammonia-oxidizers, *Acidobacteria* and *Archaea*. Highly similar band patterns obtained from replicate samples indicated that the genetic structure of the soil microflora was spatially homogeneous. Complex eubacterial community profiles reflected a high level of diversity. Group specific DGGE analysis produced simpler band patterns and facilitated the detection of differences between soils. CF and OF samples showed differences in the community structure of *Eubacteria*, *Actinomycetes*, ammonia-oxidizers and *Archaea*. In particular the ammonia-oxidizers showed clear and simple DGGE profiles and were found to be promising marker organisms for the analysis of agricultural soils. Eubacterial 16S rDNA fragments of about 640 bp were used to create a clone library of each soil. A total of 28 different inserts selected by DGGE screening were sequenced. The obtained sequences were affiliated with *Actinobacteria*, *Acidobacteria* and *Proteobacteria*.

Key words: *Acidobacteria*, *Actinomycetes*, ammonia-oxidizers, *Archaea*, DGGE, shotgun cloning, organic farming, soil.

Introduction

Many environmental problems such as erosion, groundwater pollution, soil acidification, soil salinization or the spread of antibiotic resistances have their roots in unsustainable agricultural practice¹⁻³. Over the past decades alternative agricultural practices, such as organic farming, have been developed in order to produce healthier food and to preserve soil quality and fertility⁴. Rapid and reliable scientific strategies need to be developed to enable an ecological evaluation of these farming concepts.

The importance of the soil microflora, which is involved in the decomposition of ecosystem litter, the formation of humic compounds and the release of nutrients, to soil fertility is well known⁵. Soil microorganisms are sensitive to anthropogenic disturbances and in particular to agricultural activities^{1, 6, 7}. Conventional and organic farming systems have been found to affect the soil microflora in different ways. Organically managed soils usually exhibit higher microbial biomass and activity⁸⁻¹².

For an assessment of soil fertility, microbial diversity and community structure are considered to be particularly valid microbiological parameters. The composition of the microflora gives information about both the state and the history of the soil ecosystem. It reflects the immediate displacement of organisms as well as long-term effects on processes caused by successions in the microbial community^{6, 13}. Microbial diversity has been observed to decrease as a response to environmental stress. A decrease in diversity results in the risk that the ecosystem will have a reduced ability to respond to perturbations¹⁴.

Studies addressing the microbial community composition revealed differences between conventional and organic farming systems^{8, 10, 12, 15}. These observations have been made by

using cultivation assays, Biolog metabolic fingerprinting or phospholipid fatty acid analysis (PLFA). Traditional cultivation techniques are known to cover only 0.1-1% of the soil bacteria¹⁶. Biolog is strongly selective for fast growing bacteria¹³ and PLFA, although comprising the entire active community, provides only limited information about diversity since individual fatty acids cannot be attributed to specific species⁸. Hence surveys relying on these techniques are likely to reflect the state of the soil microflora in an incomplete manner.

Culture-independent techniques based on the analysis of environmental DNA, especially the *rrs* gene (16S rDNA), have the potential to overcome these limitations and have become a powerful tool of modern microbial ecology¹⁶. The existence of databases containing thousands of ribosomal sequences allows phylogenetic affiliation of uncultured microorganisms¹⁷. The genetic structure of microbial communities as a whole can be studied by denaturing gradient gel electrophoresis (DGGE). This technique has proved to be very suitable for comparative fingerprinting of many different samples¹⁸. Moreover the number of DGGE bands gives information about the species richness of the analysed communities. However it has become evident that soil microbial communities are too complex to be comprehensively fingerprinted as a whole^{18, 19}. Ecologically relevant micro organisms of low abundance may not be detected in an universal approach.

The use of specific 16S rDNA primers focuses the fingerprinting analysis on individual phylogenetic groups and reveals details about the composition of the total community²⁰. In addition, group specific fingerprinting based on functional genes helps to link structural to functional information²¹. A simplification of the

system, by restricting the analysis to a defined subset of the microbial community, should facilitate the detection of differences between similar samples such as differently treated agricultural soils.

In this study the total eubacterial community of a conventional and an organic farming soil, named CF and OF respectively, was studied by DGGE analysis and shotgun cloning. This universal approach was combined with specific DGGE fingerprinting of four phylogenetic groups, *Actinomycetes*, ammonia-oxidizing bacteria, *Acidobacteria* and *Archaea*, relevant to soil ecosystems. The *Actinomycetes* are known to decompose organic materials in soils including recalcitrant polymers and furthermore are associated with the natural suppression of plant diseases^{15, 22}. The ammonia-oxidizing bacteria play a central role in global N cycling and plant nutrition and are also involved in the environmental issue of nitrate leaching. Because of their phylogenetic and functional coherence the ammonia-oxidizers have become a model system in molecular microbial ecology²³. The *Acidobacteria*, a novel bacterial division discovered by culture-independent techniques, are numerically significant constituents of many ecosystems, especially of soils²⁴. The domain *Archaea* was thought to be limited to extreme environments, but molecular studies gave evidence that *Archaea* are widespread in non-extreme ecosystems and form diverse populations in soil²⁵⁻²⁷.

The aim of this work was to evaluate the suitability of the DGGE-cloning approach as a rapid molecular strategy for comparing differently managed soils and to assess the sensitivity of the *Actinomycetes*, the ammonia-oxidizing bacteria, the *Acidobacteria* and the archaeal community as indicators for agricultural influences.

Materials and Methods

Field sites: Soil samples were collected from two farming sites in Deutsch-Wagram (Marchfeld, Lower Austria). Both sites were under conventional agricultural management in the past. In 1995 one site was converted into an organic farming field (OF) whereas the other site remained under conventional farming (CF). Both treatments involved tillage, irrigation and crop rotations. The CF soil was supplied with mineral fertilizers (N, P and K) and treated with chemical pesticides. CF seeds were chemically dressed against pathogens. Over the last decade wheat, maize and turnip have been grown on the CF field. The OF treatment was without mineral fertilizers, pesticides and dressing. The nutrient status of the OF soil was balanced by an elaborate crop rotation system including the cultivation of various cereals, a one-year fallow under legume cover every 5 or 6 years and annual legume-intercropping. The legumes were incorporated into the soil to compensate nitrogen depletion. Compost was added once, in fall 2000. Weeds were controlled mechanically by intensified tilling and harrowing.

The two fields were 1 km apart and did not differ in texture (loamy sand), moisture content (16% of the fresh weight), NH_4 content (15 g per g dry weight) and pH (7 in water). However the total organic carbon content, measured in an elementary analyser (EA 1110 CE Instruments) combined with a mass spectroscope (D+, Finnigan Mat.), was 3.4% in CF soil and 1.1% in OF soil.

