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The effect of sample size in studies of soil microbial community structure

Sanghoon Kang [1], Aaron L. Mills *

Laboratory of Microbial Ecology, Department of Environmental Sciences, 291 McCormick Road, P.O. Box 400123, University of Virginia, Charlottesville, VA 22904-4123, USA

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Abstract

Replicate soil samples of 0.01, 0.1, 0.25, 1.0 and 10.0 g were taken from a single, large, homogenized sample from a field maintained as continuous meadow. The samples were processed for direct enumeration of bacterial cells and community structure assays by DGGE analysis of PCR-amplified 16S-rDNA fragments from whole community extracts. The goal was to determine the sample size or size range that produced the most consistent results (i.e., mean values) and the lowest variance. Enumeration data were analyzed by ANOVA, and the community composition fingerprints were analyzed by discriminant analysis (DA). Acceptable results were obtained for sample sizes from 0.1 to 1.0 g for both enumeration and community fingerprinting, but the size that yielded the best results for both measures was 0.25 g. The results suggest that for well homogenized silt loam soils with moderate organic matter concentrations, this sample size should produce high quality consistent results. For soils that differ in organic concentrations or clay content, a reconnaissance survey similar to the present examination is recommended.

Keywords: Spatial scale; Heterogeneity; Bacteria; Community; Fungi; Sampling

1. Introduction

Soil contains the most complex and dynamic microbial assembly in the biosphere (Curtis et al., 2002). Different sizes of soil particles and aggregates in infinite combination result in a highly diverse physical environment with heterogeneity readily displayed at very fine scales (Grundmann and Debouzie, 2000; Ranjard and Richaume, 2001; Heijnen et al., 1993), at intermediate (e.g., cm) scales (Franklin et al., 2002; Franklin and Mills, 2003), and at field and landscape scales (Green et al., 2004). At the smaller scales, heterogeneity and spatial structure can mask signals that are of interest at the scales of human interest, which are most often plot scales when studying ecological processes. For example, anaerobic denitrification occurs under apparently aerobic conditions when there are anaerobic microsites available (Hutchinson and Mosier, 1979). The diversity of physical characteristics of soil associate with aggregation at small scales means that soil can contain a large diversity of microorganisms in close proximity, and the chemical composition of soil is also highly heterogeneous in both vertical and horizontal dimensions (Dighton et al., 1997; Gallardo et al.,

* Corresponding author. Tel.: +1 434 924 0564; fax: +1 434 982 2137.
E-mail address: amills@virginia.edu (A.L. Mills).
[1] Current address: Rm 286, Bldg 1505, MS 6036, PO Box 2008, Functional Genomics and Microbial Ecology Group, Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831 USA.

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2000; Bird et al., 2002; Yang et al., 1995). There are, simply, a very large number of niches contained within tiny spaces.

Sampling strategy in soil microbial ecology is, therefore, critical to the appropriate characterization of the field situation (Litchfield et al., 1975). In an attempt to account for heterogeneity, large samples, or even multiple samples are taken, pooled, and then homogenized to produce a final sample that is "homogeneous" and from which a smaller sample can be taken for analysis. That sample is thought to be representative of the entire mixed sample. Among many issues related to sampling, including how many samples and by what distance they should be separated, one of the most fundamental and critical, but often ignored is the size of the sample taken from the homogenized mixture. Indeed the high sensitivity and precision of modern molecular methods begs the question of whether a soil sample can be successfully homogenized at all. Economic parsimony suggests that a few small samples would benefit collection, storage, and analysis in terms of both effort and cost (Terry et al., 1981). However, the residual heterogeneity within a well mixed sample needs to be captured if the sub-sample is to adequately represent the environment from which the soil was originally extracted, and that dictates a larger sub-sample. Existence of patches, virtually guaranteed in the distribution of soil microbial communities, invokes the rule that sample sizes should be selected so that among-replicate variance from variable sample sizes is minimized (Green, 1979). Sample size had a significant effect on the measurement of soil microbial biomass in that standardized biomass was significantly different among different sample sizes (Christie and Beattie, 1987). More recent studies on genetic fingerprinting techniques for microbial community structures also indicate that there is a clear beneficial effect of increasing sample size in terms of better representation of the environment from which the samples were collected (Ranjard et al., 2003; Ellingsøe and Johnsen, 2002).

