Microb Ecol (2016) 72:717–724
DOI 10.1007/s00248-016-0810-0

SOIL MICROBIOLOGY

Bacterial Community Structure at the Microscale in Two Different Soils

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Received: 3 November 2015 / Accepted: 27 June 2016 / Published online: 14 July 2016

Abstract The spatial distributions of bacteria in the soil matrix have a role in ecosystem function, for example, at the small scale, through gene transfer or xenobiotic degradation. Soil bacterial biogeography has been evidenced at the large scale, but data are scarce at the small scale. The objective of this work was to determine the spatial pattern of bacterial diversity, in spatially referenced microsamples, in order to define bacterial community spatial traits. Two soils with different physical structures, moderately aggregated (La Côte St André (LCSA)) or poorly aggregated (La Dombes (LD)), were studied. The spatial distribution of bacteria was studied in microsamples (diameter 3 mm) along 10- and 20-cm transects, with a taxonomic microarray. 16S rRNA gene sequencing was used to further study the spatial characteristics of the microbial communities in LD soil. The frequency-occupancy plot, in the LCSA and LD soils, using microarray and sequencing data, followed Hanski's core-satellite theory. The frequency-occupancy distribution plots obtained in two different soils showed bimodality and indicated that the microscale spatial distributions were different, particularly core taxa percentage. Core taxa are widespread and abundant, while satellite taxa are restricted in their distribution. The spread of satellite taxa was at a distance range larger than 5 cm, whereas the core taxa were distributed in a distance range less than 3 mm. Besides, there was a positive abundancy-occupancy relationship at this fine scale. It may be interesting to further evaluate the role of the different bacterial spatial distributions at the fine scale on soil function.

Keywords Abundancy-occupancy relationship · Bacteria community structure · Microscale in soil · Frequency-occupancy relationship · Soil microbial diversity · Soil structure

Introduction

Soil is a complex system with a 3-D spatial physical structure within which substrates and bacteria are organized. The specific spatial distributions of bacteria in the soil matrix, particularly at the small scale, may have a role in ecosystem function through, e.g. gene transfer or xenobiotic degradation. The existence of bacterial biogeography has been recognized at the large scale [1–3], as well as at intermediary scales in studies targeting specific bacterial groups [4–7]. Few studies, however, have targeted the fine scale, i.e. from the cell to habitat scales, despite the fact that all biotic and abiotic interactions take place at these scales [8]. The studies that have been carried out at these scales have concentrated on the distribution of bacteria or enzyme activities [9–14], and they have determined that bacteria are characterized by patchy distributions. The studies have also suggested that spatial patterns in soils may have a functional role.

The environmental variables that are known to influence bacterial diversity and community structure, such as soil

Electronic supplementary material The online version of this article (doi:10.1007/s00248-016-0810-0) contains supplementary material, which is available to authorized users.

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physico-chemical variables, have very rarely been studied in a spatially explicit manner at the small scale, in spite of the fact that soil structure induces significant physical constraints at all scales, and particularly at the fine scale [15]. Soil characteristics have an effect on the spatial distribution of bacterial cells and on the organization of diversity through, for example, pore network connectivity, water film continuity or discontinuity [15], oxygen concentrations, pH, pore sizes available for microbial habitation and habitat surface [16]. In some studies, microsampling approaches at the sub-millimetre scale have been proposed to measure spatial relationships between microhabitats [9, 11, 12, 14, 17–19]. These spatial relationships are often overlooked as soil samples are pooled and homogenized, resulting in loss of information.

The objective of this work was to study the spatial pattern of bacterial diversity and spatial community structure in soil, at the microscale (millimetre scale). In order to determine if different spatial distributions could be identified at the small scale, the study was carried out on two soils with different physical structures: a poorly structured soil (La Dombes (LD)) and a moderately structured soil (La Côte St André (LCSA)). A high-throughput method of diversity analysis and a spatially referenced microsampling were combined. Bacterial diversity was analyzed, in LCSA and LD soils, using a 16S taxonomic microarray on 3-mm-sized undisturbed samples, sampled at the centimetre scale within a few square centimetre soil. The microsamples were spatially referenced along transects. Bacterial diversity in the LD soil was also analyzed using sequencing. The spatial distributions were different in the two soils, as indicated with the frequency-occupancy distributions, and they allowed to defining core and satellite taxa.

