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ORIGINAL ARTICLE

Seasonal Fluctuations of Bacterial Community Diversity in Agricultural Soil and Experimental Validation by Laboratory Disturbance Experiments

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Abstract Natural fluctuations in soil microbial communities are poorly documented because of the inherent difficulty to perform a simultaneous analysis of the relative abundances of multiple populations over a long time period. Yet, it is important to understand the magnitudes of community composition variability as a function of natural influences (e.g., temperature, plant growth, or rainfall) because this forms the reference or baseline against which external disturbances (e.g., anthropogenic emissions) can be judged. Second, definition of baseline fluctuations in complex microbial communities may help to understand at which point the systems become unbalanced and cannot return to their original composition. In this paper, we examined the seasonal fluctuations in the bacterial community of an agricultural soil used for regular plant crop production by using terminal restriction fragment length polymorphism profiling (T-RFLP) of the amplified 16S ribosomal ribonucleic acid (rRNA) gene diversity. Cluster and statistical analysis of T-RFLP data showed that soil bacterial communities fluctuated very little during the seasons (similarity indices between 0.835 and 0.997) with insignificant variations in 16S rRNA gene richness and diversity indices. Despite overall insignificant fluctuations, between 8 and 30% of all terminal restriction fragments changed their relative intensity in a significant manner among consecutive time samples. To determine the magnitude of community variations induced by external factors, soil samples were subjected to either inoculation with a pure bacterial culture, addition of the herbicide mecoprop, or addition of nutrients. All treatments resulted in statistically measurable changes of T-RFLP profiles of the communities. Addition of nutrients or bacteria plus mecoprop resulted in bacteria composition, which did not return to the original profile within 14 days. We propose that at less than 70% similarity in T-RFLP, the bacterial communities risk to drift apart to inherently different states.

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Introduction

The complexity of most microbial communities, both in terms of species diversity and their abundance, has eluded facile analysis. Yet, to understand their functioning and resilience to external changes, it is desirable to assess the community composition, quantify individual microbial population sizes, and study fluctuations thereof [17, 42]. Especially in complex systems like soil, determination of the microbial diversity is a daunting task [3]. Estimations of the bacterial diversity in a gram of soil vary between some thousand species, as determined from extensive clone libraries [45] and from deoxyribonucleic acid (DNA) renaturation experiments [41], to more than a million [11]. Despite important advances in genome sequencing technologies, which may one day tackle the complete sequence diversity in a soil bacterial commu-

nity, for many different purposes, such as bioremediation efforts or monitoring chemical pollution effects, it is necessary to have easy monitoring methods to track community and diversity changes over time.

Cultivation-independent molecular profiling techniques have proven to be very valuable for effect monitoring on microbial communities. Examples of useful DNA-based profiling techniques are terminal restriction fragment (tRF) length polymorphisms (T-RFLPs) [27, 29], denaturing gradient gel electrophoresis (DGGE) [7, 12, 30, 32, 43], ribosomal intergenic spacer analysis (RISA) [9], and single-strand conformation polymorphism (SSCP) [19, 24, 39]. Most frequently, these profiling techniques assess bacterial diversity via 16S ribosomal ribonucleic acid (rRNA) gene diversity in a pool of fragments amplified from total community-derived DNA in the polymerase chain reaction (PCR). Typically, DNA community profiling techniques have a genus-level resolution [8] and are thought to detect bacterial populations with at least 1% relative abundance in a sample [22]. On the other hand, PCR-based approaches can lead to amplification bias and artifacts [10]. Obviously, this 1% is lower coverage than large-scale sequencing of clone libraries can give, but profiling techniques are faster and therefore more suitable for field-scale experiments, where replication and increased sampling frequency are important [17].

Despite abundant usage of DGGE, SSCP, and T-RFLP in a wide variety of microbial ecosystems including agricultural soil, it is not very clear what the natural and temporal fluctuations in soil microbial communities are. This seems important information for subsequent effect studies to be based upon. Studies on a number of long-term field sites have suggested that soil microbial communities develop significantly different in time (5–20 years) as a function of plant type [22, 2] or agricultural practice [44]. Bacterial communities in extremely perturbed or technical environments (e.g., reactors or enrichment cultures) also change measurably but not always consistently as a function of, e.g., pollutant concentration [1, 13, 28], pollutant, or soil type [13, 25, 31]. Bacteria inoculation, on the other hand, did not lead to detectable bacterial community changes in a soil bioremediation experiment to enhance 2-nitrophenol degradation [34]. Natural or seasonal variations themselves, however, are less well described. Buckley and Schmidt [3] detected up to fourfold variations in the relative ratio of taxonomic units as determined by quantitative filter hybridizations on RNA extracted from agricultural soils sampled on four occasions during 2 years. Lipson and Schmidt [26] detected significant community shifts in alpine soil bacterial communities after snow melt by using clone library analysis, leveling up to a twofold increase or decrease per taxonomic group. Phospholipid fatty acid profiling from agricultural soil microbial communities also suggested significant seasonal changes [15, 2], although this is not necessarily due to a change in community composition but to microbial physiology.

In this paper, we focused on determining the sensitivity of T-RFLP to measure relative population variations in a regular agricultural soil bacterial community. Although the focus of this study is the bacterial community, the soil fungal communities should not be neglected [38]. The T-RFLP method for 16S rRNA gene diversity in soil was recently compared with community-level substrate utilization methods, with RISA, and with sequencing of extensive clone libraries, and it was concluded that T-RFLP was sufficiently representative and highly sensitive to detect community differences while maintaining rapidity [17]. T-RFLP profiling would thus form an ideal method to investigate the seasonal variability of soil bacterial communities, which could be used as a baseline against which potential effects of external influences might be better assessed. The first objective of our work, therefore, was to determine such a baseline of natural variability in a regular agricultural soil bacterial community. Hereto, we sampled and analyzed the bacterial community diversity of one agricultural soil on several occasions during 1 year. The second objective was to place the magnitude of community variations in perspective with experimentally induced community changes by subjecting the same soil in microcosms to artificial disturbances, notably bacteria inoculation, application of herbicide or nutrients.

Materials and Methods

Soil Sampling and Site Description

The soil used for the community analysis was collected from an agricultural field (47°24′18.03″N, 8°36′36.64″E, 432 m altitude) near the Swiss Federal Institute for Aquatic Science and Technology (Eawag, Dübendorf, Switzerland), where crop rotation is applied. Values of major soil chemical parameters were: 5.1% organic C, 60% SiO_2, 11.7% Al_2O_3, 2.4% MgO, 4.8% Fe_2O_3, pH-H_2O: 7.6, pH-KCl: 7.0. During the year of sampling (2002–2003), maize was grown in the summer season, followed by winter lettuce. For mean temperature and rainfall, consult Table S1 in the Supplementary online material. For each sampling a total of ~500 g of surface bulk soil (depth 0–5 cm, excluding plants or plant roots) was pooled from randomly collected 50-g aliquots within a 1-m^2 area and transferred to a sterile 500-mL Schott flask (Milian SA, Genève, Switzerland). The same field was sampled at the approximate same spot (within 1 m^2) at 12 occasions during the 2 subsequent years, but only eight samples produced interpretable T-RFLP data. Three aliquots of 5 g (fresh weight) were

prepared from each pooled sample. Soil was neither dried nor sieved before community DNA isolation. Total community DNA was isolated and purified from aliquots immediately after sample collection. For community disturbance experiments, the same spot was sampled on one occasion in Spring 2003.