Best approaches for obtaining statistically acceptable data with the lowest number of replicates (that is, to obtain the greatest overall reduction in total variance) involve replicate sampling at the level with the highest variance (Sokal and Rohlf, 1995; Snedecor and Cochran, 1989). Experience has shown that the highest variance is mostly associated with environmental variables as opposed to analytical variables and the variables with the largest variance components are those which are captured by sampling at higher levels (Kaper et al., 1978). For example, strong designs for comparing two fields might include replicate samples randomly collected from around each field, homogenization of each sample, then withdrawal of a single subsample for analysis from each of the homogenized samples.

In this study, we determined appropriate sample sizes for the best representation of the microbial abundance and community structure in a homogenized sample. Means and variances of abundance data were compared among different sample sizes using one-way ANOVA and Hartley's F_{max} test. Centroids were used for comparisons of microbial community structures on discriminant function (DF) plot.

2. Materials and methods

2.1. Study site and sampling

The study was conducted on a field of approximately 14 ha at Blandy Experimental Farm (BEF) in the northern Shenandoah Valley of Clarke County, Virginia (78.06° W, 39.06 °N) where a meadow comprising native temperate tallgrass species, as a part of the collection of the Orland E. White Arboretum, was being established. The soil sampled for this study was the Poplimento silt loam (Fine, mixed, mesic Ultic Hapludult) which underlies a near-monoculture of *Andropogon gerardii* (big bluestem). Approximately 1 kg of soil was collected with a clean shovel and placed in a sterile bag in June 2003. The soil was stored frozen at −80 °C at the field facility and transported on ice to the lab. Soil sample was thawed and mixed and broken up in the bag by hand while wearing sterile gloves. The mixed soil was sieved (2.5 mm), and 3 replicates for each of the analyses for each of 5 different amounts (0.01, 0.1, 0.25, 1.0 and 10.0 g) were separated and processed.

2.2. Enumeration

Total abundance of soil microorganisms was measured using epifluorescent-microscopic direct counting with acridine orange staining (Bottomley, 1994). A set of 3 replicates for each of the test sample sizes was added to each of 3 10-mL portions of distilled H_2O, except that the 10-g-sized samples were prepared in 100 mL of distilled H_2O. 1 mL of each soil suspension was diluted with 9 mL of 10% formaldehyde that had been passed through a 0.2-μm pore diameter filter. 0.1% (w/v) Acridine Orange solution was prepared with 2% formaldehyde and filtered through a 0.2-μm filter. After staining, cells were counted under epifluorescent illumination. A total of 10 fields or 200 cells were counted (whichever was reached first), except that

never were fewer than 5 fields counted, regardless of the number of cells.

2.3. DNA extraction

Microbial community DNA was extracted using the UltraClean™ Mega Soil DNA isolation kit (MoBio Laboratories, Inc., Solana Beach, CA) for 10-g samples, and the UltraClean™ Soil DNA isolation kit was used for all other samples. The extraction protocol was modified with the "alternative lysis method" as a replacement for the mechanical lysis method. To determine the DNA extraction yield, final concentrations of DNA were measured with the PicoGreen® dsDNA Quantification kit (Molecular Probes, Eugene, OR).

2.4. Microbial community characterization

Soil microbial community structure was characterized by denaturing gradient gel electrophoresis (DGGE) profiling 16S-rDNA from whole-community DNA extracts. The 16S-rDNA fragments were obtained by the amplification of 20 to 40 ng of template DNA with a universal primer set (P63f and P518r) for soil bacteria (Table 1) (El Fantroussi et al., 1999). A GC clamp of 40 bases was added to prevent complete separation of double helix DNA and subsequent loss from the gel (Sheffield et al., 1989). PCR, DGGE, and post-DGGE analysis were performed using the procedures described by Kang and Mills (2004). Two PCR additives were used in the mixture for clear and accurate results: 40 μg BSA and 5% DMSO (dimethylsulfoxide) in each reaction (Hengen, 1997; Mahbubani and Bej, 1994; Smith et al., 1990; Muyzer and Smalla, 1998).