Material and Methods

Soil Sampling

A sandy loam brown soil from LCSA, Isère (France), with a granular structure, and a loamy soil from LD, Ain (France), a poorly aggregated soil [20], were sampled as follows: a clod of each soil was brought back to the lab for subsequent microsampling. Twenty-five and 23 microsamples of 3-mm diameter (about 20 mg) were taken from a 30-cm^2 levelled surface that was parallel to the soil surface, from the LD and LCSA soils, respectively. The inter-sample separation distances ranged from 3 mm to 10 cm (Fig. 1). The bacterial community structure was analyzed in each microsample using taxonomic microarrays (see below). A second sampling effort was carried out in the same area of the LD soil 4 years later. Twenty-two soil microsamples were taken along a 22-cm transect (1-cm lag distance) using a small corer. A 1-mm-thick slice was taken 1 mm below the soil surface (100 mg), and the bacterial communities were analyzed by 16S pyrosequencing analysis. The characteristics of the two soils are presented in Table S1.

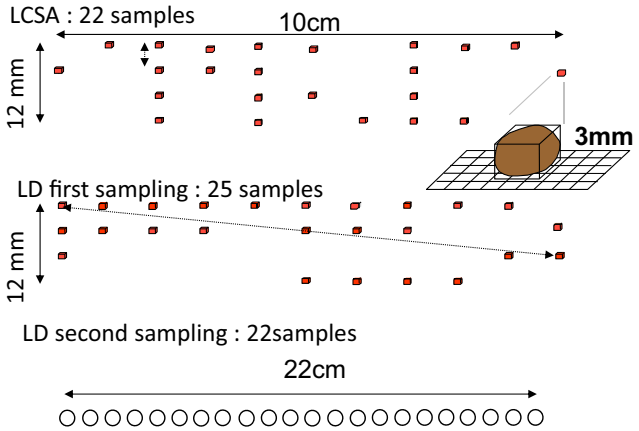

Fig. 1 Sampling map of microsamples in LCSA and LD soils. Each schema represents a levelled clod of soil. *Double arrows* between two microsamples of the first sampling represent minimum (3 mm, contiguous samples) and maximum (10 cm) distances between sample centres. The LD soil second sampling was carried out 4 years after the first sampling

DNA Extraction

DNA extraction from microsamples was performed following the protocol of Orsini and Romano-Spica [21]. Soil microsamples were suspended in 800 µL PBS 1× pH = 8 (1 g mL^{-1}) and centrifuged at 5700*g* for 1 min. The pellet was suspended in 1-mL washing solution (50 mmol L^{-1} Tris–HCl pH = 7.7, 25 mmol L^{-1} EDTA, 0.1 % SDS, 0.1 % PVP, H_2O) and centrifuged at 5700*g* for 1 min. Then, the pellet was suspended in 35-µL lysis solution (50 mmol L^{-1} Tris–HCl pH = 8, 25 mmol L^{-1} EDTA, 3 % SDS, 1.2 % PVP, H_2O) and microwaved at 600–700 W for 45 s. Four hundred-microliter pre-heated (75 °C) extraction solution (10 mmol L^{-1} Tris HCL pH = 8, 1 mmol L^{-1} EDTA, 0.3 mol L^{-1} sodium acetate, 1.2 % PVP, H_2O) was then added before a phenol chloroform extraction. The phenol chlorofrom extraction was performed by adding 1 volume of phenol choloroform isoamylic alcohol (25:24:1), vortexing the samples and centrifuging them for 10 min at 13,000 rpm. One volume of chloroform was then added. After 5-min centrifugation at 13,000 rpm, the water phase was kept. Ten percent of total volume of sodium acetate 3 M (pH 5) plus 2 volumes of cold absolute ethanol was added. After 20 min on ice, the samples were centrifuged for 30 min at 13,000 rpm and 4 °C, and the supernatant was eliminated. The DNA pellets were washed with EtOH 70 % and suspended in 20 µL water. For sequencing, the DNA pellets were suspended in 100 µL water.

