Hidden heterogeneity and co-occurrence networks of soil prokaryotic communities revealed at the scale of individual soil aggregates

Running title: Microbial diversity at soil aggregate-level

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Abstract

Sequencing PCR-amplified gene fragments from metagenomic DNA is a widely applied method for studying the diversity and dynamics of soil microbial communities. Typically DNA is extracted from 0.25 to 1 g of soil. These amounts, however, neglect the heterogeneity of soil present at the scale of soil aggregates; and thus, ignore a crucial scale for understanding the structure and functionality of soil microbial communities. Here we show with a nitrogen-depleted agricultural soil the impact of reducing the amount of soil used for DNA extraction from 250 mg to approx. 1 mg in order to access spatial information on the prokaryotic community structure as indicated by 16S rRNA-gene amplicon analyses. Furthermore, we demonstrate that individual aggregates from the same soil differ in their prokaryotic communities. The analysis of 16S rRNA gene amplicon sequences from individual soil aggregates allowed us, in contrast to 250 mg soil samples, to construct a co-occurrence network that provides insight into the structure of microbial associations in the studied soil. Two dense clusters were apparent in the network, one dominated by *Thaumarchaeota*, known to be capable of ammonium oxidation at low N concentrations, and the other by *Acidobacteria* subgroup 6 probably representing an oligotrophic lifestyle to obtain energy from SOC. Overall this study demonstrates that DNA obtained from individual soil aggregates provides new insights into how microbial communities are assembled.

Keywords

soil aggregates, soil microbial diversity, network analysis, spatial scales, soil DNA extraction, microhabitats

Introduction

One of the major challenges in microbial ecology is to gain a predictive understanding of microbial diversity through elucidating the principles, patterns, and interactions which lead to the assembly of highly diverse microbial communities as found for example in soil (Green, Bohannan, & Whitaker, 2008). To achieve this, it is essential to consider temporal and spatial variation in micro-habitat conditions. In soil microbial ecology, the latter, however, is commonly ignored (Lombard, Prestat, van Elsas, & Simonet, 2011; Vos, Wolf, Jennings, & Kowalchuk, 2013). Instead, large composite samples are favored in order to obtain an overview of microbial diversity at the scale of a plot or a field. This neglects the fine scale heterogeneity of soil structure, and thus loses much information on patterns of community assembly (Thakur et al., 2020).

Soil structure develops as primary particles of different sizes and mineral composition, i.e., clay, silt, and sand, interact with each other, and with organic material to build microaggregates and macroaggregates, that are below or above 250 µm in diameter respectively (Six, Elliott, & Paustian, 2000). Most bacterial cells occur inside aggregates rather than on their surfaces (Ranjard, Poly, et al., 2000) and biogeochemical cycles, which are key ecosystem services driven by an interacting microbial community (Smith et al., 2015), are considered to mainly take place within aggregates (Wilpiszeski et al., 2019). Soil aggregates have been regarded as “massively concurrent evolutionary incubators” (Rillig, Müller, & Lehmann, 2017), or as “microbial villages” (Wilpiszeski et al., 2019), that represent small communities separated by distance and physical barriers and connected only periodically, e.g. during wetting events.

DNA-based methods to assess microbial diversity typically start with extracting 250 mg to 1 g of soil material (Young, Rawlence, Weyrich, & Cooper, 2014). This strategy has been useful to