The fungal community was characterized by using the primers FF390 and FR1 (Vainio and Hantula, 2000) to amplify fungal SSU rDNA (Table 1). The PCR reaction mixture contained 0.5 μM primers, 2.5 mM $MgCl_2$, 200 μM dNTP mixture, PCR buffer, and two units of AmpliTaq® DNA polymerase. There were also two PCR addictives in the reaction mixture to improve the results: 40 μg BSA and 10% glycerol per reaction (Hengen, 1997; Mahbubani and Bej, 1994; Smith et al., 1990; Muyzer and Smalla, 1998). Amplification was performed with an initial denaturation of 8 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 45 s at 50 °C, and 2 min at 72 °C. After the 30 amplification cycles, a final elongation of 10 min was included at 72 °C. Amplified 18S-rDNA fragments were separated in a polyacrylamide gel with a denaturing gradient of 40 to 55% of urea-formamide denaturant. The electrophoretic separation was run for 4 h at 175 V, and product visualization was the same as the bacterial analysis. The protocols suggested by Vanio and Hantula (2000) used a much longer running time (16~18 h) at lower voltages (50~75 V), but preliminary tests of those conditions did not produce improved results. The protocols for fungal community PCR and acrylamide gel electrophoresis were empirically optimized based on those presented by Kowalchuk et al. (2003) and Vainio and Hantula (2000).

Table 1
Primers used for DGGE analysis of bacterial and fungal community structure

Name	Sequence	Use
P63f	5′-GC clamp-CAGGCCTAACACATGCAAGTC-3′	Bacteria
P518r	5′-ATTACCGCGGCTGGCTGG-3′	
FF390	5′-CGATAACGAACGAGACCT-3′	Fungi
FR1	5′-GC clamp-AICCATTCAATCGGTAIT-3′	

2.5. Statistical analysis

One-way ANOVA with post hoc separation of means using Ryan's Q test (Sokal and Rohlf, 1995) was used to assess differences in means among different sample sizes for both cultural and direct enumeration and DNA extraction yield. Centroids of bacterial and fungal community structures generated from discriminant analysis (DA) were also compared by ANOVA using SAS 8.2 (SAS Institute Inc., Cary, NC). All tests were conducted at $\alpha = 0.05$.

Quantitative determination of homogeneity of variances among measurements was desired for selecting sample size with the least variance. Hartley's F_{max} test was used in this case, because the other commonly used test (Bartlett's test) is more sensitive to non-normality of the data than heterogeneity of variance. Hartley's F_{max} test is based on F_{max} statistic of maximum and minimum variance among groups.

The band patterns on the acrylamide gel serve as fingerprints of the microbial community structure. Each position which contained a band in at least one sample was considered a unit character, and the presence or absence of a band in a given sample was recorded in a binary data format as '1' for a band and '0' for no band. The binary data were fed into analyses of microbial community structure including discriminant analysis (DA) (SPSS v. 11, SPSS Inc., Chicago, IL). The approach was used mainly for graphical representation of microbial community structures on discriminant function plots and for validation of sample identities determined by their traits.

3. Results

3.1. Abundance

The effect of sample size on measured abundance of bacterial cells was significant (ANOVA, $p=0.0001$), although the difference between the lowest recovery and the highest was only a factor of about 2.2 (Fig. 1). Pairwise comparison of the mean abundance for each sample size using Ryan's Q test separated the 10.0-g sample from all of the smaller sample sizes. Exclusion of the 10.0-g samples from the analysis did not result in acceptance of the null hypothesis ($p=0.0249$), but when the results for the 0.01-g samples were excluded from the reduced data set, the null hypothesis was accepted ($p=0.5925$). Hartley's F_{max} test on all sample sizes rejected null hypothesis of equal variance ($F_{max}=19.13$; $F_{max[5,5]}=16.3$). Exclusion of the 10.0-g sample data resulted in acceptance of the null hypothesis that variances of sample size between 0.01 and 1.0 g are homogeneous ($F_{max}=1.98$).

3.2. DNA extraction yield

The effect of sample size on the yield of extracted DNA from each sample (Fig. 2) was significant ($p=0.0004$), and the variance was not equal among replicates the size categories ($F_{max}=317.86$; $F_{max[5,2]}=202$). Pairwise comparison of the mean abundance using Ryan's Q test separated both the 10.0- and 0.01-g samples from rest of sample sizes. For samples of 0.1, 0.25 and 1.0 g, means were equal ($p=0.1322$) and variances were homogeneous ($F_{max}=14.03$).