DNA Amplification and DNA Hybridization

The taxonomic microarrays contained 1328 probes targeting a wide range of taxonomic levels, from the phyla down to the finest taxonomic level allowed [22, 23]. There were four repeats of the probe set spotted per microarray. Previous work presented DNA amplification, hybridization and data filtration protocols [22, 24]. The probes of interest for spatial analysis have been evaluated for below-family-level theoretical specificity (wmm = 1.5) [22, 24] against the SILVA database, using the web server probeCheck http://www.microbial-ecology.net/probecheck with a weighed mismatch cut-off of 1.5.

DNA Sequencing

The 16S rRNA gene V4 variable region PCR primers 515/806 with barcode on the forward primer were used in a 30-cycle PCR (5 cycles used on PCR products) using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, after which a final elongation step at 72 °C for 5 min was performed. After amplification, PCR products were checked in 2 % agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Then, the pooled and purified PCR products were used to prepare a DNA library by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq, following the manufacturer's guidelines. Sequence data were processed with the MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). Briefly, sequences were joined and barcodes, sequences <150 bp and sequences with ambiguous base calls removed. Sequences were denoised, OTUs generated and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3 % divergence (97 % similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from Greengenes, RDPII and NCBI (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu) [25].

Statistical Analysis

All statistical analyses were carried out using R [26, 27] with ade4 package [28]. Principal component analysis (PCA) was performed to explore the relationship between samples. The difference between soils was tested with a permutation test of between-class analysis. A Mantel test was carried out to determine the relationship between the community similarity and the spatial distance between microsamples. The uniformity of frequency distribution was tested with a chi2 test with R [27].

Due to the experimental procedure, each microsample was unique and there could not be any repetition. Care was taken to optimize each step during the analysis procedure so that the hybridization result was as representative of the information present in the initial sample as possible.

Results

Comparison of the two soil community structures is presented, as a first approach, with the number of hybridized probes in the two soils: 105 and 118 probes were positive in the LCSA and LD soils, respectively, 95 of which were common to both soils. The remaining 10 and 23 probes were positive only in the LCSA and LD soils, respectively.

The frequency-occupancy diagram (distribution of the numbers of species occupying different numbers of areas) is commonly used in macroecology and community ecology. The frequency-occupancy diagram (percentage of positive probes in defined number of microsamples as a function of the percent microsamples in which they are present) (Fig. 2) was constructed with the subset of "low-taxonomic-level" probes targeting taxa below the family level (43 and 40 probes in LCSA and LD, respectively). The non-uniformity of the distributions (Fig. 2) was highly significant (chi2 test $p < 0.001$) [24] for LCSA and significant for LD ($p = 0.025$). The frequency-occupancy plot was clearly bimodal in LCSA (Fig. 2). The non-uniformity cannot be rejected for LD, and the frequency-occupancy plot also showed bimodality. The probes which were positive in one microsample (one microsample represents 4.3 and 4 % of the microsamples taken along the two transects, that is 4.3 and 4 % occupancy, in LCSA and LD, respectively) were deemed to be spatially rare positive probes. Eleven probes and four probes were spatially rare in LCSA and LD, respectively (see Table S2 for details). Eleven and five probes were ubiquitous in LCSA and LD, respectively (see Table S2 for details). These results were confirmed with a sequencing approach on one of the soils due to the possibility of microarray bias. These sequence data have been submitted to the EMBL-EBI database under accession number PRJEB14534 at European Nucleotide Archive (ENA), http://www.ebi.ac.uk/ena/data/view/PRJEB14534. Sequencing results on the LD soil, analyzed at the species level (97 % similarity), led to a bimodal frequency-occupancy plot (test chi2, $p < 2.2E\text{-}16$) with a core mode (Fig. 2). The core and satellite species represented 24.8 % (426 species) and 10.6 % (182 species), respectively (see Table S2 for details), among 1716 species found in this study. The distribution of satellite taxa in each aggregate along LD transect is presented in Fig. 3. More precisely, the names of satellite taxa in each aggregate are indicated in Table S2. The