Sampling: Samples were taken from the top 15 cm of the bulk soil on fields prepared for the cultivation of maize after ploughing in spring 2002. To consider spatial heterogeneity each field was sampled at three points 100 m apart from one another. Each

replicate consisted of five 5 cm dia cores collected from a square of 10 m², that were pooled before taking out 60 g of soil for laboratory analyses. Samples were frozen within five hours and stored at -20°C.

DNA extraction and purification: DNA was extracted directly from soil by using the Fast DNA SPIN kit for soil (Bio 101). The protocol of the manufacturer was modified as follows. To remove any extracellular DNA, 1 g soil was resuspended in 1 ml sodium phosphate buffer (120 mM, pH 7.6) and incubated on a shaker at 150 rpm for 10 min. The slurry was centrifuged at 6000xg for 5 min., and 0.7 g of the soil pellet was placed in the MULTIMIX2 tissue tube, 978 μl sodium phosphate buffer and 122 μl of the MT buffer (Bio 101) were added. The mixture was then bead beaten two times for 1 min. with one intervening minute on ice and centrifuged at 14,000xg for 2 min. The PPS reagent and the binding matrix suspension (Bio 101) were applied to the supernatant following the instructions of the manufacturer. The resulting suspension was transferred to a spin filter and centrifuged at 14,000xg for 1 min. DNA was washed twice with 500 μl of the SEWS-M solution (Bio 101) and eluted from the binding matrix with 300 μl DES (Dnase/Pyrogen free water). The extracted DNA was further purified with the QIAamp Viral RNA mini kit (Quiagen) and finally eluted from the silica column with 100 μl ddH₂O. DNA concentration in the purified extracts was estimated by using the Nucleic Dot Metric TM System (Genotech). It ranged between 0.06 and 0.11 $\mu\text{g } \mu\text{l}^{-1}$.

PCR amplification: 16S rDNA from *Eubacteria*, *Archaea*, *Acidobacteria* and *Actinomycetes* as well as a fragment of the *amoA* gene of the ammonia-oxidizers were amplified from the soil extracts. Table 1 lists the primer combinations used in this study. To obtain enough PCR products for DGGE analysis, DNA of each phylogenetic group was amplified in two successive rounds of PCR.

The first amplification was carried out in 25 μl volumes containing 12.5 pmol of each primer (Table 1), 5 pmol of each deoxyribonucleotide triphosphate, 0.5 U of Taq polymerase (Roche Diagnostics), 2.5 μl 10x PCR buffer (100 mM Tris-HCl, 15 mM MgCl_2 , 500 mM KCl, pH 8.3, Roche Diagnostics), 1.25 μl DMSO (Dimethyl sulfoxide, Sigma), 10 μg BSA (bovine serum albumin, Roche diagnostics) and sterile water up to the final volume. 1.5 μl DNA extract was used as template.

PCR was performed in a Robocycler (Stratagene, La Jolla, CA). For the amplification of eubacterial 16S rDNA fragments, samples were first denatured at 95°C for 5 min., followed by 35 cycles of 95°C for 1 min., 55°C for 1 min. and 72°C for 2 min. and a final extension step at 72°C for 5 min. The *amoA* gene fragments were amplified under the same conditions, but the number of cycles was raised to 40. *Actinomycete* 16S rDNA was amplified by applying the following programme: 94°C for 5 min., followed by 35 cycles of 94°C for 1 min., 63°C for 1 min. and 72°C for 1 min. and a final extension at 72°C for 5 min. Cycling conditions for the amplification of 16S rDNA fragments of *Acidobacteria* and *Archaea* have been described previously^{24, 28}.

For DGGE analysis, the DNA was reamplified in 150 μl reaction volumes divided in 3 aliquots of 50 μl . As template 3-4 μl PCR product obtained in the first round was added to each of the 50 μl aliquots. Concentrations of reagents were as in the first round of PCR, but no BSA was added. In the second round one of the

primers carried the GC clamp described by ¹⁸. Nested PCRs were performed under the same thermal conditions as the initial amplifications except for eubacterial 16S rDNA, where the extension time was reduced to 1 min. and for archaeal 16S rDNA (see ²⁸).

DGGE analysis: 150 µl PCR product obtained by nested PCR was precipitated in 1 ml 96% ethanol, resuspended in 15 µl ddH₂O and separated by DGGE. Electrophoresis was performed as described by Muyzer et al.¹⁸ with 8% (vol vol⁻¹) polyacrylamide gels (acrylamide: bisacrylamide = 37.5:1), containing a linear gradient of formamide and urea. The following gradients were used: 30-60% or 35-60% for eubacterial 16S rDNA, 40-60% for the *Actinomyces*, 15-50% for the *amoA* sequences, 20-50% for *Acidobacteria* and 35-55% for *Archaea* (with 100% denaturant defined as 7 M urea and 40% (vol vol⁻¹) formamide).

Gels were electrophoresed in 0.5x TAE buffer [20 mM Tris, 10 mM acetate, 0.5 mM Na₂EDTA; pH 7.8] at 60°C and 200 V for 3.5 h using the D-Gene system (Bio-Rad). The DGGE band patterns were visualised by ethidium bromide staining (in a 0.5 µg ml⁻¹ solution) and UVP transillumination.

Cloning of 16S rDNA fragments and PCR-DGGE screening:

5 µl PCR products amplified with the eubacterial primer combination 341f and 984r were ligated into the pGEM-T easy vector (Promega) following the instructions of the manufacturer. The ligation product was transformed into *E. coli* XL1 blue Tet^R, which allows the identification of recombinants (white colonies) on an indicator LB medium containing ampicillin (100 mg ml⁻¹), tetracycline (10 mg ml⁻¹), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 0.1 mM) and IPTG (isopropyl-β-D-thiogalactopyranoside: 0.2 mM) ²⁹. 48 clones from each soil were obtained and resuspended in 40 µl TE buffer [10 mM Tris, 1 mM EDTA, pH 8]. Screening of the clone libraries by PCR and DGGE was performed ³⁰ with two modifications of volumes: 2.5 µl clone lysate were used as template for reamplifying the inserts and 25 µl nested PCR product were loaded on the gradient gel. Inserts of clones matching bands in the fingerprint of the original samples were sequenced.