3.3. Microbial community structure

Both bacterial and fungal community structure analysis indicated major differences in the communities recovered from different sample sizes (Fig. 3). Centroids of all sample sizes showed a significant effect in both DFs ($p<0.0001$ for DF1 and $p=0.0001$ for DF2) for the bacterial communities recovered. The larger sample sizes (0.25~10.0 g) yielded similar bacterial community structures, although pairwise comparisons using Ryan's Q test on the more closely clustered samples indicated that there were some significant differences among the three samples on both axes. Variances among replicates were not significantly different from each other in both axes ($F_{max}=9.99$, 5.36 and 4.86 for DF1, DF2 and sum of two, respectively; $F_{max[5,2]}=202$). Overall matching of original and calculated memberships was 86.7% (2 mismatches out of 15). Clustering of fungal communities was less than for the bacteria. The effect of sample size on the community structure viewing all the samples was seen strongly on both axes ($p<0.0001$ for DF1 and $p=0.0001$ for DF2). Sample sizes of 0.1 and 0.25 g were best clustered, and the 1.0-g sample size might be included in the group as well based on its position on the DF1 axis (0.1, 0.25 and 10.0 g were clustered on the DF2 axis). As for the bacteria, variances among replicates of fungal DNA were not significantly different from

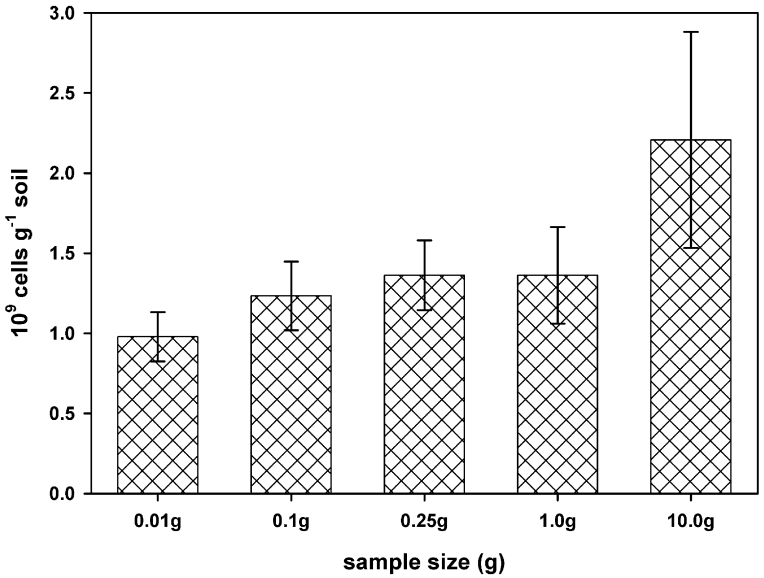

Fig. 1. Total microbial abundance in different sample sizes of soil. Error bars represent 1 standard deviation.

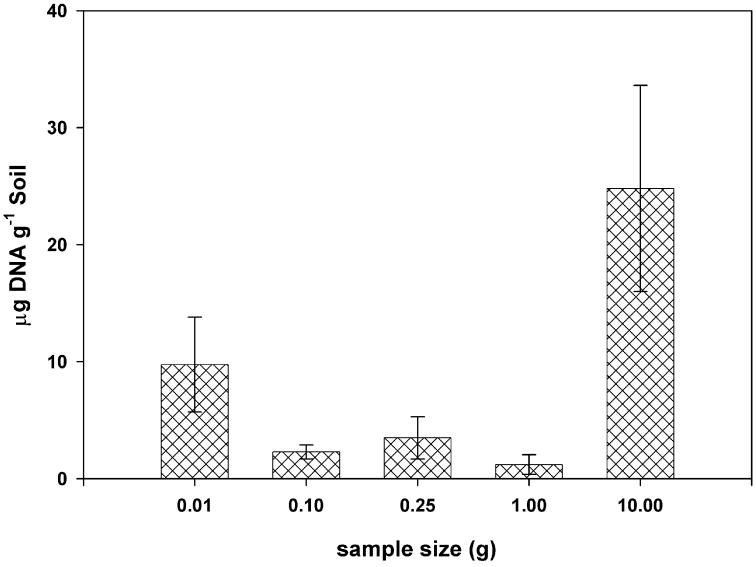

Fig. 2. DNA extraction yield from different sample sizes. Error bars represent 1 standard deviation.

