The dilution procedure to manipulate microbial biodiversity in terrestrial systems revisited

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Running title: Manipulation of microbial biodiversity

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ABSTRACT

The importance of soil microbial diversity for the functioning of terrestrial ecosystems is hard to assess experimentally. An often used approach is the so-called dilution method. This method is based on the assumption that the biodiversity of the microbial community is reduced after dilution. Although this method is generally accepted, little is known about the fate of both rare and dominant species in communities subjected to the dilution procedure and how the communities develop in soil after inoculation. To study these two questions, serial dilutions of a soil suspension were made, and re-inoculated into the original soil previously sterilized by γ-irradiation. We determined the structure of the microbial communities in the suspensions and the inoculated soils using 454-pyrosequencing of 16S rRNA gene. Upon dilution, several diversity indices all showed that, indeed, the community diversity reduced dramatically. The structure of microbial community was changed after 8 weeks incubation in soil, indicating the importance of soil related mechanisms operating in the assembly of a variety of communities that were based on inoculated communities formed after dilution. We found unique OTUs even in the highest dilution both in the suspensions and in the incubated soil samples, which illustrates that the dilution method does not allow for firm conclusions on the separation of rare *versus* dominant members of the original soil community. In summary, our results did show that the dilution procedure may be used to reduce the microbial community diversity, but the regrown community is less predictable and more complex than previously thought.

INTRODUCTION

The significance of biodiversity for terrestrial ecosystem processes continues to be a matter of much debate (1-3). Compared to the importance of plants and animals, the role of microbial biodiversity is still poorly understood. This lack of knowledge is of great concern as soil microbes, particularly bacteria, represent the majority of biodiversity in terrestrial ecosystems, and are known to carry out numerous essential ecosystem functions, including nutrient cycling and facilitating plant nutrition (4).

The biggest obstacle for a better understanding of the importance of microbial biodiversity for the functioning of terrestrial ecosystems is the lack of sound experimental approaches to make directed and predictable changes in the diversity of microbial communities in soil. One of the most interesting approaches so far is the so-called dilution method. This method involves the inoculation of sterilized soils with more or less diluted inocula derived from suspensions of the same soil (4-14). However, the above mentioned studies were often limited in sampling depth and extent and focused only on the structure of the microbial community after regrowth in the soil. As a consequence they do not provide information about the original community from which the different communities after incubation originate and can therefore not test the assumption that dilution mainly influences the diversity through reduction of the number of rare species. In reality rare species in the original community may have become common after incubation or vice versa.

High-throughput next generation sequencing technologies have led researchers to deeper sampling depths by providing large numbers of reads with cost-effective means to detect microbial phylogenetic diversity (15). This has provided new insights

into the details of microbial communities in natural ecosystems (16-18) and human bodies (19). One of the exciting possibilities provided by this technology is to estimate accurately the assembly processes and structure of microbial communities including the long tail of rare microbes, which may lead to a better understanding of their ecology and functioning in soil.

The major aim of this study was to determine the changes and the associated variation in the composition of a soil microbial community brought about by inoculation of serial dilutions of suspensions of that soil and to detect how the microbial community structure develops during regrowth in soil. This also allows for the investigation of the possibilities to separate rare species from the abundant members and for a better understanding of the behavior of microbial phyla/species after inoculation in soil as well as of the selective pressure of the soil environment on the assembly of microbial communities. We addressed three basic questions: 1) does the dilution procedure reduce the diversity of the microbial community after inoculation and subsequent incubation of soil suspensions in soil, 2) is the dilution procedure effective in separating rare and abundant species so to allow for the assessment of their specific roles? 2) does the composition of the microbial community change during incubation in soil? In order to answer these questions, we established a range of microbial communities through the inoculation of serial dilutions of microbial suspensions from non-sterilized soil samples into the same soil after sterilization.

MATERIALS AND METHODS

Soil sampling and treatment.

30 l soil was collected at a depth of around 15 cm from dune sandy soil in Meijendel, The Netherlands. The soil was sieved , homogenized and aliquots of 500 g were stored in plastic bags. The bags containing soil were γ-irradiation sterilized (> 25 kGray, Isotron, Ede, the Netherlands). One bag of soil was kept separately to serve as inoculum. Sterility was checked by spreading 0.5 g sterilized soil from the inoculum-bag onto nutrient agar. No bacterial growth on agar plates for 6 replicates was observed in the sterilized soil samples. A subsample of the fresh soil was taken to determine soil moisture (24 h, 105℃).