57 investigate the overall microbial diversity of soils (Fierer & Jackson, 2006; Roesch et al., 2007), its
58 variation across geographical regions (Griffiths et al., 2011; Karimi et al., 2018), or its response to
59 land use change at a continental scale (Szoboszlay, Dohrmann, Poeplau, Don, & Tebbe, 2017).
60 However, in order to understand the processes and interactions taking place within soil microbial
61 communities, it would be rewarding to increase the spatial resolution of the community analysis
62 to individual aggregates and investigate microbial diversity in these spatial entities. Approaching
63 the "calling distance" of microbial interactions (Nunan, 2017), this would increase the likelihood
64 of detecting interacting microbial partners. Correlation networks have increasingly been applied
65 to reveal relationships between microbial community members as detected by PCR amplicon
66 sequence analyses (Banerjee et al., 2016; Barberan, Bates, Casamayor, & Fierer, 2012; Karimi et
67 al., 2020; F. Li, Chen, Zhang, Yin, & Huang, 2017). However, to interpret a positive correlation as
68 a mutualistic and a negative correlation as an antagonistic relationship is only possible for
69 community members sharing the same microhabitat (Weiss et al., 2016). Without distinction of
70 micro-habitats it is hard to assign the presence of taxa to niche exclusion (Faust & Raes, 2012).
71 Analyzing soil DNA extracted from 250 mg to 1 g represents mixed DNA from many microhabitats.
72 In contrast, working with individual soil aggregates, should strongly enhance the ecological
73 significance of soil microbial networks analyses.

74 The potential impact of the heterogeneous soil constituents on structuring microbial
75 communities at a microscale has already been demonstrated with pooled soil primary particles,
76 where the majority of abundant bacterial and fungal taxa exhibited particular preferences for
77 clay, silt, or sand-sized fractions (Hemkemeyer, Christensen, Tebbe, & Hartmann, 2019;
78 Hemkemeyer, Dohrmann, Christensen, & Tebbe, 2018). Furthermore, comparing pooled samples

79 of micro- and macroaggregates revealed that these two size classes also differ in microbial
80 community composition (Constancias et al., 2014; Davinic et al., 2012; Fox et al., 2018), diversity
81 (Bach, Williams, Hargreaves, Yang, & Hofmockel, 2018; Ivanova et al., 2015) and their response
82 to stress (Ranjard, Nazaret, et al., 2000). However, information on the heterogeneity of microbial
83 communities of individual aggregates within specific aggregate fractions is still lacking. A major
84 limitation of analyzing individual aggregates is the difficulty of obtaining a sufficient quantity of
85 nucleic acids from small amounts of soil for molecular methods. Attempts made so far, therefore,
86 either pooled several aggregates for DNA extraction (Bach et al., 2018; Bailey, Fansler, Stegen, &
87 McCue, 2013; Ivanova et al., 2015), sampled very large aggregates weighing 20 – 70 mg
88 (Kravchenko et al., 2014), or applied whole genome amplification (WGA) (Bailey, McCue, et al.,
89 2013). These solutions, however, do not deliver data on individual aggregates, provide coarse
90 spatial resolution, or generate substantial bias in the results (Direito, Zaura, Little, Ehrenfreund,
91 & Roling, 2014; Wang et al., 2016), respectively. To our knowledge, the only study that reported
92 the bacterial community composition in smaller, i.e. below 3 mm, individual aggregates without
93 applying WGA utilized taxonomic microarrays; a method of relatively low resolution, and focused
94 solely on linking enzyme activity profiles with community structure (Kim, Nunan, Dechesne,
95 Juarez, & Grundmann, 2015). Furthermore, applying molecular methods to small samples that
96 yield very low amounts of nucleic acids require validation to prove the consistent performance of
97 the methods and rule out the possibility of contamination and stochastic effects influencing the
98 results.

99 The tremendous scientific potential that individual soil aggregate-based microbial community
100 analysis should have for characterizing the heterogeneity of soil microbial communities at a

101 biologically and ecologically more meaningful scale motivated us testing the following hypotheses
102 in this study:

103 1. Metagenomic DNA of sufficient quantity and quality for PCR-based analyses can be
104 extracted from soil samples in the mg-range, thus representing the scale of macro-
105 aggregates
106 2. Increasing spatial resolution reveals heterogeneity in soil bacterial and archaeal
107 community structure and abundance
108 3. A higher heterogeneity seen among small soil samples is not a result of contamination or
109 sub-optimal performance of molecular methods
110 4. Comparing individual aggregates from the same soil unveils patterns of microbial co-
111 occurrence within the soil microbial community not seen with the commonly used
112 250 mg soil sample size.