Soil Microbial Community Total DNA Isolation

Total DNA was isolated from 2- or 5-g quantities of soil by a bead-beating procedure essentially as described by Bürgmann *et al.* [4], followed by standard phenol/chloroform/isoamylalcohol extraction and ethanol/sodium acetate precipitation [37]. After recovery of the DNA by centrifugation for 20 min at 13,000 rpm, it was washed with ice-cold 70% ethanol, dried and resuspended in 50 μL Tris–EDTA (TE) buffer (10 mM Tris pH 8.0, 1 mM ethylenediamine tetraacetic acid [EDTA]). The DNA was further purified according to the Geneclean® III protocol (Q-Bio gene, Montréal, Québec, Canada), finally eluted in 50 μL of sterile demineralized water, after which a small aliquot was visualized by electrophoresis in ethidium bromide-stained agarose gels. DNA concentration was determined by fluorimetry by comparison to a dilution series of purified phage λ-DNA. Typical amounts recovered per gram of soil were in the range of 4 to 10 μg. The purified DNA was then used for T-RFLP and RISA analyses without further cleaning steps.

T-RFLP and RISA Analysis

For T-RFLP, 16S rRNA gene fragments were amplified from microbial community DNA by using the bacterial primers 16S 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) at position 8–27 (*E. coli* SSU rRNA, GenBank accession number J01695) and 16S 926R (5′-CCGTCAATTCCTTTRAGTTT-3′, position 926 reverse) [34]. The primers S-D-Bact-1522-b-S20 and L-D-Bact-132-a-A-18 were used to amplify the intergenic spacer between the 16S and 23S rRNA genes [36]. Primers 16S 926R and S-D-Bact-1522-b-S20 were labeled with the infrared dye IRDye™800 (hereafter, IRD-800, MWG Biotech, Engersberg, Germany), and the primer 16S 8F was marked with IRDye™ 700 (hereafter, IRD-700). Each 50-μL PCR mixture contained 9 μL of PCR buffer (Sigma Chemical, St. Louis, MO), 0.4 μL deoxynucleotide triphosphates (25 mM each), 0.25 μL *Taq* DNA polymerase (Sigma), and between 30 and 35 μL sterile demineralized H_2O. Both primers were added in 1 μL volume at 100 pmol each; 1 μL of target DNA (~50 ng) and another 7-μL sterile H_2O completed the PCR mixture. The cycling conditions for the amplification of 16S rRNA genes were the following: (1) one cycle of 3 min denaturation at 93.5°C, (2) 35 cycles, of each 30 s at 93.5°C, 30 s at 49°C, and 2 min at 72°C, and (3) a final extension cycle during 5 min at 72°C (Genius FGENO5TD thermocycler, Tecne, Burlington, NJ). The PCR conditions for RISA are described elsewhere [35]. Three independent PCR reactions were performed per soil sample. The correct size and quantity of the PCR products were verified by agarose gel electrophoresis before continuing with the T-RFLP or RISA analysis.

For T-RFLP analysis, the amplified 16S rRNA gene fragments, which were labeled at either end with IRD-800 or IRD-700, were digested using the restriction enzyme *Hae*III for 2 h at 37°C (Fermentas International, Burlington, Ontario, Canada). Subsequently, the DNA fragments were concentrated by ethanol and sodium acetate precipitation and recovered by centrifugation. The DNA pellet was resuspended in a volume of 6 (for T-RFLP) or 12 μL (for RISA) of TE buffer (pH 8.0). After the addition of 4.5 (for T-RFLP) or 9 μL (for RISA) of loading buffer (containing 95% formamide, 20 mM EDTA, and 0.05% acidic fuchsine red), the samples were denatured for 3 min at 93.5°C and immediately transferred to ice.

The DNA fragments were separated according to their size by electrophoresis on 25-cm-long polyacrylamide gels on a LI-COR 4200L sequencer (LI-COR, Lincoln, NE), using Tris–borate–EDTA buffer [37] at a voltage of 1,500 V and a running temperature of 45°C. This instrument has the advantage that both IRD-700 and IRD-800 markers can be detected simultaneously. Data were cropped and stored as TIFF image files.

Bacteria Cultivation

Sphingomonas herbicidivorans MH was used to inoculate soil aliquots for the disturbance experiments [18]. Strain MH is able to grow on phenoxyalkanoic acid herbicides, such as mecoprop (2-(4-chloro-2-methylphenoxy)propionic acid), as sole carbon and energy source [20]. The strain was grown at 30°C in 5 mL complex medium [46] in baffled Erlenmeyer flasks shaken at 180 rpm. Cells were harvested during the exponential phase, centrifuged for 5 min at 7,500×*g*, washed once with phosphate-buffered saline (PBS; the solution contains 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 $7H_2O$, 1.4 mM KH_2PO_4, pH 7.3) to remove nutrients, and pelleted again by centrifugation. Finally, the cells were resuspended in 200 μL sterile demineralized H_2O to an approximate optical density of OD=0.6 and added to 2-g soil aliquots in sterile 50-mL capped polypropylene tubes.

Disturbance Experiments and Sensitivity Analyses

To test the sensitivity of T-RFLP to detect changes in microbial community composition, soil sample aliquots

were subjected to different perturbations. Disturbance experiments were conducted with freshly collected soil (see above) divided in aliquots of 2 g in 50-mL sterile capped polypropylene tubes (Techno Plastics Products AG, Trasadingen, Switzerland), incubated at 25°C for up to 2 weeks in three replicate series at ~70% water-holding capacity. Individual tubes were sacrificed completely for total community DNA isolation after 0, 1, 2, 4, 7, and 14 days of incubation. The following treatments were applied: addition of (1) nutrient broth (NB), (2) *S. herbicidivorans* strain MH at 10^9 cells per gram soil (B, bacteria), (3) a mixture of mecoprop and strain MH (BM, bacteria and mecoprop), and (4) pure water as a control (S, soil water). For nutrient addition, 200 μL of NB solution (Biolife, Milan, Italy) were pipetted to each of the 50-mL tubes with 2 g of soil. Strain MH was supplied in 200 μL of sterile demineralized water per aliquot of 2 g of soil and 50-mL tube. Mecoprop was added as 200-μL aqueous solution of both enantiomers ([*R*,*S*]-2-[4-chloro-2-methyl-phenoxypropanoic acid]) at 100 mg/L each (Fluka, Buchs, Switzerland). In case of the addition of *S. herbicidivorans* strain MH and mecoprop simultaneously, the bacteria were resuspended in 200 μL of mecoprop solution and added to 2 g of soil. As a negative control, 200 μL of demineralized water was added per soil aliquot.