Soil suspensions for inoculation were made by mixing 20 g fresh soil and 190 ml sterilized demi-water with a blender for 2 minutes. This procedure was repeated 3 times and in between the blender was cooled down on ice for 2 minutes. This was called the 10^{-1} dilution. By mixing with sterilized demi-water serial dilutions of 10^{-6} and 10^{-9} were made. Subsequently, 25 ml of the respective dilutions were added to 500 g of soil in the bags and additional sterilized water was given to bring the moisture level of the inoculated soil at around 20%, which is roughly similar to the average level under the prevailing climatic conditions at the side from where the soil was taken. In total 36 bags of soil (6 samples × 3 dilution serials × 2 replicates) were used. The remaining suspensions were centrifuged at 3000 g for 10 min at 4℃ and the pellets were stored at -20℃ for further analysis. After inoculation, the soil bags were incubated at 20℃ using sterilized cotton plug caps to ensure gas exchange. The soils were turned regularly over once a week to ensure even microbial growth. The aim was to reach similar microbial abundances in the different dilution treatments. After 8 weeks of incubation, under laminar flow conditions, soil samples were taken to determine the microbial abundance in all treatments by real time PCR using Eub 338

(20) and Eub 518 primer (21) set for 16S rRNA gene. PCR reactions were run on a Rotor-Gene 3000 (Qiagen) and started with 15 min at 95℃, followed by 40 amplification cycles each of 95℃ for 60 s, 53℃ 50 sec and 72℃ 60 sec. A subsample of soil from each bag was stored at -20℃ for further analysis. Bacterial abundance was similar for all dilution treatments after 8 weeks of incubation as determined by qPCR (Fig. S1). We also measured fungal abundance, but the fungal biomass appeared to be rather low in these soil samples and because of the difficulties in assessing fungal abundance by qPCR due to heterogeneity in ribosomal operon number per fungal species/phylum, we decided to ignore the fungal community in the rest of our analyses.

DNA extraction, PCR reaction and 16S rRNA gene fragment pyrosequencing.

Total DNA was extracted from the soil suspensions and incubated soil to determine the composition of the respective microbial communities by 454-pyrosequencing. DNA was extracted using the MoBio Power Soil Extraction Kit according to the supplier's manual (MO BIO Laboratories, Carlsbad, CA, USA). Total DNA concentration was qualified on ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE). For DNA concentrations below 5 ng/µl, *i.e.* 5 of 10^{-6} and 4 of 10^{-9} soil suspension samples, nested PCR was performed. The general bacterial primer 27F and 1492R (20)were used for the first amplification, and then 2 µl of the amplified products from the first round was used as template for the second round PCR using barcoded primers 515F and 806R (22). 5 ng/µl of DNA/sample of the diluted samples was used as template for the first round of nested PCR with the PCR program of 95℃ for 5 min followed by 25 cycles each of 95℃ for 30 s, 55℃ 1 min and 72℃ 10 min. For PCR reactions using barcoded primers were performed using 5

μM of each forward (515F) and reverse (806R) primers, 5 mM dNTPs (Invitrogen, Carlsbad, CA), 1 unit of *Taq* polymerase (Roche, Indianapolis, IN), and 5 ng/μl of sample DNA as the template in a total volume of 25 μl. The PCR conditions for the barcoded primer were similar to the first PCR round except for 25 cycles with 52℃ annealing temperature. All PCR reactions were performed with negative control to test for contamination. PCR products of each subsample from the barcoded primers were generated in six replicates and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). Equimolar purified PCR products were mixed and sequenced using Roche Genome Sequencer FLX Titanium 454 sequencing platform (Macrogen, Seoul, Korea).

Sequence analysis.

The raw data were processed using the QIIME v.1.6.0 pipeline (23). Low-quality sequences less than 150 bp in length or with average quality score of less than 25 were removed. After denoising the sequences using Denoiser 0.91 (24), and checking for chimeras using USEARCH (25). Operational Taxonomic Units (OTUs) were identified using the UCLUST 1.2.21 algorithm (26) with a phylotype defined at the 97% sequence similarity level. The resulting OTUs were aligned against the Ribosomal Database Project database (27).