113 **Materials and methods**

114 *Overview of the experiments*

115 Three experiments were conducted in this study. In the 1st experiment, samples decreasing in size
116 from 250 mg to 1 mg taken from the same soil were subjected to DNA extraction. To address the
117 first two hypotheses, qPCR and high-throughput amplicon sequencing targeting the 16S rRNA
118 gene were conducted to characterize the bacterial, archaeal, and fungal abundance and the
119 prokaryotic diversity in these samples. The 2nd experiment addressed the third hypothesis by
120 comparing 250 mg soil samples and aliquots of a homogenized soil slurry. The volumes of the

121 aliquots were chosen to contain the amount of DNA expected from 1 mg, 5 mg, and 25 mg soil
122 samples. Since all aliquots were taken from the same thoroughly homogenized soil slurry,
123 differences in prokaryotic community structure between these soil homogenate samples must be
124 results of contamination, stochastic effects, or sub-optimal performance of the DNA extraction
125 and PCR. In the 3rd experiment, DNA was extracted from individual aggregates and 250 mg soil
126 samples taken from the same soil to address the fourth hypothesis. All experiments included
127 several control samples to test for the presence of contamination.

128 *Soil sampling and DNA extraction*

129 The soil used in all experiments was a loam topsoil of a Haplic Chernozem (FAO classification)
130 from the Bad Lauchstädt experimental research station of the Helmholtz Centre for
131 Environmental Research in Germany (51°24'N 11°53'E) (Merbach & Schulz, 2013). It originated
132 form the Static Fertilization Experiment, initiated in the year 1902, and samples were collected in
133 December from a plot without any fertilization since 1903 (treatment NIL)(Ludwig et al., 2011)
134 which was under long term sugar beet – potato – winter wheat – barley rotation. Consequently,
135 the soil was compared to its fertilized variants depleted in nitrogen (Blair, Faulkner, Till,
136 Körschens, & Schulz, 2006). The soil samples had a pH value of 7.1 (in 0.01 M $CaCl_2$) and 17.7 mg
137 kg^{-1} organic C. It was sieved (2 mm mesh size) and stored at 4°C until use.

138 Before sampling, approximately 100 g soil was incubated at room T in the dark for 24 h. The soil
139 was then spread out in a sterile petri dish and samples were taken with sterilized spatulas directly
140 into the bead-beating tubes of the DNA extraction kit. Control samples were included in all
141 experiments. They received no soil but were handled together with and the same way as the soil

142 samples. DNA was extracted with the Quick-DNA Fecal/Soil Microbe Microprep Kit (Zymo
143 Research, Freiburg, Germany) including two 45 s bead beating cycles in an MP FastPrep-24 5G
144 Instrument (MP Biomedicals, Eschwege, Germany) at 6.5 m/s speed with a 300 s break in
145 between. The DNA extracts were eluted in 30 µl elution buffer. All work was done in a biosafety
146 cabinet decontaminated with UV light to minimize the chance of contamination. Measurement
147 of the DNA yield was attempted with Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes, Life
148 Technologies, Eugene, Or) but accurate quantification was not possible for many of the small
149 samples due to results close to the background fluorescence in the blank controls. In preparation
150 of this study, several DNA extractions methods were tested but were not found suitable. This
151 included the Fast DNA Spin kit for soil (MP Biomedicals, LLC, Illkrich, France) and variations of the
152 phenol-chloroform protocol (Miller, Bryant, Madsen, & Ghiorse, 1999).