Data Analysis

The TIFF image files containing the community T-RFLP or RISA profiles were further analyzed using the BASE IMAGIR™ and GENE IMAGIR™ 4.03 software (Scanalytics, Fairfax, VA) to determine the number, length, and relative intensity of each of the tRFs in a sample. Output text files were further processed in Microsoft Excel. Accurate determination of tRFs was only possible in the size range of 100 to 700 bp, and bands outside this range were excluded from the analysis. Band intensities ("peak area") were normalized by dividing each band's absolute intensity by the total intensity of bands per lane. Peaks below 0.01% of the sum of all peak intensities were not considered. Diversity indices were calculated from the presence/absence and relative band intensities per sample using the formula, diversity index $= 1 - \sum (p_i)^2$, where p_i is the relative intensity of each individual band divided by the sum of the relative intensities of all fragments per lane. Principal components analysis (PCA) was performed using the subroutines "dudi.pca" and "prcomp" in the statistical program R (http://www.r-project.org). Sample clustering was performed on Euclidean distance matrices calculated from the dataset of relative band intensities by using "agnes" in the package "cluster" of R. Distance matrices were compared in a Mantel test using Monte Carlo permutations with help of the program "mantel.rtest," which is part of the package "ade4" [5]. To test significance of clusterings, we performed bootstrapping by resampling distance matrices produced by "agnes" in a small subroutine written for this purpose. Consensus clusters were calculated from the bootstrapping output in the program "consense," which is a part of the Phylip 3.66 package. Branching frequencies were then added to the original "agnes"-input cluster for bootstrapping.

Results

Seasonal Bacterial Community Variability Studied from Polymorphisms in Amplified 16S rRNA Gene Fragments

The variability in bacterial community structure in an agricultural soil was studied by T-RFLP and RISA during 1 year by sampling eight times at approximately the same location (Table 1). If the bacterial community of the site would fluctuate in response to the seasons or be affected by human activities (e.g., plowing, cultivation) during the year, we expected that this would result in a change of individual bacterial population sizes making up the community and, thus, in different relative amounts of population DNA and 16S rRNA genes among the total community-derived DNA. A double-end-labeled T-RFLP system was used here to simultaneously extract a more optimal amount of data from each sample. Around 37 tRFs could be detected in the IRD-800 gel images produced with labeled primer 926R (Fig. 1a, Table 1), whereas 74–82 bands were distinguishable on gels with IRD-700-labeled fragments (primer 8F) albeit seemingly concentrated in a smaller size "region" (Fig. 1b). At first sight, the profiles did not show much variability in samples taken at different time points, except for an intensity increase of the fragments at 290 bp in panel a1 (lanes 6ab) and at 610 bp in panel b1 (lanes 6ab). It cannot be excluded that some tRFs would be artifacts from PCR [10]. Pearson's similarity indices within replicates varied between 0.835 and 0.997, the IRD-700 analysis being overall less reproducible than the IRD-800-based one (Table 1). Examples of the development of relative average peak intensity for selected individual bands throughout the time period of analysis (Fig. 1c) reinforced the visual trend that little variation occurred in many of the bands but that other individual tRFs varied significantly throughout the season. Pairwise comparisons between subsequent sampling pairs in time resulted in 10 to 41 bands out of 125 of the total combined dataset (IRD-700) or between 9 and 12 from 37 total tRFs (IRD-800) being significantly different in intensity ($P<0.05$, t test, Table 2). Pairwise sample similarities were lower than within sample replicate similar-

Table 1 Diversity statistics calculated from T-RFLP and RISA profiles of 16S rRNA genes amplified from agricultural soil DNA

Method	Index	Sample, time (days)[a]							
		A, 0	B, 14	C, 39	D, 50	E, 65	F, 168	G, 299	H, 317
T-RFLP[b]	S(700)[c]	77	76	76	74	76	74	79	82
	S(800)	36	36	36	36	36	36	37	37
RISA	S(800)	–[d]	43	–	–	42	46	–	44
T-RFLP	D(700)[e]	0.966	0.970	0.974	0.970	0.965	0.968	0.967	0.965
	D(800)	0.937	0.934	0.931	0.934	0.931	0.936	0.936	0.929
RISA	D(800)	–	0.944	–	–	0.932	0.943	–	0.938
T-RFLP	Sim(700)[f]	0.987±0.004	0.918±0.040	0.835±0.072	0.857	0.977±0.011	0.973	0.927±0.039	0.927
	Sim(800)	0.997±0.001	0.996±0.000	0.996±0.001	0.992	0.980±0.012	0.994	0.987±0.007	0.979

[a] Time points of soil collection starting from day 0 on July 1 2002 (A). Sampling dates of other samples: B, July 15, C, August 9, D, August 30, E, September 14, F, December 12, G, February 25 2003, H, May 14 2003

[b] *T-RFLP* Agricultural soil DNA analyzed with T-RFLP to study the natural variation of microbial community, *RISA* same soil DNA analyzed with RISA.

[c] Phylotype richness, *S*, calculated as the total number of distinct tRFs and IGS sizes (between 100 and 700 bp) in a profile

[d] Not determined

[e] Simpson's index, *D*, calculated as follows: $D = 1 - \sum (p_i)^2$, where p_i is the individual relative band intensity divided by the sum of all band intensities per lane

[f] Average Pearson's similarity coefficient, Sim, among replicates±average deviation (if more than two replicates)

ities and fluctuated between 0.417 and 0.989, the IRD-800 dataset bearing overall higher similarities (Table 2). Intensity fluctuations were also higher in IRD-700- than IRD-800-derived tRFs (from 8 to 19 tRFs being 200-fold less to 358-fold more intense in subsequent samples for the IRD-700 channel, Table 2). Very few bands consistently varied throughout all samples except one tRF (i.e., IRD-700 320 bp, more than fivefold in five of eight datasets). Between 1 and 13 newly appearing or disappearing tRFs were scored between consecutive time samples (IRD-700 data, Table 2) with a tendency of increasing differences around sample F.