Alpha diversity calculation was performed based on the raw (non-rarefied) OTU table since alpha diversity is highly dependent on the number of singletons in a sample (28). Chao1 richness, Simpson, Shannon and evenness diversity indices were determined with the “vegan” package (29) in R (The R Foundation for Statistical Computing). The percentage of coverage was calculated by Good's method using the

formula % = [1-(n/N)] × 100, where n means the number of phylotypes represented by singletons and N is the total number of sequences (30). Good's method equation gives an idea of the coverage of an entire sampled community by the data obtained from a limited data set.

The OTU table was rarefied to 1535 reads by "single rarefaction" QIIME script since this number was the lowest number of reads for all samples. To compare the communities from the different dilution treatments, principal coordinate analysis was applied to reduce the dimensionality of the distance matrices. This Partial Least Square - Discriminant analysis (PLS-DA) was performed using SIMCA-P software (version 12.0 Umetrics, Umeå, Sweden) to visualize the variation in microbial communities. Scaling was based on the Pareto method. The models were validated by default validation method with permutation test through 20 applications and CV-ANOVA.

RESULTS

Effect of dilution and incubation on bacterial community diversity.

Alpha diversity was significantly reduced by increasing dilution both in the soil suspensions and in incubated soil samples (Table 1, ANOVA soil suspensions: S.obs: $F = 48.51$; $P = 0.000$; S.chao1: $F = 96.675$; $P = 0.000$; Shannon: $F = 54.055$; $P = 0.000$; Simpson: $F = 3.963$; $P = 0.043$; Evenness: $F = 12.530$; $P = 0.001$, ANOVA incubated soil samples: S.obs: $F = 38.376$; $P = 0.000$; S.chao1: $F = 44.699$; $P = 0.000$; Shannon: $F = 395.076$; $P = 0.000$; Simpson: $F = 94.289$; $P = 0.000$; Evenness: $F = 119.426$; $P = 0.000$]. Although Good's estimator coverage did not level off even for

199 the 10^{-1} dilution of the soil suspensions and incubated soil samples, it increased with
200 increasing dilution showing that microbial species were lost through dilution.
201
202 **Effect of dilution and incubation on bacterial community composition.**
203 After classifying the OTUs with the RDP database, the soil microbial community
204 consisted of 18 phyla (Fig. 1A and B). Phylum-level taxonomic assignments indicated
205 that *Proteobacteria*, followed by *Actinobacteria*, *Bacteroidetes*, *Acidobacteria*,
206 *Verrucomicrobia*, *Planctomycetes* and *Firmicutes* dominated the microbial
207 communities in the original non-diluted (10^{-1}) soil suspension (> 90% of all
208 sequences). The variance in the abundance of the 7 dominating phyla among the
209 replicated samples increased from the low dilution treatments to the high dilution
210 treatments in soil suspensions (Table 2 and Fig. S2). The same was true for the
211 incubated samples while in general the variance among the replicates of the incubated
212 samples was much lower than the variance among the replicated samples of the soil
213 suspensions (Table 2 and Fig. S3). This difference indicates that selective processes in
214 the soil lead to more equal communities. Largest relative changes between the
215 occurrences of specific phyla were detected for the phylum of *Proteobacteria* that was
216 highly dominant in the higher suspension dilutions but less dominant in the incubated
217 soil samples, for the phylum of *Bacteroidetes* that decreased slightly with increasing
218 dilution in the soil suspensions but outgrew and increased significantly in the samples
219 after incubation, and for the phylum of *Verrucomicrobia* that was not detected in the
220 higher soil suspension dilutions but showed up in high numbers after incubation (Fig.
221 1).
222

To compare the overall community structure of the different dilution treatments, PLS-DA analyses was performed to determine the distances at the OTU level between communities of the soil suspensions (Fig. 2A) versus those of the incubated soil samples (Fig. 2B). The first two principle components explain 93.1% and 97.7% of variation in the microbial community structure of soil suspension and incubated soil.

Effect of dilution on rare/abundant OTUs.