153 In the 1st experiment, five different size-groups of soil samples were collected: 250 mg, 125 mg,
154 25 mg, 5 mg, and 1 mg, respectively. Eight samples were taken from each size-group along with
155 six control samples. Sample weights from all experiments are listed in Table 1. In the 2nd
156 experiment, ten samples of 250 mg soil and six control samples were taken. Eight of the soil
157 samples were processed normally in the DNA extraction, while for the other two, the DNA
158 extraction was interrupted after the centrifugation following the bead beating. By this point, the
159 samples had been turned into homogenized slurry by the bead beating, and the soil debris had
160 been separated from the supernatant that contained the metagenomic DNA. The supernatant
161 from the two samples were pooled and homogenized by vortexing. The mass of the resulting
162 suspension was 1 112 mg and it originated from 497 mg soil in total. Accordingly, a 55.9 mg aliquot
163 of this suspension contained the amount of DNA extractable from 25 mg soil, an 11.2 mg aliquot

164 the amount from 5 mg soil, and a 2.2 mg aliquot the amount from 1 mg soil. Eight aliquots from
165 each of these sizes, hereafter referred to as 25 mg, 5 mg, and 1 mg soil homogenate samples,
166 were taken and each mixed with 350 µl bashing bead buffer from the DNA extraction kit to
167 continue the DNA extraction. In the 3rd experiment, 37 individual soil aggregates, weighing 5.3
168 mg on average and similar in size (ca. 2 mm), were taken for DNA extraction along with 35 samples
169 of 250 mg soil and nine control samples.

170 *Abundances of microbial groups assessed by qPCR*

171 The abundance of *Bacteria*, *Archaea*, and *Fungi* were assessed by qPCR targeting the 16S rRNA
172 gene and the ITS region as previously described (Hemkemeyer, Christensen, Martens, & Tebbe,
173 2015). Archaeal and fungal abundance was only investigated in the 1st experiment. Reactions
174 were run in a Bio-Rad CFX96 real-time PCR system in duplicates from different dilutions of the
175 DNA extracts. In case of the 250 mg and 125 mg soil samples, 50- and 100-fold dilutions were
176 taken; from the 25 mg samples 10- and 20-fold dilutions; and from the 5 mg, 1 mg, individual
177 aggregate, and control samples undiluted DNA extracts and two-fold dilutions were used. PCR
178 efficiencies were 95.9 – 104.6% for the bacterial 16S rRNA gene, 94.2 – 96.2% for the archaeal
179 16S rRNA gene, and 83.3 – 84.3% for the fungal ITS with $R^2 \geq 0.995$ in all cases. Results were
180 compared with Tukey's HSD tests, or Welch's t-test in case of the data from the 3rd experiment.
181 The analysis was carried out in R 3.4.4 (www.r-project.org). One of the 250 mg samples from the
182 1st experiment yielded a magnitude higher copy number in the fungal ITS qPCR assay than the
183 others. It was deemed to be an outlier and excluded from the analysis.

184 *High-throughput sequencing of 16S rRNA gene amplicons and data processing*

185 To characterize the bacterial and archaeal communities in the samples, DNA extracts were
186 subjected to high-throughput amplicon sequencing of the V4 region of the 16S rRNA gene
187 following the protocol of Kozich *et al.* (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013) with
188 primers updated to match the modified 515f and 806r sequences according to Walters *et al.*
189 (Walters et al., 2016). In case of the small soil samples and control samples, due to the low DNA
190 yield, 10 µl DNA extract was used as template in the PCRs. Paired-end sequencing was done on
191 Illumina MiSeq instruments at StarSEQ (Mainz, Germany). Samples from the same experiment
192 were sequenced in the same run. All data is available at the European Nucleotide Archive under
193 the accession numbers PRJEB36881 (1st experiment), PRJEB36883 (2nd experiment), and
194 PRJEB36887 (3rd experiment).