Sequencing of cloned inserts and phylogenetic affiliations: For sequencing, the inserts were amplified with primers Sp6 and T7 in a 100 µl PCR mixture. The amplicons were subsequently purified and sequenced ³⁰. Sequencing errors were minimized by combining a medium run sequence analysis using the primer Sp6 with a short run using T7. The obtained sequences were compared to known sequences using the Fasta search option for the EMBL database to find close evolutionary relatives ³¹.

Results

DGGE analysis of eubacterial community structure: The eubacterial community structure was analysed by separating 200 bp 16S rDNA fragments obtained by nested PCR in DGGE analysis. The central lanes in Fig. 1 show the genetic structure of the eubacterial communities in the CF and OF soil. Obtained band patterns showed about 15 predominant and numerous faint bands, indicating high bacterial diversity in investigated agricultural soils. Samples from CF and OF soil produced similar band patterns. However, differences could be detected particularly in the lower part of the gel, where OF samples showed additional bands.

The spatial community structure of *Eubacteria* was assessed by comparing the profiles of ten random samples taken from each

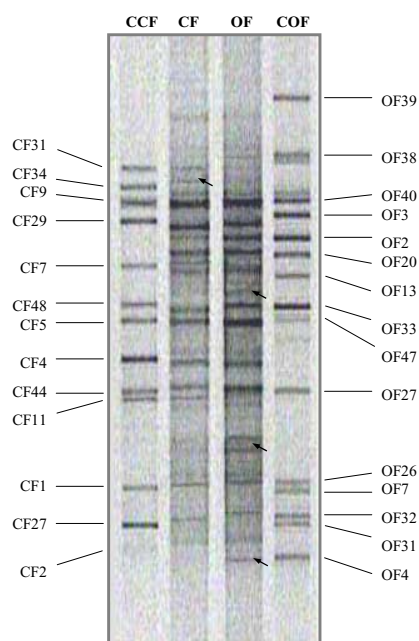


Figure 1. DGGE analysis of the eubacterial community. Negative image of ethidium bromide-stained DGGE separation patterns of 16S rDNA fragments amplified with eubacterial primers. The central lanes show original fingerprints obtained from DNA extracts of soils under conventional (CF) and organic farming (OF). The peripheral lanes represent fingerprints of all sequenced and pooled clones derived from the conventional (CCF) and the organic farming soil (COF).

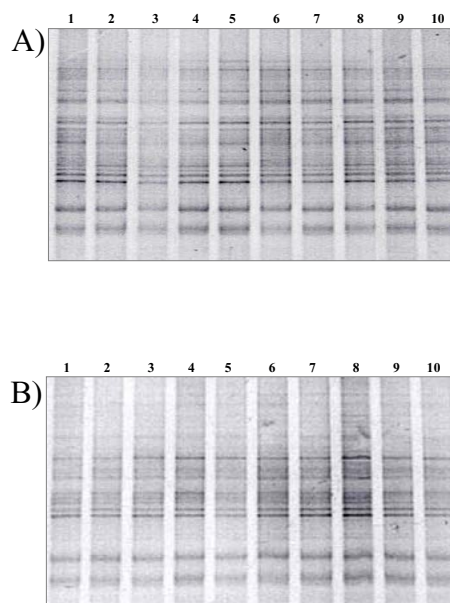


Figure 2. Spatial distribution of the Eubacterial community. Negative image of ethidium bromide-stained DGGE separation patterns of 16S rDNA fragments amplified from soil DNA extracts. A) Eubacterial community in ten random samples from a soil under conventional management. B) Eubacterial community in ten random samples from a soil under organic management.

site. For both CF and OF soil band patterns were reproducible (Fig. 2). Therefore random samples can be considered representative for the eubacterial community in a whole field.

Sequence analysis of eubacterial 16S rDNA clones: Shotgun cloning was performed in order to obtain phylogenetic information about the composition of the soil bacterial communities. 16S rDNA

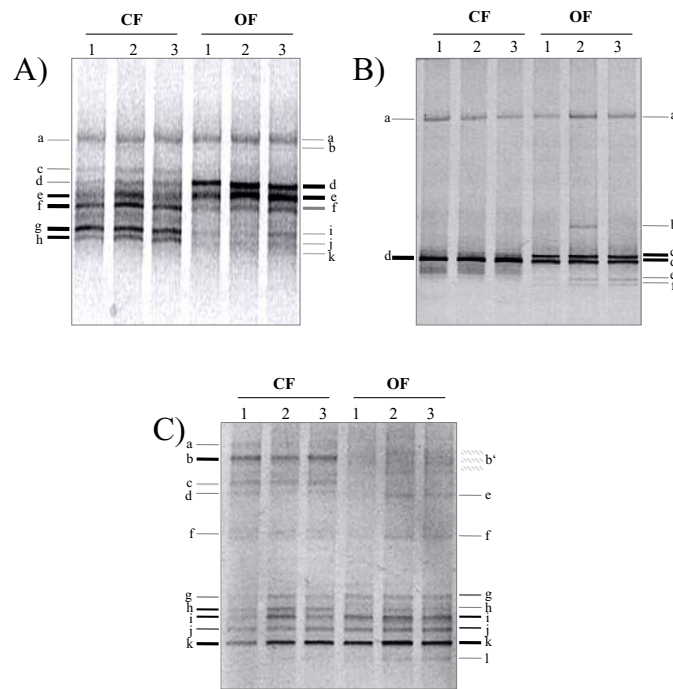


Figure 3. Group-specific fingerprinting. Negative image of ethidium bromide-stained DGGE separation patterns of PCR amplified DNA fragments derived from DNA extracts of soils under conventional (CF) and organic farming (OF). Three replicates from each soil were analysed (lanes 1-3). **A)** *Actinomycete* community (16S rDNA). **B)** Ammonia-oxidizing community (*amoA* gene). **C)** Archaeal community (16S rDNA).

sequences amplified with eubacterial primers from a CF and an OF soil extract were used to create the clone libraries CCF and COF respectively. Clones were grouped by DGGE screening. Thirteen different inserts of the CCF library and 15 inserts of the COF library corresponding to most of the intense and to some of the faint bands in the DGGE community fingerprints were sequenced.

The peripheral lanes in Fig. 1 (marked CCF and COF) show artificial band patterns made up of identified sequences. The length of the sequenced fragments ranged between 622 and 648 bp with the exception of clone CF 31 where only 304 bp were available for sequence analysis. The sequences are accessible under the accession numbers given in Table 2, where also the results of comparison to the EMBL database are summarized. CF as well as OF clones affiliated with 3 bacterial divisions commonly associated with soil: *Actinobacteria* (high G+C gram positive bacteria), *Acidobacteria* and *Proteobacteria*. Two sequences were phylogenetically ambiguous and represent either chimeras or members of unknown bacterial groups.