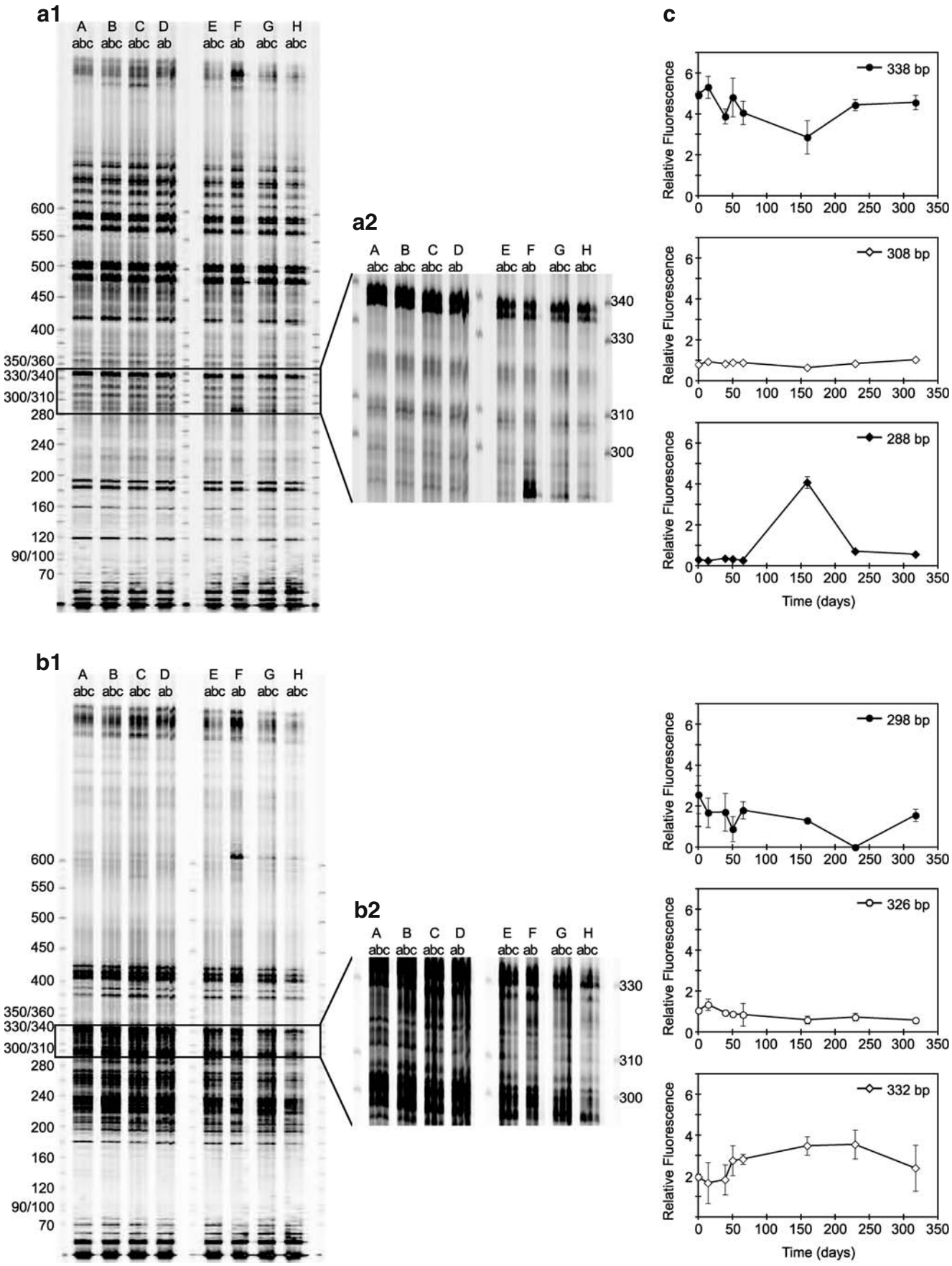
To consolidate the observations made with T-RFLP, a subset of the same soil DNA samples was again analyzed via RISA, which targets a different region as T-RFLP. The four samples showed a highly consistent number of 42 to 46 discrete bands (Fig. S1). The Simpson's diversity indices and phylotype richness calculated from T-RFLP and RISA data were remarkably stable for all samples, except for some tiny but insignificant variations (Table 1). Despite individual fragment intensity fluctuations, therefore, both T-RFLP and RISA data suggested that no changes in the total diversity of the soil microbial community took place during the sampling period.

Principal Components and Clustering Analysis

To analyze overall community fluctuations in more detail, we performed PCA and clustering for each of the datasets (IRD-700 and IRD-800 derived relative band intensities in T-RFLP). Two components were sufficient to explain 86.7 (IRD-700) and 97.6% (IRD-800) of the total variation (Fig. 2). With few exceptions, sample replicates grouped relatively close together in the PCA plot, showing that there was on average little variation when all tRFs in the sample were taken into account (Fig. 2). Clustering analysis of distance matrices on both datasets confirmed that most triplicates grouped very closely together with a very small Euclidean distance between triplicates (Fig. 3). Bootstrapping analysis showed that most branchings between sample triplicates were not statistically significant (Fig. 3) and thus that samples taken at different points in time cannot be said to differ. Notable outliers in PCA were the samples F1/F2 for the IRD-700 dataset and H1/H2 for the IRD-800 dataset. Because there is too little congruency between both datasets (Mantel test observation 0.42, $P=0.03$, Table 3), we cannot conclude whether the H or F samples significantly differ in T-RFLP or represent outliers in the analysis.

To analyze the sensitivity of the PCA for such outliers further, we tested whether one prominent band could change the position of the sample in the PCA plot. Hereto, we decided to artificially change the intensity of the

Fig. 1 Bacterial community structure of agriculture topsoil sampled at ▶ different time points during 1 year. **a** T-RFLP profiles of amplified 16S rRNA gene fragments in the different samples (*A–H*), restricted with *Hae*III and labeled with IRD-800 at the primer 926R. **a2** Magnification of the region between 280–360 bp. **b** T-RFLP profiles of the same samples using restricted amplified 16S rRNA gene fragments labeled with IRD-700 at the forward primer 16S 8F and its magnified region (**b2**). Molecular size markers (in bp) are indicated on the *left*. **c** Examples of average relative intensities (among triplicates) of selected tRFs throughout the sampling year. Lanes *A–H* denote different time points of soil sampling and correspond to those of the PCA (Fig. 3, Table 1); with (*abc*) representing replicates


a1
A B C D E F G H
abc abc abc ab abc ab abc abc
600
550
500
450
400
350/360
330/340
300/310
280
240
200
160
120
90/100
70
a2
A B C D E F G H
abc abc abc ab abc ab abc abc
340
330
310
300
c
338 bp
308 bp
288 bp
Relative Fluorescence
0 50 100 150 200 250 300 350
Time (days)
b1
A B C D E F G H
abc abc abc ab abc ab abc abc
600
550
500
450
400
350/360
330/340
300/310
280
240
200
160
120
90/100
70
b2
A B C D E F G H
abc abc abc ab abc ab abc abc
330
310
300
298 bp
326 bp
332 bp
Relative Fluorescence
0 50 100 150 200 250 300 350
Time (days)

visually strong band at 288 bp in the F samples (IRD-800) from a relative intensity of 4.3 and 3.9 to 0.40 (average intensity value of the other samples). In the PCA plot, this resulted in the clustering of the F samples with the others (*F1 and *F2 in Fig. 2a), suggesting that its original position was solely determined by the intensity of this individual band. Similarly, when the relative band intensities at 614 bp in the samples F1 and F2 (IRD-700) were substituted by the average values of the other samples, the F data points shifted to a position closer to the rest of the samples (*F1 and *F2 in Fig. 2b).