each other in both axes (F_{max}=5.35, 14.42 and 2.04 for DF1, DF2 and sum of the two, respectively; $F_{max[5,2]}$=202). Overall matching of membership was 93.3% (one mismatch out of 15). The overall patterns of separation of samples for each community type (bacteria vs. fungi) on discriminant function space were quite similar even though the identity of the community within the individual centroids was different, and the scales at which the centroids were most similar to one another differed between the bacterial and fungal communities.

4. Discussion

The present work demonstrates that a soil sample can, indeed, be homogenized adequately to allow subsamples taken for analysis to adequately capture the residual heterogeneity that exists at scales representing the inter- and intra-aggregate variability. Obviously, the degree of homogenization is important, as is the final aggregate size. It seems reasonable to assume that a few of the residual aggregates should be included in the subsample used for analysis, and that suggests that the soil should be divided into very fine units. Assuming a bulk density of 1.2, a 0.25 g sample occupies about 0.21 cm^3, or a cube of 0.59 cm on each side.

Microbial measurements for the present study were analyzed systematically and formal statistical analyses were employed to determine the amount of sample to be withdrawn from a homogenized soil mixture that best represents the microbial community. All microbial measurements showed some difference among sample sizes examined, but a consistent pattern of acceptable results was obtained for the range of sample sizes between 0.1 and 1.0 g, based on the greatest reduction in between-subsample variance and consistency of mean values among the different sample sizes. Both sample size and distance between sampling locations are critical for proper interpretation of the results from highly heterogeneous soil systems (Grundmann and Gourbière, 1999), but when considering the issues in a sampling strategy for soil microbiogy, the appropriate size of the sample collected for analysis is often ignored (Ranjard and Richaume, 2001).

4.1. Enumeration

Abundance is expressed as the average of cell counts from the highly heterogeneous microbial habitats in the soil. Therefore, small sample sizes are expected to have lower abundance per sample, with occasional very high abundance leading to higher variance. Larger sample sizes tend to have more counts, but because the samples integrate more of the original material, they should have lower variance (Grundmann and Gourbière, 1999). The most suitable sample size was determined statistically with regards to both mean and variance because sample sizes that produced both the most consistent counts (across sample sizes) and smallest variance were accepted as the best representation of the overall abundance.