A possibility to determine if the dilution approach is appropriate to separate rare species from abundant ones is to make Venn diagrams to assess the shared and unique OTUs between dilution treatments in the soil suspensions (Fig. 3A) and incubated soil (Fig. 3B). We found 954, 77 and 10 unique OTUs in the 10^{-1}, 10^{-6} and 10^{-9} samples of the soil suspensions and 383, 91 and 71 unique OTUs in the respective dilution treatments of the incubated soil samples.

DISCUSSION

A number of studies have used the approach to artificially change microbial diversity by the dilution method (4-13). This approach is one of the few available methods to manipulate microbial biodiversity of complex natural ecosystems such as the soil. And, indeed, our results show that dilution reduces the microbial biodiversity in the soil suspension and the soil after incubation of more or less diluted suspensions.

The previous studies mostly based their conclusions on community measurements with limited resolution only detecting the more abundant species since those species can be detected in the easiest way. However, compared to the rare biosphere, the abundant members are only a small fraction of microbial diversity (16), and thus, the real microbial biodiversity in these ecosystems may not be accounted for.

Furthermore, none of those studies focused on the changes in the community structure from the original more or less diluted inoculated suspensions into the communities of the incubated soil and on the degree of variation in the suspensions after dilution and consequences for the variances in the incubated soils. Therefore, we aimed here to detect the variation in community structure between replicate samples and how the regrown microbial community developed during incubation and regrowth in soil. This allows for testing if we can separate the rare species from the abundant members by the dilution procedure. Although the rare microbes should be more prone to be lost from the original microbial community at increasing dilution, our results show that unique OTUs still do show up in the highest dilution treatment in the suspensions and even increased in the incubated soil. The reason for this phenomenon is unknown. Most likely certain microbial species are suppressed or masked by the abundant members in the low dilution samples and only show up in the higher dilution treatments. However, for the incubated soil samples the latter is not the case because the total density was equal for all dilution treatments. Most likely rare species that were suppressed by the abundant members in the low dilution samples acquired an opportunity to develop in the higher dilution samples because bacterial densities were low in those samples after dilution and had more space to use. It is not possible to conclude that these hidden species are rare species, and thus, the conclusion is warranted that the dilution approach does not guarantee the identification of rare versus abundant species.

Interestingly in the higher dilution (10^{-9}) of soil suspensions a relative low number of cells of certain species were obviously present which were not detected in the suspensions, presumably because they were potentially better adapted (rare/abundant)

273 species to the prevailing conditions of the soil environment (31) than other ones that
274 were detected in the suspensions. Other taxa were lost during incubation since they
275 probably needed special ecological requirements, in particular substrate. Although all
276 inoculated organisms returned into the same environment where they came from
277 originally, the actual conditions for the individual organisms could have changed
278 dramatically due to the difference in spatial arrangements and the large heterogeneity
279 in soil. So, the structure of the microbial community changed significantly during
280 incubation and regrowth. This may have important ecological consequences, as this
281 may have also changed the functioning of the microbial community, including
282 resilience against adverse conditions. In a next publication we will show that such
283 changes do alter the relationship between microbes in the rhizosphere and secondary
284 metabolite production in plants.
285 In this study we have considered several taxonomic diversity indicators. All indicated
286 that the dilution procedure has a strong reducing effect on the microbial diversity
287 (Table 1). We have used these different diversity indices because they give different
288 insights in the diversity of complex communities such as soil microbial communities.
289 Comparing to richness index (Chao estimator), diversity indices (Shannon and
290 Simpson) focus on both the richness and evenness of a community. Shannon diversity
291 is often sensitive to the presence of rare species, while Simpson index emphasize the
292 dominant members (32). Haegeman et al. (33) suggests that community diversity is
293 best estimated by Shannon and Simpson indices, whereas Chao's estimator was not a
294 reliable estimator of richness in the presence of rare species. Despite the differences in
295 the focus of the diversity indices used here, all indices showed a similar trend. This
296 strongly suggests that the alpha diversity reduces in response to dilution of microbial
297 communities and that this reduction is reflected in the diversity of the communities

after incubation in soil.

Yet the diversity indices in the soil communities after regrowth slightly converged. This demonstrates that biotic homogenization took place during incubation and regrowth in soil. The mechanisms behind this homogenization are not known but it points to the selective power of the soil environment towards microbial community assembly processes.