Artificial Disturbance of the Microbial Community in Soil

To better interpret the magnitudes of seasonal variations observed in the soil microbial community of the agricultural field, we analyzed community changes in artificially perturbed laboratory microcosms containing the same soil by T-RFLP. These were short-period incubations with the only purpose of producing bacterial community changes and not to mimic seasonal developments in the field. As for the field samples, the tRF patterns of the control microcosm (S, soil with water added only) did not show major changes in number of detected bands, the calculated diversity index, and the Pearson's pairwise similarity coefficient (Table 4), showing that despite small microcosm size, no changes were induced by the system setup. Compared to the control, the addition of the bacteria *S. herbicidivorans* MH (B) without or with mecoprop (BM) caused an increase in phylotype richness (Table 4) but a significant temporary decrease in diversity index, between days 1 and 7, after which the diversity index returned to a similar level as at the beginning of the incubation. In addition, similarity coefficients decreased strongly for samples taken at days 1 and 2, compared to the time zero sample, after which, however, the similarities recovered (Table 4). Soil samples to which *S. herbicidivorans* strain MH was inoculated (B) showed a large increase in relative intensity of a fragment at 382 bp in the IRD-700 profile and at 480 bp in the IRD-800 profile after 1 and 2 days of incubation (Figs. S2a, c and S3). The 480-bp band corresponds to an obtained fragment size (490 bp) when performing T-RFLP for the 16S rRNA gene on *S. herbicidivorans* genomic DNA (not shown). This effect was also observed in the tRF patterns of soils treated with mecoprop and inoculated with bacteria (BM, Fig. S4). Phylotype richness and diversity index in the nutrient-amended soil (NB) decreased strongly at day 1 but returned to a similar value as on day 0. Even visually, nutrient amendment resulted in large tRF differences (Fig. S5). On the contrary, profile similarities compared to the time zero sample decreased at day 1 and remained strongly dissimilar hereafter (Table 4).

PCA and bootstrapping analysis of the control soil community showed a strong clustering of all samples with very slight time variability (Fig. 4, Fig. S6). Inoculation of *S. herbicidivorans* strain MH quickly resulted in a strong

Table 2 Comparative statistical data on bacterial 16S rRNA gene profiles obtained with T-RFLP at different time points during the year

Dataset	IRD-700					IRD-800			
	Pearson's pairwise similarity coefficient	Number of significant band differences[a]	Number of bands with less than 0.2-fold change [minimum][b,d]	Number of bands with more than fivefold change [maximum][c,d]	Number of new or disappearing bands in subsequent sample	Pearson's pairwise similarity coefficient	Number of significant band differences[a]	Number of bands with less than 0.2-fold change [minimum][b,d]	Number of bands with more than fivefold change [maximum][c,d]
A×B	0.928	28 (n=114)	8 [0.021]	8 [18.2]	1/2	0.989	12 (n=37)	0	0
B×C	0.832	41 (n=111)	8 [0.011]	11 [18.7]	3/3	0.980	9 (n=37)	0	0
C×D	0.823	–	14 [0.008]	19 [290]	4/6	0.976	–	0	0
C×E	–	10 (n=125)	–	–	–	–	0 (n=37)	–	–
D×E	0.958	–	8 [0.003]	13 [130]	4/2	0.989	–	0	0
E×F	0.417	–	13 [0.053]	9 [358]	5/7	0.953	–	1 [0.06]	0
E×G	–	18 (n=125)	–	–	–	–	12 (n=37)	–	–
F×G	0.494	–	12 [0.002]	15 [111]	13/9	0.952	–	0	3 [13.1]
G×H	0.871	–	15 [0.011]	15 [51]	8/5	0.909	–	0	1 [6.5]
H×A	0.866	–	–	–	2/7	0.913	–	–	–

[a] $P<0.05$, pairwise t test, number of corresponding significant changes in two datasets: 12, in three datasets: 5, in four datasets: 0
[b] Number of bands less than 0.2-fold in two datasets: 15, in three datasets: 3, in four or more: 0
[c] Number of bands more than fivefold in two datasets: 24, in three or more: 0
[d] Number of bands less than 0.2-fold and more than fivefold in two datasets: 12, three datasets: 17, four datasets: 9, five datasets: 1

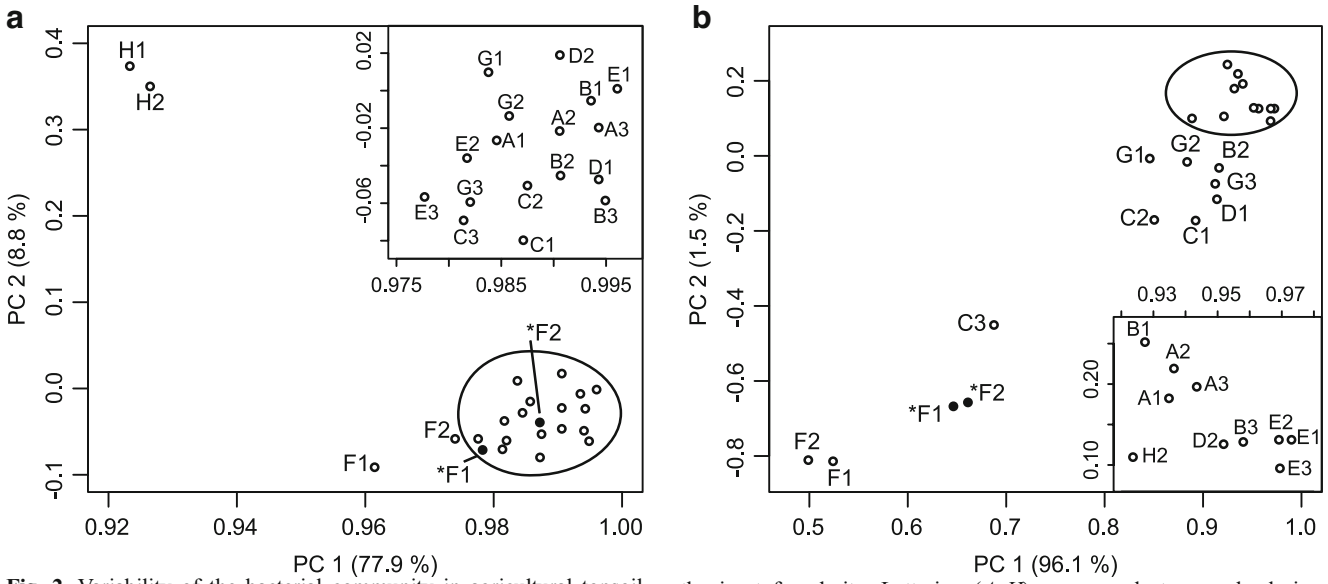


Fig. 2 Variability of the bacterial community in agricultural topsoil over time during 1 year. **a** Principle components analysis of the relative band intensities of all tRFs using the IRD-800-primed amplicons. The first two components contribute to 86.7% of the total variation. **b** PCA of relative band intensities of tRFs using the IRD-700 primed amplicons; components 1 and 2 are responsible for 97.6% of the total variation. The regions within the ellipses are enlarged in the inset for clarity. Lettering (*A–H*) corresponds to sample designations as in Fig. 1 and Table 1. Indices refer to the different replicates per sample. *F1 and *F2 in panels **a** and **b** show the shifts of the data points after the artificial relative intensity change of the bands at 288 (IRD-800) and 614 bp (IRD-700) as explained in the main text. Note the scale differences between **a** and **b** and between the enlarged regions