DGGE analysis of the Actinomycete community: The *Actinomycete* community profiles consisted of 2-4 predominant and 3-5 faint bands (Fig. 3A). CF samples produced 4 bands that were also present in the OF fingerprints (bands a, d, e, f). In addition CF soils showed two rapidly migrating bands (bands g and h) and one slowly migrating band (band c), that had no equivalent in the OF fingerprints. On the other hand four very faint bands (bands b, i, j and k) were only visible in the OF samples.

DGGE analysis of the ammonia-oxidizer community: The structure of the ammonia-oxidizing community was studied by DGGE analysis of a 491 bp fragment of the *amoA* gene encoding the a-subunit of the ammonia monooxygenase (Fig. 3B). The profiles based on the functional gene were simple. Two

predominant bands were visible in all samples (bands a and d). OF samples showed higher diversity and more pronounced spatial heterogeneity of ammonia-oxidizing bacteria: An additional strong band (band c) was present in all OF samples. Two additional faint bands were visible in replicates 2 and 3 (bands e and f), and one fine band (band b) was detected in replicate 2 of the OF soil.

DGGE analysis of the Acidobacterium community: DGGE analysis of 16S rDNA amplified with a primer combination specific for the *Acidobacterium* division resulted in complex band patterns with at least two predominant bands in the top region of the gel and a cluster of extremely fine bands below (data not shown). Due to the complexity of the obtained band patterns differences between CF and OF soil could not be described.

DGGE analysis of the archaeal community: The genetic structure of the archaeal communities in the analysed fields showed 7-10 bands with some variations between the replicate samples of the OF soil (Fig. 3C). CF and OF fingerprints had six bands in common positions (bands f, g, h, i, j, k). In the upper section of the CF band patterns one additional strong band (band b) and three additional weak bands (bands a, c and d) were found. The OF samples showed only a smear (smear b') in the corresponding position. However, two faint bands (e and l) were only visible in replicates 2 and 3 of the OF soil.

Discussion

Microbial community characteristics of the Marchfeld arable soils

Spatial distribution of the bacterial community: The results suggest that the genetic structure of the microflora in arable soils is spatially homogeneous. Eubacterial community fingerprints did not vary significantly among 10 random samples taken from the same field (Fig. 2). The community structure of *Actinomycetes*,

ammonia-oxidizers, *Acidobacteria* and *Archaea* was analysed in three replicate samples from either soil and was spatially reproducible apart from minor variations in the ammonia-oxidizer and the *Archaea* profiles of the OF soil (Fig. 3A and 3C). Spatial homogeneity has been observed previously in presently and historically cultivated soils³²⁻³⁴. It might be a soil feature promoted by regular anthropogenic influence. In this study samples were taken shortly after ploughing which might have disrupted any temporally formed microzones.

Structure and composition of the bacterial community: DGGE analysis of the total eubacterial community produced complex band patterns (Figs 1 and 2) indicating the presence of a diverse bacterial microflora in accordance with previous findings on soil ecosystems³⁵. The differences between Figs 1 and 2 resulted from a modification of the denaturing gradient. For the analysis of spatial heterogeneities (Fig. 2) a gradient of 30-60% was used. To improve the resolution the gradient was narrowed down to 35-60% in the gel presented in Fig. 1.

To obtain phylogenetic information, eubacterial clone libraries were created from the CF and the OF soil. The eubacterial community profiles were too complex to allow an excision of single bands for sequencing. Moreover short DNA fragments as used for DGGE analysis are considered insufficient for an accurate phylogenetic placement³⁶.

DGGE screening of clones allowed grouping and selection of inserts matching bands in the original soil fingerprints³⁷. Taking the two clone libraries together a total of 28 different inserts were sequenced. Fifteen of the identified sequences affiliated with the division *Actinobacteria*, 6 with the *Acidobacteria* and 4 with the *Proteobacteria* (Table 2). These divisions usually represent major constituents of soil clone libraries^{36,38,39}. Six of the clones attributed to the *Actinobacteria* (CF5, CF11, CF29, CF31, OF13, OF33), five of the *Acidobacterium*-like sequences (CF7, OF20, OF26, OF39, OF47), two of the sequences affiliated with the *Proteobacteria* (CF34, OF3) and both unclassified sequences (CF9, CF27) clustered in the neighbourhood of other soil and rhizosphere clones^{32,40-43}. The remaining sequences (CF4, CF44, OF2, OF27, OF40) had their closest recognized relatives among clones and isolates from other environments such as activated sludge, antarctic sandstone, marine environments or a prehistoric cave^{37,44-47}. The presence of one sequence related to rumen bacteria in the OF soil (OF38) cannot be attributed to the amendment practice, since no animal manure was used.

Summarizing the DGGE screening results of both soil clone libraries, the clones grouped as follows: 42 of the 96 analyzed inserts comigrated with sequences affiliated with *Actinobacteria*, 24 inserts comigrated with *Acidobacterium*-like sequences and 8 inserts comigrated with sequences related to the division *Proteobacteria*. Due to the limited resolution of DGGE such

attributions must be interpreted with caution, since comigration of different sequences, as observed by⁴⁸, may have lead to mistakes in grouping clones and in assigning them to bands in the original fingerprints.

In any case the number of clones analysed was too low to reflect the composition of the entire community. As it is visible in Fig. 1 even some of the predominant bands of the soil profiles representing abundant species had no equivalent in the clone libraries. Furthermore the assignment of cloned sequences to bands in the original soil fingerprint was hampered due to complexity of the eubacterial community. However, the cloning assay gave evidence that a majority of the bacteria present in these soils were more closely related to other soil clones than to cultured species. This in turn highlights the importance of culture-independent techniques for studying agricultural influences on the soil microflora.

Differences between the conventionally and the organically managed soil: In this study conventional farming involved the use of mineral N, P and K fertilizers and of herbicides. In the organic farming system nutrient supply was provided by legume intercropping, weeds were controlled by intensive tillage, and rotations comprised a wider range of crops than in the conventional system. In molecular surveys addressing the effects of these agricultural measures individually, each of them was found to influence the composition of the soil microflora in a different way^{19,39,49-55}.

Eubacterial community: The eubacterial community fingerprints indicated slight structural differences between the two soil communities analysed (Fig.1). Differences in the eubacterial community composition of conventionally and organically farmed soils have been detected previously by culture-dependent techniques¹⁵, Biolog 12 and PLFA^{8,10}. However, no differences have been found when RFLP genetic fingerprinting was used for a comparative analysis⁷. Comparisons between different studies are difficult, since neither conventional nor organic farming practice is standardised. Furthermore the use of different lysis protocols and primer sets results in varying findings⁵⁶.

