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Revisiting the dilution procedure used to manipulate microbial biodiversity in terrestrial systems revisited

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1 The dilution procedure to manipulate microbial biodiversity in terrestrial
2 systems revisited

3

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8 Running title: Manipulation of microbial biodiversity

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26 **ABSTRACT**

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

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52 **INTRODUCTION**

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

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

72

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

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

81

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

97

98 **MATERIALS AND METHODS**

99 **Soil sampling and treatment.**

100 30 l soil was collected at a depth of around 15 cm from dune sandy soil in Meijendel,
101 The Netherlands. The soil was sieved , homogenized and aliquots of 500 g were
102 stored in plastic bags. The bags containing soil were γ -irradiation sterilized (> 25
103 kGray, Isotron, Ede, the Netherlands). One bag of soil was kept separately to serve as
104 inoculum. Sterility was checked by spreading 0.5 g sterilized soil from the inoculum-
105 bag onto nutrient agar. No bacterial growth on agar plates for 6 replicates was
106 observed in the sterilized soil samples. A subsample of the fresh soil was taken to
107 determine soil moisture (24 h, 105°C).

108

109 Soil suspensions for inoculation were made by mixing 20 g fresh soil and 190 ml
110 sterilized demi-water with a blender for 2 minutes. This procedure was repeated 3
111 times and in between the blender was cooled down on ice for 2 minutes. This was
112 called the 10^{-1} dilution. By mixing with sterilized demi-water serial dilutions of 10^{-6}
113 and 10^{-9} were made. Subsequently, 25 ml of the respective dilutions were added to
114 500 g of soil in the bags and additional sterilized water was given to bring the
115 moisture level of the inoculated soil at around 20%, which is roughly similar to the
116 average level under the prevailing climatic conditions at the side from where the soil
117 was taken. In total 36 bags of soil (6 samples \times 3 dilution serials \times 2 replicates) were
118 used. The remaining suspensions were centrifuged at 3000 g for 10 min at 4°C and the
119 pellets were stored at -20°C for further analysis. After inoculation, the soil bags were
120 incubated at 20°C using sterilized cotton plug caps to ensure gas exchange. The soils
121 were turned regularly over once a week to ensure even microbial growth. The aim
122 was to reach similar microbial abundances in the different dilution treatments. After 8
123 weeks of incubation, under laminar flow conditions, soil samples were taken to
124 determine the microbial abundance in all treatments by real time PCR using Eub 338

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

135

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

137 Total DNA was extracted from the soil suspensions and incubated soil to determine
138 the composition of the respective microbial communities by 454-pyrosequencing.
139 DNA was extracted using the MoBio Power Soil Extraction Kit according to the
140 supplier's manual (MO BIO Laboratories, Carlsbad, CA, USA). Total DNA
141 concentration was qualified on ND-1000 spectrophotometer (Nanodrop Technology,
142 Wilmington, DE). For DNA concentrations below 5 ng/μl, *i.e.* 5 of 10⁻⁶ and 4 of 10⁻⁹
143 soil suspension samples, nested PCR was performed. The general bacterial primer
144 27F and 1492R (20) were used for the first amplification, and then 2 μl of the
145 amplified products from the first round was used as template for the second round
146 PCR using barcoded primers 515F and 806R (22). 5 ng/μl of DNA/sample of the
147 diluted samples was used as template for the first round of nested PCR with the PCR
148 program of 95°C for 5 min followed by 25 cycles each of 95°C for 30 s, 55°C 1 min
149 and 72°C 10 min. For PCR reactions using barcoded primers were performed using 5

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

160

161 **Sequence analysis.**

162 The raw data were processed using the QIIME v.1.6.0 pipeline (23). Low-quality
163 sequences less than 150 bp in length or with average quality score of less than 25
164 were removed. After denoising the sequences using Denoiser 0.91 (24), and checking
165 for chimeras using USEARCH (25). Operational Taxonomic Units (OTUs) were
166 identified using the UCLUST 1.2.21 algorithm (26) with a phylotype defined at the
167 97% sequence similarity level. The resulting OTUs were aligned against the
168 Ribosomal Database Project database (27).