and significant shift of the community (compare data points B0 with B1 and B2 in Figs. 4a and S6), slowly returning to the original composition and that of the community in the control incubations, on days 7 and 14 (Fig. 4a). Both IRD-700 and IRD-800 datasets were in good agreement in Mantel permutation tests (0.87, P=0.01, Table 3). Inoculation with bacteria and application of mecoprop (BM) immediately resulted in a detectable community change, which augmented after day 2 and continued to be different from both control and time zero and day 1 samples (Figs. 4b, S7). Note, however, that the magnitude of the temporarily change in Euclidean distance between the

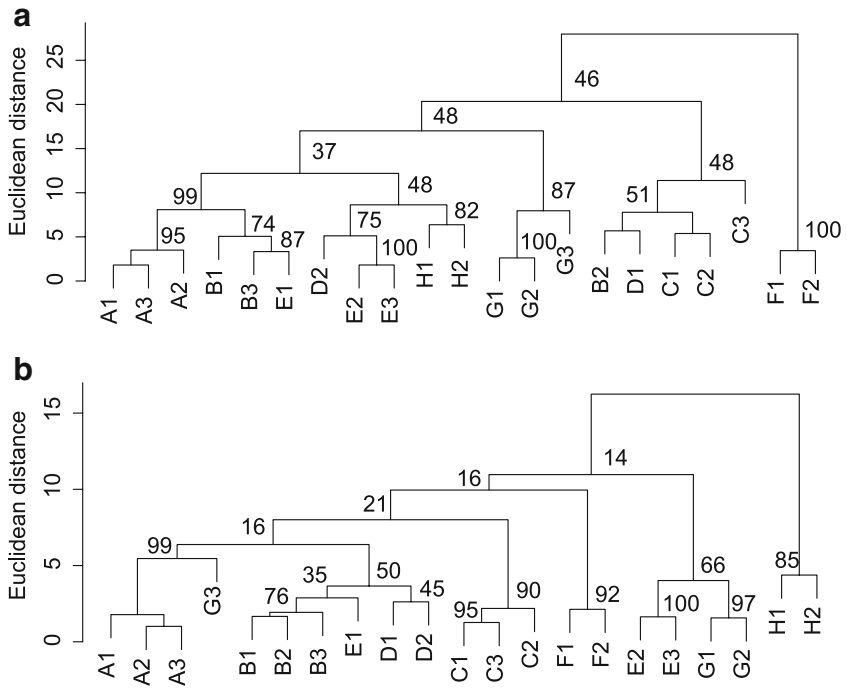


Fig. 3 Sample clustering of the agricultural topsoil bacterial community profiles using distance matrix calculations and bootstrapping. **a** Clustering based on the T-RFLP dataset obtained with the 8F IRD-700 forward-primed 16S rRNA gene amplicons. **b** Idem with the 927R IRD-800 reverse-primed amplicons. The *ordinate* shows the Euclidean distance, and *numbers at the nodes* refer to the percentage of trees carrying this branching order as obtained via bootstrapping (n=100). Sample designations (*lettering plus index*) correspond to those of Figs. 1 and 2 and Table 1

Table 3 Correlation of distance matrices of bacterial 16S rRNA gene profiles obtained with T-RFLP

Dataset	Simulated similarity[a]	Simulated *P* value[b]
dd700×dd800[c]	0.42	0.01
s700×s800	0.29	0.02
nb700×nb800	0.87	0.01
b700×b800	0.87	0.01
bm700×bm800	0.84	0.01

[a] Similarity value produced from 999 repetitions in a Monte Carlo permutation test
[b] Simulated significance value
[c] Datasets: *dd700* T-RFLP data IRD-700 channel (primer 6F) from soil community DNA, *dd800* idem IRD-800 (primer 926R), *s700* soil microcosm, *nb700* soil microcosm with nutrient broth added, *b700* soil microcosm with bacteria inoculated, *bm700* soil microcosm with bacteria plus mecoprop added

incubation with bacteria only (B) was larger than with mecoprop plus bacteria (BM). Cluster analysis reinforced the visual observations that nutrient addition resulted in an immense and immediate effect on the bacterial community composition and did not return to the control composition either (Figs. 4c, S8). Surprisingly, the variation between communities from the NB treatment completely surpassed that of the others. Both IRD-800 and IRD-700 datasets for the NB treatment samples were again in good agreement (0.87, P=0.01, Table 3). In terms of pairwise dataset comparisons, almost 25% of the tRFs between NB treatment and control were unique to either dataset, vs up to 5% for bacterial inoculation with or without mecoprop compared to the control (Table 5).

Discussion

Natural Variability

In this paper, we studied bacterial community fluctuations in a regular agricultural soil as a function of seasonal influences and human activities. Detecting such "natural" variations is important because they form the baseline against which changes in microbial diversity can be detected. For example, we might be interested in inoculating specific bacteria for bioremediation of agricultural chemicals, but possible negative effects on microbial diversity must be related to a background of natural variability. Various authors have reported that the inherent relative band intensity deviation among replicate T-RFLP analyses is in the order of between 5 and 15% [1, 16, 33], with sample replicate similarity indices of between 0.85 to 0.97, which is similar to what we found here. Fluctuations in community profiles, which are smaller than the inherent method variability, can thus not be discerned, except when

Table 4 Diversity statistics calculated from T-RFLP of 16S rRNA genes amplified from agricultural soil samples exposed to artificial disturbances