Due to the high level of bacterial diversity differences in the community profiles are difficult to detect. Therefore specific fingerprinting of four groups of soil micro organisms was performed to allow a more detailed comparison of differently treated soils. *Actinomycetes*, ammonia-oxidizers, *Acidobacteria* and *Archaea* were tested for their sensitivity to agricultural influences and for their suitability as biological indicators.

Actinomycetes: Specific DGGE analysis of the *Actinomycetes* community group produced patterns of 4-7 bands (Fig. 3A). These patterns were very simple in comparison with *Actinomycetes* community profiles of other soils²⁰ and thus may not represent

Table 1. Primer combinations used for the specific analysis of different phylogenetic groups.

Phylogenetic group	Reaction	Forward primer (reference)		Reverse primer (reference)	
Eubacteria	1 st round	341f	Muyzer et al. ⁶⁴	984r	Heuer et al. ⁶⁹
	nested PCR	341f ^a	Muyzer et al. ⁶⁴	518r	Neefs et al. ⁷⁰
Actinomycetes	1 st round and nested PCR	243f	Heuer et al. ⁶⁵	513r ^a	Heuer et al. ⁶⁵
Ammonia-oxidizers	1 st round and nested PCR	AmoA-1f ^a	Stephen et al. ⁶⁶	AmoA-2r	Stephen et al. ⁶⁶
Acidobacteria	1 st round	31f	Barns et al. ⁶⁷	1492r	Lane ⁷¹
	nested PCR	31f	Barns et al. ⁶⁷	518r ^a	Neefs et al. ⁷⁰
Archaea	1 st round	ARC344f	Raskin et al. ⁶⁸	ARC 915	Raskin et al. ⁶⁸
	nested PCR	ARC344f	Raskin et al. ⁶⁸	518r ^a	(Neefs et al. ⁷⁰)

^aprimers carrying the CG clamp in nested PCR

Table 2. Phylogenetic affinities of eubacterial 16S rDNA sequences obtained from soils under conventional (CF) and organic farming (OF).

Clone [sequence length (bp)]	Accession no.	Closest EMBL database match [Accession no]	Similarity (%)	Putative division
Clone library CCF				
CF1 [627]	AJ535740	Nocardioides simplex [AF005010]	98.1	Actinobacteria
CF2 [629]	AJ535735	<i>Blastococcus</i> sp. BC412 [AJ316574]	99.4	Actinobacteria
CF5 [646]	AJ535739	uncultured bacterium #0319-7H2 [AF234151]	98.5	Actinobacteria
CF11 [624]	AJ538044	uncultured bacterium clone [AY102335]	97.0	Actinobacteria
CF29 [645]	AJ535736	uncultured bacterium #0649-1G9 [AF234119]	85.6	Actinobacteria
CF31 [304]	AJ535737	<i>Blastococcus aggregatus</i> [AJ430193]	98.6	Actinobacteria
CF44 [630]	AJ538048	uncultured bacterium [AY093463]	92.0	Actinobacteria
CF48 [627]	AJ535738	Nocardioides sp [AF253509]	96.7	Actinobacteria
CF7 [648]	AJ538049	uncultured <i>Holophaga</i> sp. [Z95717]	95.7	Actinobacteria
CF4 [624]	AJ538047	<i>Hyphomicrobium</i> sp. PMC [AF279787]	95.7	Proteobacteria
CF34 [645]	AJ538046	agricultural soil bacterium [AJ252621]	94.9	Proteobacteria
CF9 [648]	AJ538050	unidentified eubacterium [AF009988]	89.1	unclassified
CF27 [635]	AJ538045	uncultured <i>Actinobacterium</i> [AY124381] / uncultured candidate division BD bacterium [AF545656]	99.8/ 99.1	unclassified
Clone library COF				
OF4 [646]	AJ535745	uncultured soil bacterium [AF324245]	95.4	Actinobacteria
OF7 [627]	AJ535746	<i>Blastococcus</i> sp. BC412 [AJ316574]	98.4	Actinobacteria
OF13 [645]	AJ538051	uncultured bacterium #0649-1G9 [AF234119]	96.3	Actinobacteria
OF27 [627]	AJ535742	<i>Microlunatus phosphovorius</i> [Z78207]	98.3	Actinobacteria
OF31 [624]	AJ538055	uncultured bacterium [AY102335]	97.1	Actinobacteria
OF32 [627]	AJ535743	Nocardioides sp [X90830]	97.5	Actinobacteria
OF33 [645]	AJ538056	uncultured bacterium #0649-1G9 [AF234119]	96.1	Actinobacteria
OF2 [622]	AJ538052	uncultured bacterium [AJ421205]	95.8	Acidobacteria
OF20 [625]	AJ535741	bacterial species [Z95727]	95.2	Acidobacteria
OF26 [646]	AJ538053	bacterial species [Z95731]	98.6	Acidobacteria
OF39 [645]	AJ535744	bacterial species [Z95722]	99.4	Acidobacteria
OF47 [645]	AJ538059	uncultured bacterium DA008 [Y12597]	97.7	Acidobacteria
OF3 [645]	AJ538054	agricultural soil bacterium [AJ252621]	90.7	Proteobacteria
OF40 [647]	AJ538058	unidentified bacterium [AB021333]	96.9	Proteobacteria
OF38 [633]	AJ538057	uncultured rumen bacterium 4C [AB034018]	93.7	Firmicutes

the entire *Actinomycetes* diversity. However, the fingerprints indicated differences between the *Actinomycetes* communities of the two soils, since both CF and OF samples showed characteristic bands that were not visible in the respective other soil. Since *Actinomycetes* are involved in the decomposition of organic substrates the low organic carbon content in OF soil could be responsible for variations. Furthermore certain *Actinomycetes* may have had a competitive advantage in the herbicide treated CF soil, due to their ability to metabolize such xenobiotic compounds^{42, 57}.

Ammonia-oxidizers: Fingerprints of the amoA gene encoding the active-site polypeptide of the ammonia monooxygenase showed only 2-5 different bands (Fig. 3B). Such simple community profiles are not unusual for arable soils^{58,59} although more complex communities have been observed as well^{60,61}. Higher diversity of ammonia-oxidizing bacteria was found in the organically managed soil. One explanation might be the sensibility of these nitrogen metabolizing organisms to the mineral nitrogen fertilization involved in the conventional treatment.