195 The sequencing data from the three experiments were analyzed separately. Raw reads were
196 processed with the dada2 (version 1.6.0) pipeline (Callahan et al., 2016) in R 3.4.4. Forward and
197 reverse reads were truncated at positions 240 and 90, respectively. Reads with any ambiguous
198 bases were discarded as well as forward reads with over two and reverse reads with over one
199 expected error. The data from the 2nd experiment had higher quality allowing the reverse reads
200 to be truncated at position 130 and keeping those with two or less expected errors. Error models
201 were constructed from 10^6 randomly selected reads. Sequence variants (SVs) were inferred using
202 the pool option. Forward and reverse SVs were merged trimming overhangs, and the
203 removeBimeraDenovo function was employed to detect chimeras. The SVs were classified
204 according to the SILVA reference version 132 (Pruesse et al., 2007) accepting only results with
205 ≥70% bootstrap support. SVs shorter than 220 nt or longer than 275 nt, or identified as chimeric,

206 mitochondrial, or chloroplast sequences, or not classified into *Bacteria* or *Archaea* were deleted.
207 Good's index was calculated to estimate the coverage of the SVs. SVs with ≥0.1% relative
208 abundance in any of the control samples of an experiment were regarded as potentially
209 contaminant and removed from the dataset of that experiment.

210 *Analysis of the sequencing results*

211 Principal component analysis (PCA) plots were created in R using the rda function of the vegan
212 package version 2.5-2 (Oksanen et al., 2018). To decrease the sparsity of the data, SVs not
213 reaching 0.1% relative abundance in any of the samples included in the PCA were removed.
214 Zeroes were replaced with the count zero multiplicative (CZM) method using the zCompositions
215 package version 1.1.1 (Palarea-Albaladejo & Martin-Fernandez, 2015) and centered log-ratio
216 (CLR) transformation was applied to the data to correct for compositional effects and differences
217 in sequencing depth (Gloor, Wu, Pawlowsky-Glahn, & Egozcue, 2016).

218 Aitchison distances between the samples were calculated as Euclidean distances in the CLR
219 transformed dataset (Gloor, Macklaim, Pawlowsky-Glahn, & Egozcue, 2017). SVs that didn't have
220 at least 0.1% relative abundance in any of the compared samples were removed and zeroes were
221 replaced with the CZM method to allow CLR transformation before calculating Euclidean
222 distances with the 'vegdist' function of the vegan package. Results were compared with Tukey's
223 HSD tests.

224 Plots illustrating the prevalence of abundant SVs among the soil samples were prepared in
225 Cytoscape 3.7.1 (www.cytoscape.org). CoNet 1.1.1 beta (K Faust & Raes, 2016) in Cytoscape was
226 used to construct co-occurrence networks from the data from the 3rd experiment. To limit the

227 number of parallel significance tests and the sparsity of the data, only SVs with ≥0.2% relative
228 abundance in at least one of the samples were included. Separate networks were constructed for
229 the 250 mg soil samples and the soil aggregates. However, the selection of SVs was done on the
230 joint data matrix to ensure that both networks include the same SVs. The data was relativized to
231 the total sequence count of each sample. Pearson and Spearman correlations, mutual
232 information (jsl setting), and Bray-Curtis and Kullback-Leibler (with a pseudo count of 10^{-8})
233 dissimilarities were calculated and the 1 000 highest and 1 000 lowest scoring edges from each of
234 the five metrics were kept. The ReBoot method (Faust et al., 2012), which mitigates compositional
235 effects, was used to assess the significance of the edges based on 1 000 permutations with
236 renormalization and 1 000 bootstrap iterations. In the network of the soil aggregates, edges with
237 scores below the 2.5^{th} and over the 97.5^{th} percentile of the bootstrap distribution or not
238 supported by at least three of the five metrics were considered unstable and removed. Brown's
239 method of p-value merging was applied followed by Benjamini-Hochberg correction. Only edges
240 with $q \leq 0.05$ were included in the final network. In the network of the 250 mg samples, unstable
241 edges were not removed and the Benjamini-Hochberg correction was not applied as otherwise
242 no edges were retained. The networks were visualized in Cytoscape using the compound spring
243 embedder layout. Topological parameters were calculated with NetworkAnalyzer version 2.7
244 (Assenov, Ramirez, Schelhorn, Lengauer, & Albrecht, 2008).