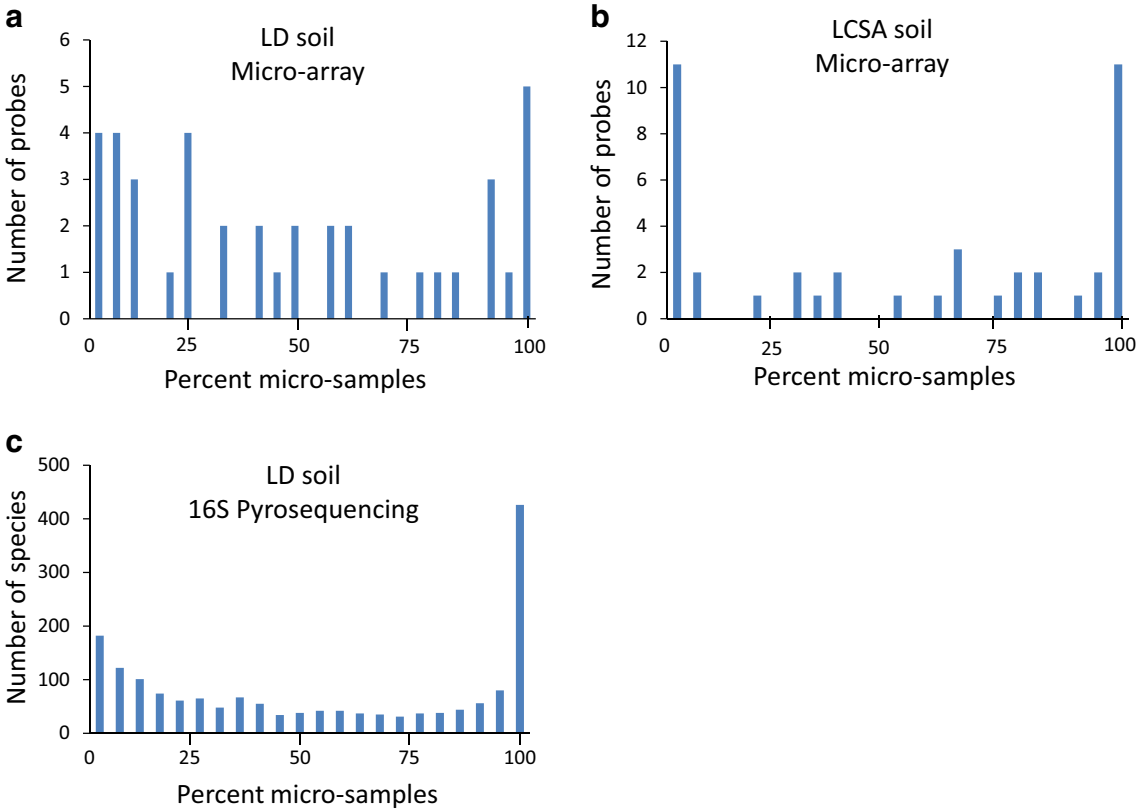

Fig. 2 Frequency-occupancy diagram using 16S microarray with low-taxonomic-level probes and pyrosequencing, (**a**) LD soil first sampling; (**b**) LCSA soil, both studied on about 20-mg soil samples and with 16S microarray; and (**c**) LD second sampling soil studied with 16S rRNA gene V4 variable region pyrosequencing, at the species level, on about 100-mg soil samples. The occupancy is the percent of microsamples in which a probe was positive or a species was found. They all showed significant bimodal distributions with $p < 0.025$, $p < 0.001$ and $p < 2.2E\text{-}16$, respectively

sequencing results presented a positive abundancy-occupancy relationship (Fig. 4). Average percent presence of satellite species (4.5 % occupancy) was 0.0014 %, and it was 0.22 % for core species (Fig. 4). Looking at individual species, 0.05 % of the core species presented more than 1 % presence on average in all microsamples, with a maximum of 3.98 % (*Sphingomonas* spp.), whereas all satellite species presented an average percent presence below 0.039 % in microsamples (data not shown). Besides *Sphingomonas* spp., among the most common core taxa in LD soil were *Acidobacterium* spp.,