Indeed, mineral fertilizers caused structural shifts and a loss of diversity in the ammonia-oxidizing population of grassland soils⁵⁹. Apart from fertilization, tillage has been observed to reduce the diversity of ammonia-oxidizer populations^{55,60}. However, in this study more complex community fingerprints have been obtained from the OF soil, which has been tilled more frequently. Differences visible between the band patterns of the OF replicate samples demonstrate that spatial variation can occur in the ammonia-oxidizing communities of arable soils. By contrast spatial stability has been observed in grassland and forest soils^{60,62}. The low overall diversity of the ammonia-oxidizers and their obvious sensitivity to agricultural influences made this group of bacteria

particularly suitable for assessing differential effects of the two farming systems.

Acidobacteria: Among the phylogenetic groups analysed by specific fingerprinting the *Acidobacteria* exhibited the highest level of diversity. Due to the complexity of the DGGE profiles it was not possible to enumerate bands and to describe differences between soil samples. To date little is known about the ecological functions of *Acidobacteria* in soil, however, a correlation between the nutrient state of soils and their *Proteobacteria/Acidobacteria* ratio have been found⁶³. This was the first attempt to separate 16S rDNA fragments amplified with an *Acidobacteria*-specific primer by DGGE. Further optimization of the electrophoresis conditions is required to provide a better insight in the community structures formed by these numerically important soil micro organisms.

Archaea: The archaeal community profiles obtained in this work showed up to 10 different bands (Fig. 3C) and indicated structural differences between the conventionally and the organically managed soil. Differences might be explained by low organic carbon content and intensified tillage of the OF soil.

Complex archaeal communities have been observed previously in soil²⁵⁻²⁷. Since none of the soil-borne *Archaea* have been physiologically characterized so far, it is difficult to predict their ecological functions and specific reactions to agricultural influences. Øvreås and Torsvik¹⁹ found that a sandy frequently tilled soil was different from an organic rarely tilled soil with respect to the community structure of the *Archaea*.

In general our results confirm the sensibility of soil *Archaea* to agricultural activities and thus contrast the observations of

Buckley et al.²⁶ who did not find any treatment effects when analyzing the archaeal community of a native and a cultivated soil.

Conclusions

Due to its limited resolution DGGE analysis cannot completely characterize complex microbial communities. However, it has proved to be sensitive to differences between differently managed soils. Group specific fingerprinting simplified the DGGE band patterns and facilitated the detection and evaluation of differences. The assignment of cloned sequences to bands in eubacterial soil fingerprints was hampered by the complexity of the bacterial community. Therefore shotgun cloning should be combined with group specific fingerprinting. Such an approach simplifies the original band patterns and should allow the identification of characteristic sequences that discriminated between differently managed soils.

The comparison of two farms gave the first indication that management-dependent differences in the community structure of many microbial groups can be detected on the farm level. The occurrence of detectable differences in four different phylogenetic groups (*Eubacteria*, *Actinomycetes*, ammonia-oxidizers and *Archaea*) suggests that the two farming systems have multiple differential effects on the soil microflora. Extensive research including the analysis of more than one site of each management system are required to corroborate these observations.

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References

- ¹Kennedy, A.C. and Smith, K.L. 1995. Soil microbial diversity and the sustainability of agricultural soils. *Plant and Soil* **170**: 75-86.
- ²Hansen, B., Alrøe, F.H. and Kristensen, E.S. 2001. Approaches to assess the environmental impact of organic farming with particular regard to Denmark. *Agriculture, Ecosystem and Environment* **83**: 11-26.
- ³Nwosu, V.C. 2001. Antibiotic resistance with particular reference to soil microorganisms. *Research in Microbiology* **152**(5): 421-30.
- ⁴Stolze, M., Piore, A., Häring, A. and Dabbert, S. 2000. The environmental impact of organic farming in Europe. *Organic farming in Europe: Economics and Policy*. Hohenheim, Germany, University of Hohenheim **6**: 1473-6513.
- ⁵Atlas, R.M. and Bartha, R. 1998. *Microbial ecology*. Benjamin/Cummings Science Publishing, Redwood City, California, pp. 99-140, 332-459.
- ⁶Atlas, R.M. 1984. *Diversity of microbial communities*. Advances in microbial ecology. K.C. Marshal. New York, Plenum Press. pp. 1-47.
- ⁷Buckley, D.H. and Schmidt, T.M. 2001. The structure of microbial communities in soil and the lasting impact of cultivation. *Microbial Ecology* **42**(1): 11-21.
- ⁸Bossio, D.A., Scow, K.M., Gunapala, N. and Graham, K.J. 1998. Determinants of soil microbial communities: Effects of agricultural management, season and soil type on phospholipid fatty acid profiles. *Microbial Ecology* **36**(1): 1-12.
- ⁹Gunapala, N. and Scow, K.M. 1998. Dynamics of soil microbial biomass and activity in conventional and organic farming systems. *Soil Biology and Biochemistry* **30**: 805-816.
- ¹⁰Lundquist, E.J., Scow, K.M., Jackson, L.E., Uesugi, S.L. and Johnson, C.R. (1999). Rapid response of soil microbial communities from conventional, low input and organic farming systems to a wet/dry cycle. *Soil Biology and Biochemistry* **31**: 1661-1675.
- ¹¹Fliessbach, A. and Mäder, P. 2000. Microbial biomass and size density fractions differ between soils of organic and conventional agricultural systems. *Soil Biology and Biochemistry* **32**: 757-768.
- ¹²Mäder, P., Fliessbach, A., Dubois, D., Guns, L., Padrou, F. and Niggli, U. 2002. Soil fertility and biodiversity in organic farming. *Science* **296**: 1694-1697.
- ¹³Johnsen, K., Jacobsen, C.S., Torsvik, V. and Sørensen, J. 2001. Pesticide effects on bacterial diversity in agricultural soils - a review. *Biology and Fertility in Soils* **33**: 443-453.
- ¹⁴Eckschmitt, K. and Griffith, B. 1998. Soil biodiversity and its implications for ecosystem functioning in a heterogeneous and variable environment. *Applied Soil Ecology* **10**: 201-215.
- ¹⁵Workneh, F. and van Bruggen, A.H.C. 1994. Microbial density, composition and diversity in organically and conventionally managed rhizosphere soil in relation to suppression of corky root of tomatoes. *Applied Soil Ecology* **1**: 219-230.
- ¹⁶Amann, R.I., Ludwig, W. and Schleifer, K.H. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**(1): 143-69.
- ¹⁷Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M. and Tiedje, J.M. 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Research* **31**(1): 442-3.
- ¹⁸Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**(3): 695-700.
- ¹⁹Øvreås, L. and Torsvik, V.V. 1998. Microbial diversity and community structure in two different agricultural soil communities. *Microbial Ecology* **36**(3): 303-315.
- ²⁰Heuer, H., Krsek, M., Baker, P., Smalla, K. and Wellington, E.M. 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology* **63**(8): 3233-41.
- ²¹Rothhauwe, J.-H., Witzel, K.-P. and Liesack, W. 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and Environmental Microbiology* **63**: 4704-4712.
- ²²Crawford, D.L. 1988. Biodegradation of agricultural and urban wastes. In Goodfellow, M., Williams, S.T. and Mordarski, M. (eds). *Actinomycetes in biotechnology*. Academic Press Ltd., London, UK, pp. 433-439.
- ²³Kowalchuk, G.A. and Stephen, J.R. 2001. Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annual Review of Microbiology* **55**: 485-529.
- ²⁴Barns, S.M., Takala, S.L. and Kuske, C.R. 1999. Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. *Applied and Environmental Microbiology* **65**(4): 1731-1737.
- ²⁵Bintrim, S.B., Donohue, T.J., Handelsman, J., Roberts, G.P. and Goodman, R.M. 1997. Molecular phylogeny of *Archaea* from soil. *Proceedings of the National Academy of Sciences of the USA* **94**(1): 277-282.
- ²⁶Buckley, D.H., Graber, J.R. and Schmidt, T.M. 1998. Phylogenetic analysis of nonthermophilic members of the kingdom *Crenarchaeota* and their diversity and abundance in soils. *Applied and Environmental Microbiology* **64** (11): 4333-4339.
- ²⁷Sandaa, R.A., Enger, Ø. and Torsvik, V. 1999. Abundance and diversity of *Archaea* in heavy-metal-contaminated soils. *Applied and Environmental Microbiology* **65**(8): 3293-7.