245 **Results**

246 *Microbial DNA can be extracted from soil samples in the mg-range*

247 DNA could be extracted from soil samples as little as 0.87 mg as well as from intact soil aggregates.
248 In all cases, the extracted DNA was sufficient for 16S rRNA gene amplicon sequencing and the
249 qPCR assays. Accurate quantification of the DNA yield with PicoGreen assays was not possible
250 because the fluorescence readings from many of the small samples were close to the background
251 fluorescence in the blank controls.

252 To assess whether the DNA extraction could recover microbial DNA with similar efficiency from
253 small quantities of soil as from 250 mg samples, estimates of the abundances of *Bacteria*,
254 *Archaea*, and *Fungi* in one gram of soil were calculated from the qPCR results (Figure 1;
255 Supplementary data file #1). Similar estimates were obtained from the 250 mg and 5 mg soil
256 samples from the 1st experiment. Estimates from the 1 mg samples tended to be lower but were
257 not significantly different. In contrast, the estimates of fungal abundance in a gram of soil were
258 significantly lower from the 1 and 5 mg than from the 250 mg samples. Estimates of bacterial
259 abundance obtained from the 25 mg and 125 mg samples and archaeal abundance from the 25
260 mg samples were significantly higher than from the 250 mg samples. Bacterial abundance
261 estimates from the single aggregates and 250 mg soil samples of the 3rd experiment covered the
262 same range (Figure S1), but the mean of the estimates from the single aggregates (2.27×10^9
263 copies/g soil) was lower ($p < 0.001$) than from the 250 mg samples (4.69×10^9 copies/g soil).

264 The control samples amplified in the qPCR assay targeting the bacterial 16S rRNA gene but yielded
265 only 3 to 356 copies per µl DNA extract. In comparison, the 1 mg soil samples had 8 777 to 45 534

266 copies per µl DNA extract (Supplementary file 1). The control samples from the 1st experiment
267 had 0 to 13 fungal ITS copies per µl DNA extract and none of them showed amplification in the
268 archaeal 16S rRNA gene qPCR assays.

269 *Removing potentially contaminant SVs from the sequencing results*

270 It was possible to generate sequencing results from all control samples (the complete dataset
271 with the taxonomic classification of the SVs is in Supplementary file 2). Good's coverage index of
272 the SVs was over 0.993 in all of them indicating that almost their complete prokaryotic community
273 was captured by the sequencing (Table S1). They were very similar to each other in their
274 prokaryotic community structures but very different from the soil samples (Figure S2). Every SV
275 that reached 0.1% relative abundance in any of the control samples of an experiment was
276 considered as a potential contaminant. There were 450, 77, and 591 such SVs in the datasets of
277 the 1st, 2nd, and 3rd experiments respectively. In the data from the 1st experiment, these SVs
278 together covered 98.9 – 99.8% of the sequences obtained from the control samples and 2.5 –
279 6.6% of the sequences from the soil samples. In the 2nd and 3rd experiments, 92.5 – 99.5% and
280 98.6 – 99.6% of the sequences from the control samples, and 2.9 – 17.3% and 5.9 – 9.4% of the
281 sequences from the soil samples, respectively, were covered by the potentially contaminant SVs.
282 To mitigate the effect of contamination on the results, the potentially contaminant SVs were
283 deleted from the data matrices before further analysis.

284 *Increasing spatial resolution reveals heterogeneity in soil bacterial and archaeal*
285 *community structure but not in their abundance*

286 The yield of high-quality 16S rRNA gene amplicon sequences was somewhat lower from the 1 mg
287 samples than from the 5 mg and 25 mg samples in the 1st experiment (Figure 2A). Apart from this,
288 however, the sequencing yield did not differ between the sample groups within any of the
289 experiments. Thus, it is possible to compare the number of SVs detected in the samples without
290 rarefying the data. The number of SVs in the 1st experiment was not significantly different
291 between the 250 mg, 125 mg, and 25 mg samples, but decreased significantly in the 5 mg and
292 even further among the 1 mg samples (Figure 2B). An opposite trend was apparent in the Good's
293 coverage index (Table S1). Similarly, significantly lower numbers of SVs were detected in the
294 single aggregates than in the 250 mg soil samples of the 3rd experiment.