Sample[a]	Channel[b]	Time (days)[c]																	
		0			1			2			4			7			14		
		R[c]	D[d]	P[e]	R	D	P	R	D	P	R	D	P	R	D	P	R	D	P
S[b]	IRD-700	70	0.971	1	70	0.968	0.975	70	0.965	0.947	nd[f]	nd	–	70	0.969	0.926	70	0.970	0.938
	IRD-800	36	0.933	–[f]	36	0.931	–	36	0.941	–	nd	nd	–	36	0.938	–	36	0.939	–
B	IRD-700	70	0.974	1	73	0.966	0.758	73	0.951	0.579	nd	nd	–	73	0.969	0.835	73	0.969	0.837
	IRD-800	36	0.935	–	37	0.910	–	37	0.898	–	nd	nd	–	37	0.943	–	37	0.940	–
BM	IRD-700	70	0.977	1	74	0.974	0.892	74	0.970	0.830	nd	nd	–	73	0.974	0.900	73	0.972	0.873
	IRD-800	36	0.938	–	37	0.932	–	36	0.923	–	nd	nd	–	36	0.939	–	36	0.937	–
NB	IRD-700	70	0.968	1	64	0.964	0.299	71	0.970	0.158	75	0.974	0.456	76	0.968	0.340	75	0.973	0.321
	IRD-800	37	0.929	–	33	0.876	–	36	0.899	–	38	0.922	–	38	0.928	–	38	0.922	–

[a] Sample types: *S* control soil plus water, *B* addition of bacteria (*S. herbicidivorans* MH), *BM* addition of bacteria and mecoprop, *NB* addition of nutrient broth
[b] IRD-700, primer 6F; IRD-800, primer 926R
[c] Time of total soil DNA extraction after incubation start
[d] Phylotype richness, *R*
[e] Simpson's index, *D*
[f] Pearson's similarity coefficient, *P*, with respect to t=0 sample
[g] *nd* No data, – not calculated

the number of replicate analyses is increased. It is interesting to note that the bacterial community in the soil as examined via variability of the 16S rRNA genes and 16S–23S rRNA intergenic spacer region using T-RFLP and RISA, respectively, remained overall highly constant during the seasons (with changing temperature and rainfall) and despite human activities (plowing, maize and winter lettuce, fertilization). The highly stable bacterial community is well documented by the overall community parameters richness and Simpson's diversity indices and by PCA, clustering, and bootstrapping on complete tRF datasets. There was a tendency for the December sample ("F") to be different from the rest (Table 2), but IRD-700 and IRD-800 datasets did not consistently place this sample outside the rest. One component in PCA was largely sufficient to explain the variability among the samples but did not correlate with the sampling date or season itself. Clustering and bootstrapping of distance matrices suggested that none of the clustering branches was significant (i.e., occurring in more than 50% of the bootstrapped trees) or occurred similarly in both IRD-700 and IRD-800 datasets, implying that the sampled communities do not follow a clear trend and thus remain the same in statistical terms. Because we sampled more or less randomly in time, we may have missed changes resulting from rainfall, which were already leveled out at the time of sampling. However, other authors have reported that normal soil moisture fluctuations (drought and rewetting) have relatively little effect on bacterial community composition as measured by 16S rRNA gene or transcript diversity [14].

Nevertheless, a significant number of individual tRFs did vary in relative intensity (Fig. 1, Table 2), both for the IRD-700 and the IRD-800 datasets, and there was a slight tendency that samples E, F, and G (fall, winter, and spring) were more variable in number of unique differences (Table 2). This and the magnitude of some tRF fluctuations (up to 358-fold) leads us to conclude that individual populations within the soil microbial community do vary, whereas this may be leveled out at the variance level of all tRFs. We are aware that a single tRF in the analysis can actually be comprised from different bacterial populations, which happen to have the same restriction cleavage position in their 16S rRNA gene with respect to the PCR primer-binding site and that, therefore, even a nonchanging tRF in the analysis may be the consequence of fluctuating subpopulations. In addition, misinterpretations may occur in band designations in particular among similarly sized bands [33] and as a result of PCR artifacts [10]. For the time being and without further information on the tRFs sequences, therefore, we do not assign specifically fluctuating tRFs to particular bacterial populations. This problem, although, is partially overcome at the level of the complete datasets by the use of both end-labeled-amplified 16S rRNA gene fragments.

Detecting Community Changes

We could thus conclude that the soil bacterial community overall did not change significantly (within the limits and sensitivity set by the inherent method variability and accuracy of T-RFLP or RISA), whereas a limited number of individual bacterial populations did vary (Table 2). Not many studies have specifically addressed natural community fluctuations in soil microbiota, and those which did reported contradicting results or applied different methodologies and statistical analyses. For example, the soil microbial communities in a pine forest soil in Java were shown to remain stable throughout a wet and a dry season but differed according to soil organic carbon content [21], whereas Laverman *et al.* [23] concluded that populations of ammonium-oxidizing bacteria remained stable throughout the year in a temperate, acid forest soil, although the total bacterial community structure dramatically differed. In the previously mentioned studies of Buckley and Schmidt [3] and Lipson and Schmidt [26], significant changes in taxonomic group sizes of soil bacterial communities were detected during the seasons, amounting to approximately twofold increase or decrease per taxonomic group. However, of the four reports, Krave *et al.* [21] employed rRNA rather than 16S rRNA gene abundance, whereas none of the other reports included community-wide statistical analysis, making comparisons about the magnitude of changes rather difficult. Because clear community differences were observed in long-term studies between differently treated soils, several authors concluded that soil communities respond at time scales of 10 or more years [3, 22, 44], which would also suggest that seasonal changes in community composition remain small.

To relate the magnitudes of observed T-RFLP variations in the agricultural soil bacterial community to some experimental parameter and perhaps define a "bandwidth" of natural variability, we subjected soil in microcosms to a number of artificial laboratory-induced perturbations, which we expected would lead to a measurable difference in community composition. Inoculation of 10^9 bacteria (*S. herbicidivorans* MH) to 2 g soil resulted in a very clearly differentiated community profile compared to a control soil, incubated under the same conditions. On the other hand, addition of water only and incubating for 14 days at 25°C did not significantly change the bacterial community profile. It is interesting to note that bacterial communities perturbed with inoculated bacteria returned to their original community profile after 7 days and the 480-bp band attributed to the 16S rRNA gene of *S. herbicidivorans* MH disappeared, which may indicate that the inoculated bacteria did not survive well in the soil. Adding both bacteria and mecoprop (a frequently used herbicide in agricultural practice) changed the community profile to a