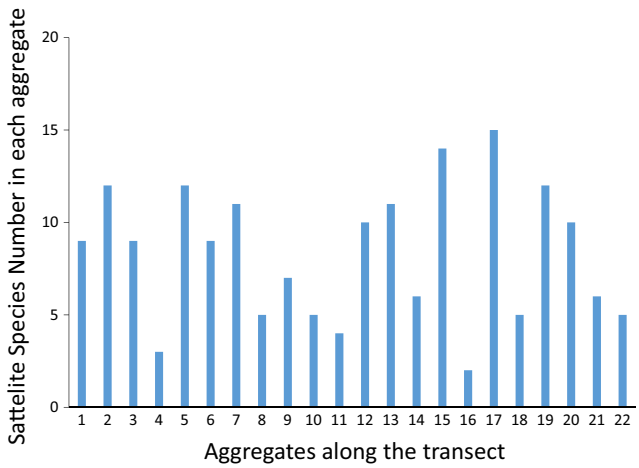

Fig. 3 Number of satellite taxa in each aggregate along LD transect as obtained with sequencing approach

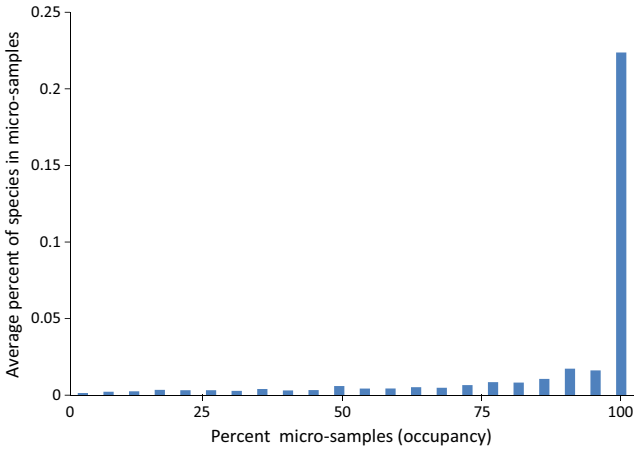

Fig. 4 Abundancy-occupancy diagram built with 16S rRNA gene V4 variable region pyrosequencing data on LD soil: average percentage of species present in microsamples for each occupancy value. The occupancy is the percent of microsamples in which a species was found. OTUs were defined at 97 % similarity

Candidatus Solibacter spp., *Conexibacter* spp., *Gemmatimonas* spp., *Rubrobacter* spp. and *Singulisphaera* spp.

The diversity of the two soils was compared using a PCA. The first two axes of the PCA ordination map explained 18 and 13 % of total variability among all microsamples and including all probes (Fig. 5). The two soils were separated (permutation test $p < 0.001$). The community structure patterns contributed to the separation. No spatial organization of diversity was observed in relation to geographical separation distance between microsamples (Mantel test p = NS for microarray and sequencing results). There was no relationship between community similarity and the spatial distance between microsamples. There was an important diversity at the microscale level. The PCA carried out using the 18 very high-taxonomic-level probes only, also separated the two soils (permutation test $p < 0.001$).

Discussion

The type of organism distribution is an important parameter in community ecology [29]. Some spatial distributions, such as the frequency-occupancy distributions and the abundancy-occupancy distributions, are central to community ecology studies. These distributions were discussed in several studies [29–32]. It has long been known that species can be divided into core and satellite species, as mentioned in Hanski's core and satellite theory [29–31]. The original core-satellite distinction in this theory referred to the spatial distribution (patch occupancies) of species where high numbers of species in the first and last occupancy class cause bimodal species number-occupancy distribution [29]. Core species are widespread and abundant, while satellite species are restricted in their distribution and are rare [30]. Distributions can be core mode or satellite mode [32]. In our study, the frequency-occupancy distribution observed in both soils (Fig. 2) was in agreement with Hanski's core and satellite theory [29, 33]. Such bimodal frequency-occupancy relationship has not yet been shown in bacteria at such fine spatial scales. In our study, the definition of satellite taxa as being in less than 4.3 and 4 % of the sampled locations in LCSA and LD, respectively, was linked to the sampling grain. The satellite taxa probes likely give an underestimation of the number of satellite taxa at the species level, as several low-taxonomic-level taxa, at species level for example, may potentially hybridize the probe. The core taxa probes may target true core taxa, or they may target the combining of hybridized taxa which, due to the possible relatively low probe specificity, could lead to the observation of a dispersed distribution. Importantly, the frequency-occupancy distribution obtained with microarray results and defining core and satellite taxa were confirmed with sequencing results in LD soil, and their relative abundance was quantified. The results confirmed the bimodal frequency-occupancy plot in LD, with a core mode with 24.7 % species present in all microsamples (100 % occupancy) and 10.6 % species present in one microsample only (4.5 % occupancy). The slightly larger sample size used for the sequencing approach compared to the microarray approach, and the finer resolution power of sequencing, probably increased the number of core species. Core and satellite taxa may change with changing environmental conditions. These satellite taxa might be different from taxa constituting the real rare biosphere defined at a larger scale in Sogin et al. [34]. Our sequencing results also indicated that the positive abundancy-occupancy relationship (Fig. 3), which is one of the most robust patterns in macroecology and is observed on different scales, also