295 Principal component analysis (PCA) from the sequencing results from the 1st experiment arranged
296 all 250 mg and 125 mg samples, and most 25 mg samples into a single, tight group, indicating high
297 similarity in their prokaryotic community structures (Figure 3A). In contrast, samples from the 5
298 mg and more so from the 1 mg categories, showed higher heterogeneity in community structure.
299 Similarly, PCA indicated heterogeneity between individual aggregates that was not seen among
300 the 250 mg samples in the 3rd experiment (Figure 3B).

301 The abundant SVs ($\geq$ 0.1% relative abundance in at least one of the samples) in the 250 mg, 125
302 mg, and 25 mg samples from the 1st experiment were almost all detectable in each individual
303 sample, showing that the composition of the soil prokaryotic community appears very uniform
304 when investigated at such a coarse spatial resolution (Figure 4). In contrast, 172 of the abundant
305 SVs detected in the 1 mg samples were unique to just one or two of these samples. Among these

306 SVs, representatives of *Planctomycetes*, *Proteobacteria*, and *Acidobacteria* were especially
307 numerous, while *Thaumarchaeota* and *Actinobacteria* were dominant among the SVs present in
308 all samples. The 5 mg samples represented a level of spatial resolution at which some
309 heterogeneity in the prevalence of the abundant SVs was already apparent with 22 of them
310 detectable in two or only in a single sample.

311 In total, 5 620 SVs were detected in the eight 1 mg soil samples of the 1st experiment
312 (Supplementary file 2). Of these, 4 764 (85%) were also present in at least half of the 250 mg
313 samples. The remaining 856 SVs had low relative abundance in the 1 mg samples with only 59
314 reaching more than 0.1% relative abundance in any of them. The 5 mg samples together
315 contained 8 010 SVs, of which 6 443 (80%) were also detectable in at least half of the 250 mg
316 samples. Of the remaining 1 567 SVs, only 26 reached more than 0.1% relative abundance in any
317 of the 5 mg samples.

318 The qPCR results did not confirm our hypothesis that increasing spatial resolution would reveal
319 heterogeneity in microbial abundance. Bacterial and archaeal 16S rRNA gene, and fungal ITS copy
320 numbers did not show a larger variation among the 1 and 5 mg samples than between the 250
321 mg samples from the 1st experiment (Figure 1). Similarly, in the 3rd experiment, bacterial
322 abundance did not vary more in the single aggregates than in the 250 mg samples (Figure S1).

323 *Impact of stochastic effects and inconsistent performance of the methods*

324 The soil homogenate samples from the 2nd experiment served to test the influence of stochastic
325 effects and sub-optimal performance of the DNA extraction and PCR when extracting small
326 amounts of soil. The estimated bacterial abundance in a gram of soil based on the qPCR results

327 was in general higher in the samples from the 2nd experiment but followed the same pattern as
328 in the samples from the 1st experiment with no differences between the 1 mg, 5 mg, and 250 mg
329 samples but significantly higher values in the 25 mg samples (Figure S3). The small soil
330 homogenate samples did not show the degree of heterogeneity in prokaryotic community
331 structure we observed among the small soil samples of the 1st experiment. The abundant SVs (≥
332 0.1% relative abundance in at least one sample) in the 25 mg soil homogenate samples were all
333 detectable in at least five of the eight replicates. Out of the 354 abundant SVs in the 5 mg soil
334 homogenate samples, one was present in only three of the samples but the others were
335 detectable in at least six. The 1 mg soil homogenate samples harbored 446 abundant SVs. None
336 of them was unique to a single sample and 442 were present in five or more of the eight samples.
337 The Aitchison distances of community structure were much higher among the 5 mg, and
338 especially among the 1 mg soil samples of the 1st experiment compared to the distances between
339 the 250 mg soil samples (Figure 5). In contrast, the distances between 5 mg or 25 mg soil
340 homogenate samples of the 2nd experiment were very similar to the distances among the 250 mg
341 soil samples indicating no difference in the heterogeneity of prokaryotic community structure
342 between these sample groups. The distances between the 1 mg soil homogenate samples were
343 only slightly increased.