- ²⁸Piñar, G., Saiz-Jimenez, C., Schabereiter-Gurtner, C., Blanco-Varela, M.T., Lubitz, W. and Rölleke, S. 2001. Archaeal communities in two disparate deteriorated ancient wall paintings: detection, identification and temporal monitoring by denaturing gradient gel electrophoresis. *FEMS Microbiology Ecology* **37**: 45-54.
- ²⁹Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular cloning: A laboratory manual*. 2nd edn. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory.
- ³⁰Schabereiter-Gurtner, C., Piñar, G., Lubitz, W. and Rölleke, S. 2001. An advanced molecular strategy to identify bacterial communities on art objects. *Journal of Microbiological Methods* **45**(2): 77-87.
- ³¹Pearson, W.R. 1994. Rapid and sensitive sequence comparison with FAST and FASTA. *Methods in Enzymology* **183**: 63-98.
- ³²Felske, A., Wolterink, A., Van Lis, R. and Akkermans, A.D. 1998. Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). *Applied and Environmental Microbiology* **64**(3): 871-879.
- ³³Gelsomino, A., Keijzer-Wolters, A.C., Cacco, G. and van Elsas, J.D. 1999. Assessment of bacterial community structure in soil by polymerase chain reaction and denaturing gradient gel electrophoresis. *Journal of Microbiological Methods* **38**(1-2): 1-15.
- ³⁴Felske, A., Wolterink, A., van Lis, R., de Vos, W.M. and Akkermans, A.D. 1999a. Searching for predominant soil bacteria: 16S rDNA cloning versus strain cultivation. *FEMS Microbiology Ecology* **30**(2): 137-145.
- ³⁵Torsvik, V., Goksøyr, J. and Daae, F.L. 1990. High diversity in DNA of soil bacteria. *Applied and Environmental Microbiology* **56**(3): 782-7.
- ³⁶Hugenholtz, P., Goebel, B.M. and Pace, N.R. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology* **180**(18): 4765-4774.
- ³⁷Schabereiter-Gurtner, C., Saiz-Jimenez, C., Piñar, G., Lubitz, W. and Rölleke, S. 2002. Phylogenetic 16S rRNA analysis reveals the presence of complex and partly unknown bacterial communities in Tito Bustillo cave, Spain, and on its Palaeolithic paintings. *Environmental Microbiology* **4**(7): 392-400.
- ³⁸Dunbar, J., Takala, S., Barns, S.M., Davis, J.A. and Kuske, C.R. 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Applied and Environmental Microbiology* **65**(4): 1662-1669.
- ³⁹McCaug, A.E., Glover, L.A. and Prosser, J.I. 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. *Applied and Environmental Microbiology* **65**(4): 1721-1730.
- ⁴⁰Ludwig, W., Bauer, S.H., Bauer, M., Held, I., Kirchhof, G., Schulze, R., Huber, I., Spring, S., Hartmann, A. and Schleifer, K.H. 1997. Detection and in situ identification of representatives of a widely distributed new bacterial phylum. *FEMS Microbiology Letters* **153**: 181-190.
- ⁴¹Holmes, A.J., Bowyer, J., Holley, M.P., O'Donoghue, M., Montgomery, M. and Gillings, M.R. 2000. Diverse, yet-to-be-cultured members of the *Rubrobacter* subdivision of the *Actinobacteria* are widespread in Australian arid soils. *FEMS Microbiology Ecology* **33**(2): 111-120.
- ⁴²Topp, E., Mulbry, W.M., Zhu, H., Nour, S.M. and Cuppels, D. 2000. Characterization of S-triazine herbicide metabolism by a *Nocardioideis* sp. isolated from agricultural soils. *Applied and Environmental Microbiology* **66**(8): 3134-3141.
- ⁴³Valinsky, L., Della Vedova, G., Scupham, A.J., Alvey, S., Figueroa, A., Yin, B., Hartin, R.J., Chrobak, M., Crowley, D.E., Jiang, T. and Borneman, J. 2002. Analysis of bacterial community composition by oligonucleotide fingerprinting of rRNA genes. *Applied and Environmental Microbiology* **68**(7): 3243-3250.
- ⁴⁴Schumann, P., Prauser, H., Rainey, F.A., Stackebrandt, E. and Hirsch, P. 1997. *Friedmanniella antarctica* gen. nov., sp. nov., an LL-diaminopimelic acid-containing actinomycete from Antarctic sandstone. *International Journal of Systematic Bacteriology* **47**(2): 278-283.
- ⁴⁵Watanabe, K., Teramoto, M. and Harayama, S. 1999. An outbreak of nonflocculating catabolic populations caused the breakdown of a phenol-digesting activated-sludge process. *Applied and Environmental Microbiology* **65**(7): 2813-2819.
- ⁴⁶Urzi, C., Brusetti, L., Salamone, P., Sorlini, C., Stackebrandt, E. and Daffonchio, D. 2001. Biodiversity of *Geodermatophilaceae* isolated from altered stones and monuments in the Mediterranean basin. *Environmental Microbiology* **3**(7): 471-479.
- ⁴⁷Reed, D.W., Fujita, Y., Delwiche, M.E., Blackwelder, D.B., Sheridan, P.P., Uchida, T. and Colwell, F.S. 2002. Microbial communities from methane hydrate-bearing deep marine sediments in a forearc basin. *Applied and Environmental Microbiology* **68**(8): 3759-3770.
- ⁴⁸Gomes, N.C., Fagbola, O., Costa, R., Rumjanek, N.G., Buchner, A., Mendona-Hagler, L. and Smalla, K. 2003. Dynamics of fungal communities in bulk and maize rhizosphere soil in the tropics. *Applied and Environmental Microbiology* **69**(7): 3758-3766.
- ⁴⁹Atlas, R.M., Horowitz, A., Krichevsky, M. and Bej, A.K. 1991. Response of microbial populations to environmental disturbance. *Microbial Ecology* **22**: 249-256.
- ⁵⁰Engelen, B., Meinken, K., von Wintzingerode, F., Heuer, H., Malkomes, H.P. and Backhaus, H. 1998. Monitoring impact of a pesticide treatment on bacterial soil communities by metabolic and genetic fingerprinting in addition to conventional testing procedures. *Applied and Environmental Microbiology* **64**(8): 2814-21.
- ⁵¹Fantroussi, S., Verschuere, L., Verstraeta, W. and Top, E.M. 1999. Effect of phenylurea herbicides in soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. *Applied and Environmental Microbiology* **65**: 982-988.
- ⁵²Yang, Y.H., Yao, J., Hu, S. and Qi, Y. 2000. Effects of agricultural chemicals on DNA sequence diversity of soil microbial community: A study with RAPD marker. *Microbial Ecology* **39**: 72-79.
- ⁵³Crecchio, C., Curci, M., Pizzigallo, M.D.R., Ricciuti, P. and Ruggiero, P. 2001. Molecular approaches to investigate herbicide-induced bacterial community changes in soil microcosms. *Biology and Fertility in Soils* **33**: 460-466.
- ⁵⁴Sessitsch, A., Weilharter, A., Gerzabek, M.H., Kirchmann, H. and Kandeler, E. 2001. Microbial population structures in soil particle size fractions of a long-term fertilizer field experiment. *Applied and Environmental Microbiology* **67**(9): 4215-24.
- ⁵⁵Ibekwe, A.M., Kennedy, A.C., Frohne, P.S., Papiernik, S.K., Yang, C.-H. and Crowley, D.E. 2002. Microbial diversity along a transect of agronomic zones. *FEMS Microbiology Ecology* **39**: 183-191.
- ⁵⁶Felske, A. 1999b. Reviewing the DA001-files: a 16S rRNA chase on suspect #X99967, a *Bacillus* and Dutch underground activist. *Journal of Microbiological Methods* **36**: 77-93.
- ⁵⁷Esposito, E., Paulillo, S.M. and Manfio, G.P. 1998. Biodegradation of the herbicide Diuron in soil by indigenous *Actinomycetes*. *Chemosphere* **37**(3): 541-548.
- ⁵⁸Phillips, C.J., Harris, D., Dollhopf, S.L., Gross, K.L., Prosser, J.I. and Paul, E.A. 2000. Effects of agronomic treatments on structure and function of ammonia-oxidizing communities. *Applied and Environmental Microbiology* **66**(12): 5410-5418.
- ⁵⁹Webster, G., Embley, T.M. and Prosser, J.I. 2002. Grassland management regimens reduce small-scale heterogeneity and species diversity of beta-proteobacterial ammonia oxidizer populations. *Applied and Environmental Microbiology* **68**(1): 20-30.
- ⁶⁰Bruns, M.A., Stephen, J.R., Kowalchuk, G.A., Prosser, J.I. and Paul, E.A. 1999. Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils. *Applied and Environmental Microbiology* **65**(7): 2994-3000.
- ⁶¹Avrahami, S., Conrad, R. and Braker, G. (2002). Effect of soil ammonium concentration on N₂O release and on the community structure of ammonia oxidizers and denitrifiers. *Applied and Environmental Microbiology* **68**(11): 5685-5692.