169

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

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

179

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

189

190 **RESULTS**

191 **Effect of dilution and incubation on bacterial community diversity.**

192 Alpha diversity was significantly reduced by increasing dilution both in the soil
193 suspensions and in incubated soil samples (Table 1, ANOVA soil suspensions: S.obs:
194 $F = 48.51$; $P = 0.000$; S.chao1: $F = 96.675$; $P = 0.000$; Shannon: $F = 54.055$; $P =$
195 0.000 ; Simpson: $F = 3.963$; $P = 0.043$; Evenness: $F = 12.530$; $P = 0.001$, ANOVA
196 incubated soil samples: S.obs: $F = 38.376$; $P = 0.000$; S.chao1: $F = 44.699$; $P = 0.000$;
197 Shannon: $F = 395.076$; $P = 0.000$; Simpson: $F = 94.289$; $P = 0.000$; Evenness: $F =$
198 119.426 ; $P = 0.000$]. 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

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

228

229 **Effect of dilution on rare/abundant OTUs.**

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

236

237 **DISCUSSION**

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

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

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

269

270 Interestingly in the higher dilution (10^{-9}) of soil suspensions a relative low number of
271 cells of certain species were obviously present which were not detected in the
272 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

298 after incubation in soil.

299 Yet the diversity indices in the soil communities after regrowth slightly converged.

300 This demonstrates that biotic homogenization took place during incubation and

301 regrowth in soil. The mechanisms behind this homogenization are not known but it

302 points to the selective power of the soil environment towards microbial community

303 assembly processes.

304 Analysis of the overall microbial community revealed that the community changed

305 through dilution treatment of the soil suspensions and incubated soil at both phylum

306 (Fig. 1) and OTU levels (Fig. 2), but the largest shifts were observed in the occurrence

307 of the major taxonomic groups. A detailed look at the assembled microbial

308 community in the original non-diluted (10^{-1}) soil suspension revealed that the core

309 groups comprised the well-known soil microbial groups of *Proteobacteria*,

310 *Actinobacteria*, *Bacteroidetes*, *Acidobacteria*, *Verrucomicrobia*, *Planctomycetes* and

311 *Firmicutes* (34, 35). During incubation, the same core groups were observed again,

312 but the abundances of each group changed substantially. Upon dilution, the proportion

313 of each major phylum decreased but with one exception, *i.e.* *Proteobacteria* (36, 37).

314 which are dominant members in various soils and being mostly fast-growing r-

315 strategists and thus its relative abundance increases sharply upon dilution.

316 Remarkably, their relative abundance decreased during incubation in soil. It may be

317 caused by the oligotrophic conditions in prevailing in our test soil. However, the same

318 observations after incubation in soil was made for *Acidobacteria*, which are generally

319 considered to be soil-adapted oligotrophic K- strategists (38). The same holds for

320 other well-known soil inhabitants such as *Actinobacteria*. It is interesting to see that

321 relatively low abundant groups such as *Verrucomicrobia* grew out significantly in all

322 dilution treatments during incubation in soil. This is contradictory with what is known

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

327

328 The present experimental set-up with only one soil does not allow for conclusions on
329 the generality of these observations for other soils/incubation conditions. An issue that
330 may have played an important role in the preparation of the diluted soil suspensions is
331 the adsorption of cells on soil particles. Bakken (39) claimed that a satisfactory
332 separation of microorganisms and soil particles is not possible, and thus this could
333 have influenced the structure of the microbial communities in the suspensions and in
334 particular the large variation therein. If, for instance, certain rare species are more
335 often attached to soil particles than common ones because they differ in physical or
336 physiological properties, then dilution may select microbes based on the other aspects
337 than abundance only. The variance among replicated samples increased with
338 increasing dilution both in the suspensions and the incubated soil. We assume that
339 there is much more space or less limitation for the communities of the higher dilutions
340 (10^{-6} or 10^{-9}) in the very heterogeneous environment of the soil providing better
341 opportunities for microbes to establish a variety of different communities in these
342 diluted samples.

343

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

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

358

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

367

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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.