Analysis of the overall microbial community revealed that the community changed through dilution treatment of the soil suspensions and incubated soil at both phylum (Fig. 1) and OTU levels (Fig. 2), but the largest shifts were observed in the occurrence of the major taxonomic groups. A detailed look at the assembled microbial community in the original non-diluted (10^{-1}) soil suspension revealed that the core groups comprised the well-known soil microbial groups of *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Acidobacteria*, *Verrucomicrobia*, *Planctomycetes* and *Firmicutes* (34, 35). During incubation, the same core groups were observed again, but the abundances of each group changed substantially. Upon dilution, the proportion of each major phylum decreased but with one exception, *i.e. Proteobacteria* (36, 37). which are dominant members in various soils and being mostly fast-growing r-strategists and thus its relative abundance increases sharply upon dilution. Remarkably, their relative abundance decreased during incubation in soil. It may be caused by the oligotrophic conditions in prevailing in our test soil. However, the same observations after incubation in soil was made for *Acidobacteria*, which are generally considered to be soil-adapted oligotrophic K- strategists (38). The same holds for other well-known soil inhabitants such as *Actinobacteria*. It is interesting to see that relatively low abundant groups such as *Verrucomicrobia* grew out significantly in all dilution treatments during incubation in soil. This is contradictory with what is known

about *Verrucomicrobia* which are usually considered as low abundance phyla in soil (34). *Verrucomicrobia* may highly depend on C availability due to their slow-growing life strategy (22), and thus it may indicate that *Verrucomicrobia* is a potential indicator of the response of these taxa to environmental factors (36).

The present experimental set-up with only one soil does not allow for conclusions on the generality of these observations for other soils/incubation conditions. An issue that may have played an important role in the preparation of the diluted soil suspensions is the adsorption of cells on soil particles. Bakken (39) claimed that a satisfactory separation of microorganisms and soil particles is not possible, and thus this could have influenced the structure of the microbial communities in the suspensions and in particular the large variation therein. If, for instance, certain rare species are more often attached to soil particles than common ones because they differ in physical or physiological properties, then dilution may select microbes based on the other aspects than abundance only. The variance among replicated samples increased with increasing dilution both in the suspensions and the incubated soil. We assume that there is much more space or less limitation for the communities of the higher dilutions (10^{-6} or 10^{-9}) in the very heterogeneous environment of the soil providing better opportunities for microbes to establish a variety of different communities in these diluted samples.

Rare taxa may raise their numbers when the environmental conditions change (16, 40). Our results support this idea because the unique 10 OTUs in 10^{-9} of suspension were considered to be rare in the original non-diluted (10^{-1}) of soil suspension (Fig. 3), at least they were not identified as abundant and dominant members of the

community in the non-diluted samples. The number of unique OTUs in the 10^{-9} treatment of the incubated soil increased to 71, which means that even more OTUs were under the detection limit in the soil suspension. Comparing to the multivariate analyses that often give more weight to the dominant OTUs such that the observed patterns are driven by the abundant species, the Venn analysis represents all OTUs equally and provides presence/absence data set of the whole community (41). So, our results suggest that the microbial community diversity reduced by dilution procedure, but not only diluting out the rare species. They could still be present but hidden for modern community analysis approaches including modern next generation sequencing methodologies in the undiluted samples.

In summary, our results indicate that the dilution procedure leads to reduction of the bacterial diversity, but that after incubation the regrown microbial communities are less predictable and are more complex than previously thought. A key issue is that the dilution approach does not separate abundant from rare species and so does not allow for firm conclusions on the role of these rare species in terrestrial ecosystems. In future studies we hope to be able to know more about the functional responses of more or less diluted samples and the consequences of these changes for the soil ecosystem functioning.

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519 **FIGURES**

520 **FIG 1** Bacterial community composition at phylum level of (A) soil suspensions and
521 (B) incubated soil samples; the absolute reads of the abundance at 7 major phylum of
522 (C) soil suspension and (D) incubated soil samples.

523 **FIG 2** Score plot of PLS-DA based on bacterial operational taxonomic units (OTUs)
524 composition of (A) soil suspensions and (B) incubated soil samples.

525 **FIG 3** Venn diagram of shared and unique OTUs in each dilution of (A) soil
526 suspension and (B) incubated soil samples.