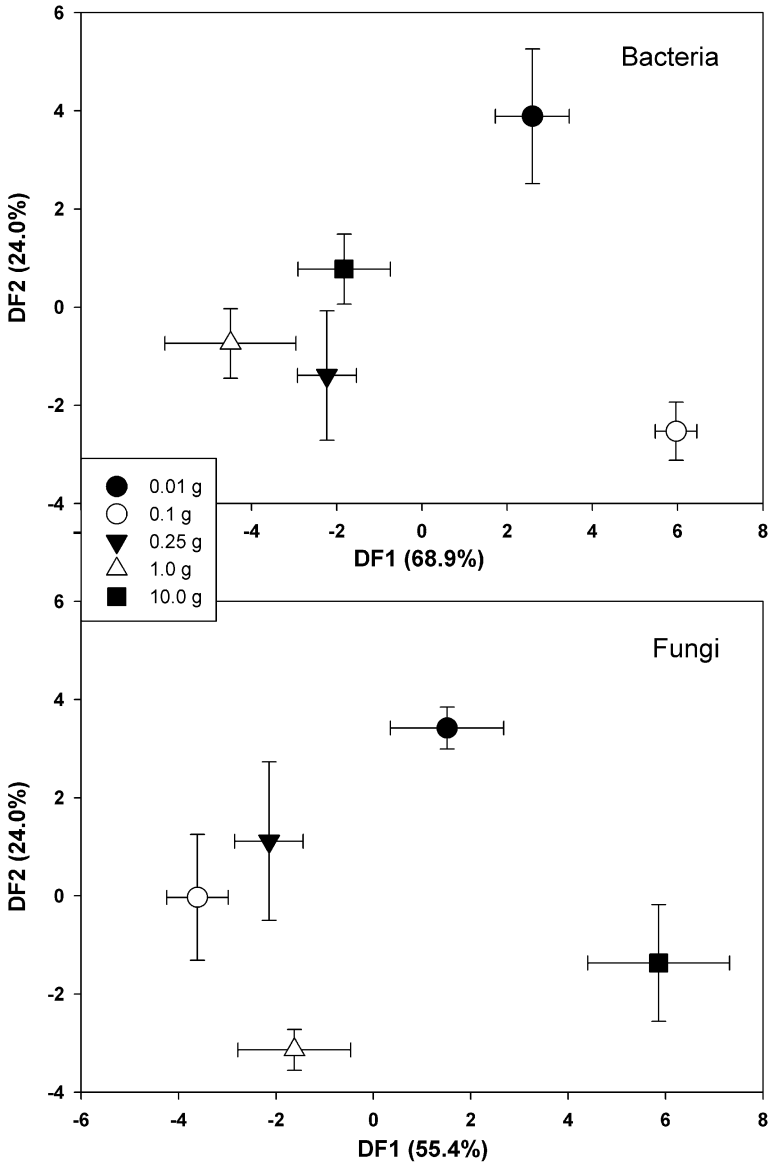

Fig. 3. Discriminant function plot of bacterial and fungal community structure using first two functions. Major portions of the variances were explained by the first two functions for both the bacterial and fungal community (92.9% and 79.4%, respectively). Points indicate centroids that are means of triplicates and error bars represent 1 standard deviation.

4.2. Microbial community structure

Due to the limited extraction efficiency of the UltraClean™ soil DNA isolation kit (MoBio Laboratories) for these soil samples, there were some differences in the extraction procedures for different sample sizes; two tubes were used for the1.0-g sample followed by pooling of the extract, whereas the UltraClean™ Mega Soil DNA isolation kit was used for the 10.0-g sample. The DNA yields ranged between 0.5–32.7 μg DNA g^{-1} of soil, a value in agreement with information provided by the manufacturer (Braid et al., 1999). Yields from the UltraClean™ kit were 0.5–14.1 μg g^{-1} soil, but the mean values for the 0.1, 0.25, and 1.0 g samples ranged from 2.1 to 5.3 μg DNA g^{-1} soil and were not significantly different, despite the fact that the 1.0-g sample was obtained with a modified protocol from the others. The recovery from the UltraClean™ Mega kit (24.8 ± 8.8 μg g^{-1} soil) was significantly higher than the other samples, and it is not known how that difference related to the different extraction kit used. Overall, the yields were about the same or slightly lower than

most of the reported yields for several different soil DNA-extraction protocols (Zhou et al., 1996; Cho et al., 1996; Blagodatskaya et al., 2003; Miller et al., 1999). From this we conclude that the use of samples of 0.1 or 0.25 g each with the manufacturer's recommended procedure yields indistinguishable differences.

Centroids and associated among-replicate variances from DA were used to determine the sample size for the best representation of the microbial community structure (Fig. 3). As was done for means and variances from the enumeration and DNA-yield data, centroids (means) and variances were tested using ANOVA and Hartley's F_{max} test. Clustering on 2-D DF plot and statistical tests on each axis was used to identify ranges of sample sizes for consistent analysis of microbial community structure. The optimal ranges were slightly different between the two microbial components of the soil community; the bacterial community structures were clustered in the larger sample size (0.25–10.0 g), and the fungal community structures were clustered in the intermediate sample sizes (0.1–0.25 g). It is considered that larger samples, because of their averaging effect, are often more suitable for generally describing the overall communities, as most available methods can only detect the several most abundant microorganisms in the communities, whereas smaller samples are more heterogeneous and are therefore better for use for a diversity inventory when the samples are handled separately (Grundmann and Gourbière, 1999). The overall patterns of tighter clustering in larger sample sizes; therefore, make sense, but the unique location of the 10.0-g sample for the fungal community is difficult to interpret.