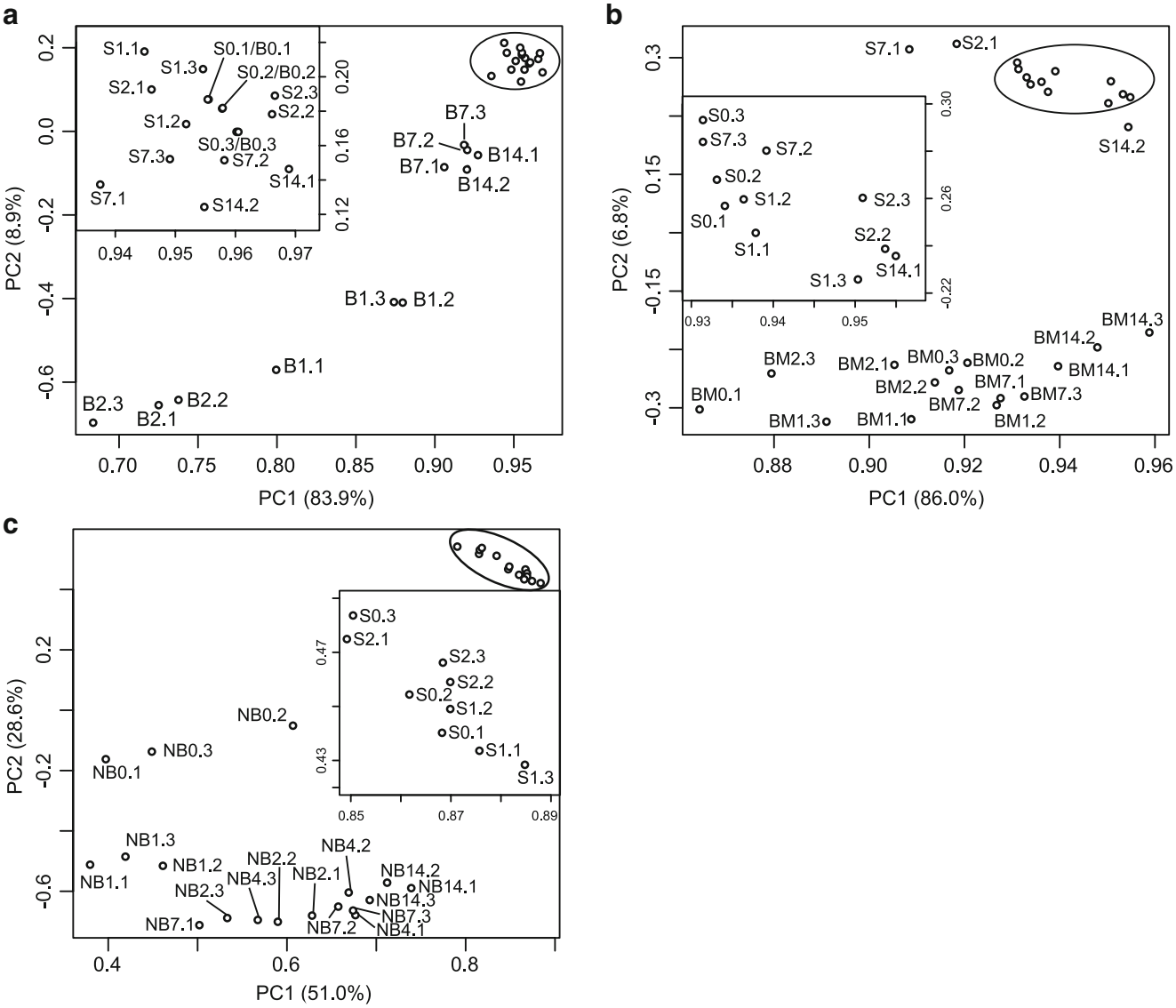


Fig. 4 PCAs of relative band intensities of the IRD-700 datasets from the soil microcosms. Different individual treatments are compared. **a** *S* (control soil plus water) vs *B* (addition of *S. herbicidivorans* bacteria), **b** *S* vs *BM* (bacteria and mecoprop), and **c** *S* vs *NB* (addition of nutrient broth). PC 1 is plotted against PC 2. The two main components explain 92.8 (**a**), 92.8 (**b**), and 79.6% (**c**) of the total variation. *Encircled data points* are enlarged in separate plots. Note the scale differences of the components among the three plots

Table 5 Number of changing labeled terminal restriction fragments between different treatments to soil in microcosms

Dataset	IRD-700[a]		IRD-800	
	Number of new tRFs[b]	Disappearing[c]	Number of new bands	Disappearing
S×NB	25 (n=95)[d]	13	11 (n=47)	8
S×B	4 (n=75)	0	1 (n=38)	0
S×BM	5 (n=75)	0	0 (n=38)	1

[a] *IRD-700* primer 6F, *IRD-800* primer 926R
[b] tRFs appearing in NB, B, or BM vs S across all time samples
[c] Idem disappearing in NB, B, or BM vs S
[d] Total number of tRFs in both datasets

lesser extent than bacteria only but to a permanently different state after 14 days. Both these treatments, therefore, suggested that the soil community in the field did not fluctuate as much as the equivalent of inoculation of one strain at 10^9 cells or addition of 4 μg mecoprop per gram soil. To put this in perspective, in a recent semifield-scale bioremediation study, Paul *et al.* [34] used 10^6 *Arthrobacter* cells per gram of soil and found no significantly different community profiles compared to a noninoculated control. Perhaps as an extreme, addition of nutrients changed the community composition immediately and for the full duration of the incubation. This perturbation is similar in effect as plating soil bacteria suspensions on nutrient agar in which case the resulting colony diversity no longer corresponds to the original community diversity [40].

Can a bandwidth of natural variability thus be defined, outside which the community will develop into a different composition? The first difficulty in this respect is to identify the proper parameter for the bandwidth definition. Zhou *et al.* [45] described no diversity differences in a soil polluted with 20% chromium using the reciprocal of the Simpson's and the log series index, whereas soils from different depths and locations did differ. The results of this work and that of others [16] suggest that classical diversity parameters (species richness, Simpson's diversity) are not sufficient for this purpose, at least not in combination with T-RFLP data that generate some 100 observations per sample (i.e., number of tRFs in IRD-700 plus IRD-800 datasets). On the contrary, the use of a pairwise similarity index was proposed by De La Cochetière *et al.* [6], who studied community fluctuations in human intestinal flora in response to antibiotic treatment. It is interesting to note that their data suggested that at ~70% community similarity (calculated from TGGE diversity of amplified 16S rRNA gene fragments), the communities still recover to their original composition [6]. Our perturbation experiments suggested that temporary similarities of 58% still recover, at least in the case of bacteria inoculation, whereas at 30% or less, the communities will differ in the long term (Table 4, Fig. 4). However, the use of similarity indices is not completely satisfying because it cannot differentiate composition trends very well (viz. the different trends between communities perturbed with bacteria alone or bacteria plus mecoprop). In this paper, the use of coordinate analysis (or PCA) or other types of clustering become more appropriate [1, 16, 17], although quantitative boundary definitions become much more difficult. In terms of "pragmatic" definition, therefore, the use of similarity indices or correlation coefficients between distance matrices may be favored in which case the 70% similarity boundary may be proposed as a rule of thumb for bacterial communities starting to drift apart. It may be interesting to test further types of experimental perturbations to better define the resilience of complex microbial communities via similarity or correlation coefficients, which may have a more general character for any complex bacterial community. Furthermore, we can conclude that soil microbial communities appear to fluctuate insignificantly at the level of T-RFLP analysis during the various seasons, which offers good possibilities to detect future aberrant trends.

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