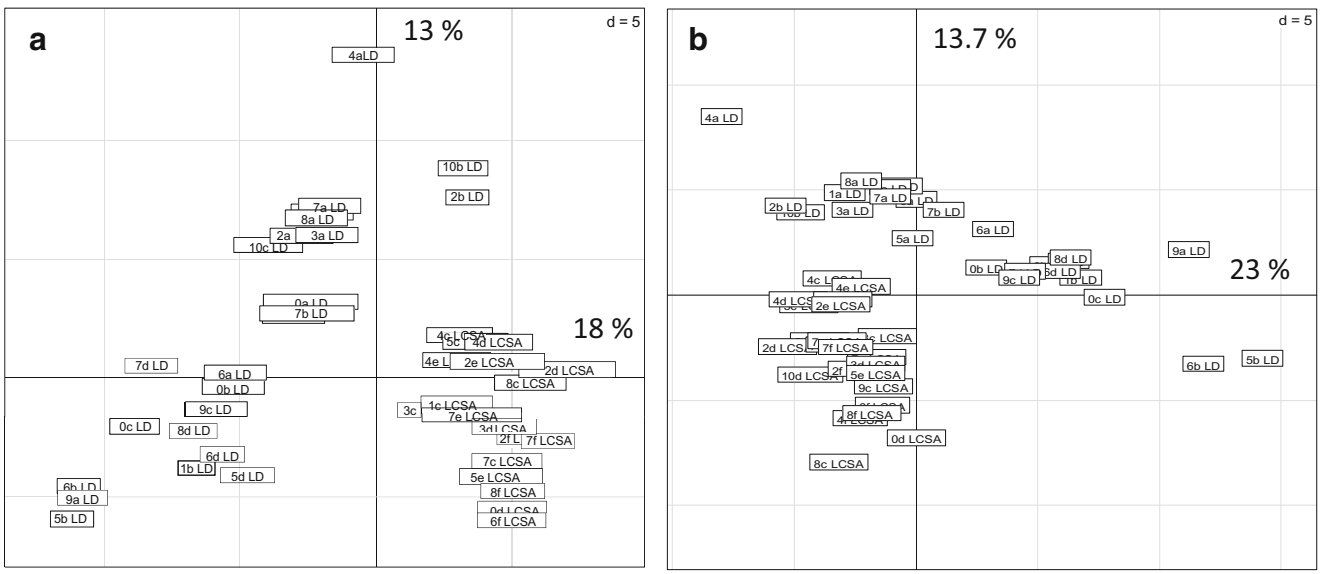

Fig. 5 PCA analysis separates LCSA and LD soils: **a** map of LCSA and LD samples using all probes and **b** map of LCSA and LD samples, using 18 highest-taxonomic-level probes only

applies to bacteria, at the small scale. Microarray results indicated the same tendency on both soils (data not shown). To our knowledge, the observation of different spatial distribution types at fine scale was never mentioned before for bacteria.