- ⁶²Laverman, A.M., Speksnijder, A.G., Braster, M., Kowalchuk, G.A., Verhoef, H.A. and Van Verseveld, H.W. 2001. Spatiotemporal stability of an ammonia-oxidizing community in a nitrogen-saturated forest soil. *Microbial Ecology* **42**(1): 35-45.
- ⁶³Smit, E., Leeftang, P., Gommans, S., van den Broek, J., van Mil, S. and Wernars, K. 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. *Applied and Environmental Microbiology* **67**(5): 2284-2289.
- ⁶⁴Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**(3): 695-700.
- ⁶⁵Heuer, H., Krsek, M., Baker, P., Smalla, K. and Wellington, E.M. 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology* **63**(8): 3233-3241.
- ⁶⁶Stephen, J.R., Chang, Y.J., Macnaughton, S.J., Kowalchuk, G.A., Leung, K.T., Flemming, C.A. and White, D.C. 1999. Effect of toxic metals on indigenous soil beta-subgroup proteobacterium ammonia oxidizer community structure and protection against toxicity by inoculated metal-resistant bacteria. *Applied and Environmental Microbiology* **65**(1): 95-101.
- ⁶⁷Barns, S.M., Takala, S.L. and Kuske, C.R. 1999. Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. *Applied and Environmental Microbiology* **65**(4): 1731-1737.
- ⁶⁸Raskin, L., Stromley, J.M., Rittmann, B.E. and Stahl, D.A. 1994. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. *Applied and Environmental Microbiology* **60**(4): 1232-1240.
- ⁶⁹Heuer, H., Hartung, K., Wieland, G., Kramer, I. and Smalla, K. (1999). Polynucleotide probes that target a hypervariable region of 16S rRNA genes to identify bacterial isolates corresponding to bands of community fingerprints. *Applied and Environmental Microbiology* **65**(3): 1045-1049.
- ⁷⁰Neefs, J.M., Van de Peer, Y., Hendriks, L. and De Wachter, R. 1990. Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Research* **18** Suppl: 2237-2317.
- ⁷¹Lane, D.J. (1991). 16S/23S rRNA sequencing. In Stackebrandt, E. and Goodfellow, M. (eds). *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons. Chichester, UK, pp.115-175.