Surprisingly, there have not been extensive studies of sample size effects on microbial community analysis, although two recent papers approached the topic with some rigorous methodology. Ranjard et al. (2003) evaluated the effect of sample size on microbial community study using ARISA (automated ribosomal intergenic spacer analysis) from three agricultural fields in France. Their sample sizes were 0.125, 0.25, 0.5, 1.0, 2.0, and 4.0 g. The DNA was extracted using the "bead beating" method, and the results were not consistent among fields. Their PC (principal component) plot was drawn to include all the individual samples (no averaging), and they concluded that the bacterial community structures represented were not influenced by sample sizes. Fungal communities, on the other hand, were very variable in sample sizes smaller than 1.0 g. Ellingsøe and Johnsen (2002) published the first paper dealing with the effect of sample size on the recovered microbial community structure. They subsampled 0.01, 0.1, 1.0 and 10.0 g from soil collected from a virgin forest soil in Denmark. Although they did not perform any formal statistical analysis on the polyphasic DGGE patterns of the bacterial community they obtained, they concluded bacterial community structures of larger sample sizes (1.0 and 10.0 g) were very similar among replicates. Non-quantitative evaluation of differences in patterns on gels is a common, but dangerous, practice among microbial ecologists. The ease of running ordination analyses to obtain quantitative interpretations of the band patterns is simple and rapid, and reliance on visual interpretation should be avoided.

The extraction procedure used for obtaining soil DNA can influence both DNA yields and the qualitative results of subsequent analysis (e.g., by DGGE) (de Lipthay et al., 2004; Niemi et al., 2001). de Lipthay et al. (2004) compared three different methods of physical cell disruption for DNA extraction; sonic disruption, grinding–freezing–thawing, and bead beating (FastDNA® Spin Kit for Soil, Bio101, Vista, CA) for six different Danish soils. Niemi et al. (2001) compared five different soil DNA extraction procedures, including the commercial kit from Mo Bio Laboratories, on rhizosphere soil. DNA yields were not significantly different among the different extraction methods in either study, except for one soil from Denmark in the work by de Lipthay et al. (de Lipthay et al., 2004). Both studies concluded that the results of DGGE, including DNA band patterns, number, and intensity of bands were substantially different among different extraction methods, although again, neither study used any formal statistical evaluations upon which to base their conclusions. In the study of Niemi et al. (2001), DNA extracted with the MoBio kit yielded the broadest and the most intense band patterns among the five extraction methods tested. Therefore, we now know that both sample size and method of DNA extraction influence the analysis of soil microbial community structure, and it is important to be very consistent in all the preparation steps for such studies. In addition, it is also necessary to note the preparation steps along with the setting of the studies when comparing results with those from other studies. Otherwise, the results may be incomparable because differences from sample sizes or extraction methods could create artifacts in the results that are not related to real differences in the communities.

Finally, it is essential to consider how this information might be used in a field sampling design. The goal of any sampling design is to reduce the overall variance with the smallest increase in sample numbers. This can

often be accomplished by collecting several samples throughout the area under examination, then analyzing a single portion from each of those samples (Green, 1979; Sokal and Rohlf, 1995; Kaper et al., 1978). Such an approach maximizes the number of independent samples taken from a specific sampling area, and therefore maximizes the degrees of freedom used for hypothesis testing.

4.3. Suggestions

The purpose of this study was to find the (sub)sample size to be drawn from a homogenized soil sample that best represents the microbial community. Therefore, tightly clustered centroids, i.e., smaller variance, was the target. The sample sizes determined in the current study were for internal comparisons of the general structure of the soil microbial community. Sample sizes excluded from the selected ranges were more diverse in the measurements and inconsistent among replicates, and that suggests higher chances of finding rare microorganisms. Molecular approaches to microbial ecology are very popular; however, community profiling is limited to visualizing only the most dominant types of microorganism in the community inhabiting the particular sample (Chandler et al., 1997). To capture larger numbers of microbial types, therefore, it seems more appropriate to use larger numbers of very small samples.

While there were some differences in the ranges of optimal sizes for the different variables examined, there was a common overlap among all the variables in 0.25-g samples. Along with the fact that optimal sample size ranges should be chosen based on the technique to be used, different environmental settings (Ranjard et al., 2003) and different DNA extraction methods can also generate inconsistent results. We would expect, for example, that soils richer in clay or containing high or low concentrations of organic matter might yield results very different from those obtained with the Poplimento silt loam used here. Certainly, the ease and efficacy of homogenization could be very different. Therefore, one should consider performing a reconnaissance study to determine the best range of sample sizes at the beginning of any project, especially when researchers are examining unfamiliar locations.

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