It was shown in this work, particularly in the sequencing results, that a very large number of taxa presented this high occupancy with distance lags between occupancy spots in the order of magnitude of 3 mm, as these taxa were found in contiguous microsamples. This is compatible with the distances obtained by Nunan et al. [35] for total bacteria. They demonstrated the presence of spatial patterns in the distribution of bacteria at the microscale, with ranges of spatial autocorrelation of 1 mm and below. In the case of satellite taxa, the fact that they were detected in one microsample only can suggest that the separation distance between them was over 5 cm, which is half the maximum distance between two microsamples in the studied clod. In a small-scale approach [19], a sequence analysis carried out on four adjacent 1-g sediment samples showed that 47.7 % OTUs were unique, which would suggest that there are numerous spatially rare members. A study of the PAH distribution at some site [36] suggested that the spatial variation in PAH degradation potential was present at a scale smaller than 9 mm. Other specialized bacterial groups, such as nitrifiers and 2,4-dichlorophenoxyacetic acid degraders, have been shown to be patchily distributed at the millimetre scale [17, 37]. Using denaturing gradient gel electrophoresis (DGGE) with small soil samples in a heavy metal-polluted soil [11], no band class was found to occur at every sampling locus from 40- to 90-cm depth and only one band class occurred in over 50 % of the samples. Seventy-five percent of the total band classes were found in 20 % or less of the 128 samples analyzed. As this experiment was carried out on samples ten times larger than in our work, it means that there was a large proportion of satellite taxa.

On the PCA sample map, LCSA and LD soil microsamples were separated, which is not surprising given the difference in soil characteristics, but remarkably, there were no misclassified microsamples. The amount of organic matter and soil structure characteristics were the most obvious differences between the two soils that were likely to have affected the resident bacterial communities. The physico-chemical characteristics in the microsamples of a same soil were probably similar as they similarly influenced microbial community structure, although it is well known that physico-chemical characteristics vary spatially. The community structure patterns contributed to the separation. In a work carried out at a larger scale [2], the microbial community composition was largely independent of geographic distance. It was suggested that microbial biogeography is controlled primarily by edaphic variables, mainly pH, and differs fundamentally from the biogeography of macroorganisms [38]. These parameters may also govern bacterial spatial organization at the microlandscape scale. Bacterial assemblages, as observed with the microarray approach, would thus reflect a very specific and rather stable combination of physico-chemical and biological parameters at the 3-mm scale. In line with this, it has been suggested that bacterial community composition obtained with DGGE analysis was semi-specific to the type of soil particles [39]. Soil separation based on bacterial diversity patterns was shown at the large spatial scale in soils [40], but this is the first instance of a complete separation at the microscale. The relationship between diversity and specific physico-chemical characteristics in microsamples was not studied in this work.

Furthermore, soils could be also discriminated using phyla-level probes. This high-taxonomic-level discriminative power would indicate that the community structure, as analyzed with diversity pattern, was rather specific in each microsample and also that high-taxonomic-level probe pattern could reflect ecological conditions [38, 41].

At the studied scale in this work, community structure was not spatially organized as shown with the Mantel test. There was no relationship between community similarity and the spatial distance between microsamples. There was an important diversity at the microscale level which corroborates the hypothesis of invasion/recess cycles based on some specific taxa study [10, 36]. In another work [11], substantial centimetre-scale spatial heterogeneity in community composition was observed among 150-mg samples of non-perturbated soil. PCR-DGGE analysis revealed that diverse communities were present in the soils with a random distribution of phylotypes throughout the sampling zone. The authors argue that this pattern implies either that the selective forces driving community structure vary at these spatial scales or that stochastic events such as immigration and extinction are important forces structuring microbial communities in spatially isolated sites, at these scales. It was shown in the present work that general ecological traits of bacterial community structure, i.e. core mode or satellite mode distribution, emerge from fine-scale approach. It can be hypothesized that these community structures, such that these shown with frequency-occupancy relationships, may sustain different types of biological soil functioning and thus represent integrative parameters of soil function. This approach should be developed to provide inferences of community structure types on soil function.

Acknowledgments For their involvement with sample processing, we thank Florent Gaudel and Jacqueline Haurat. Audrey Dubost helped with data processing. We thank Naoise Nunan for his valuable comments and suggestions on the manuscript.

This work was supported by the Specific Internal Program of the UMR 5557 Ecologie Microbienne.

Compliance with ethical standards We confirm that all of the reported work is original. The material has not been submitted for publication elsewhere.

Conflict of interest The authors declare that they have no conflicts of interest.

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