344 *Bacterial and archaeal co-occurrence patterns in 250 mg soil samples and aggregates*

345 Networks of prokaryotic co-occurrence were constructed using the 272 SVs that reached ≥0.2%
346 relative abundance in at least one of the samples of the 3rd experiment. No network was obtained
347 from the 250 mg samples unless the removal of unstable edges and the correction of the p-values
348 for multiple testing were skipped. The resulting network has 78 edges between 35 nodes (Figure

349 6A). Thus, this spatial resolution revealed only a small number of putative associations many of
350 which are likely false discoveries. In contrast, a network of 137 edges and 67 nodes (with the
351 removal of unstable edges and control of the false discovery rate) was obtained from the
352 individual soil aggregates (Figure 6B). A total of 54 of the nodes are part of a connected
353 component in which there are three nodes with high betweenness centrality: SV15
354 (*Verrucomicrobia*, candidatus *Udaeobacter*), SV31 (*Actinobacteria*), and SV36 (*Acidobacteria*
355 subgroup 6). Their relative abundance in the aggregates were 0.46 ± 0.16%, 0.33 ± 0.13%, and
356 0.30 ± 0.11%, respectively. These SVs potentially serve a keystone function by connecting two
357 clusters in the network. One of the clusters contains several SVs of *Thaumarchaeota*,
358 *Verrucomicrobia*, and *Actinobacteria*. The other is dominated by *Acidobacteria* subgroup 6. The
359 hub of the latter cluster is SV58 (*Acidobacteria* subgroup 6) with a relative abundance of 0.23 ±
360 0.16% that has the highest degree in the network being connected to 19 nodes. SV399
361 (*Chloroflexi*) (0.06 ± 0.05%) and SV123 (*Acidobacteria* subgroup 6) (0.13 ± 0.06%) are linked with
362 negative associations to several members of this cluster.

363 **Discussion**

364 The large disparity between the scale in which the soil microbiota is usually studied with
365 molecular methods (0.25 to 1 g of soil) and the distance over which microbial interactions occur,
366 impede the detection of interacting partners (Nunan, 2017). In order to gain information on the
367 soil microbial diversity at an increased spatial resolution that considers soil structure, in this study
368 we reduced the amount of soil used for DNA extraction from 250 to 1 mg and also extracted
369 individual soil aggregates. Bacterial and archaeal DNA was recovered with not a significantly

370 different efficiency from the 250 mg and the 5 mg and 1 mg samples as shown by the qPCR results.
371 This was not true for fungal DNA. Either the DNA-extraction kit was not efficient in isolating fungal
372 DNA from samples below 25 mg, or fungi may preferentially colonize larger soil aggregates. Our
373 results show that the DNA extraction kit was most efficient in recovering bacterial and archaeal
374 DNA from 25 to 125 mg soil, although the variation in the yield of 16S rRNA gene copies was large
375 among these samples. Since this increased variation was apparent among the 25 mg soil
376 homogenate samples of the 2nd experiment as well, it is not an indication of an uneven
377 distribution of bacterial cells at the scale of 25 mg samples but must be due to this particular DNA
378 extraction method not working with consistent efficiency with this amount of soil.

379 As a consequence of sampling small amounts of soil, the DNA extracts had low template
380 concentrations for the subsequent PCR analyses. Thereby we had to anticipate a high risk of
381 contamination affecting the results (Weiss et al., 2014). In fact, quantifiable amounts of *Bacteria*
382 and *Fungi*, but not *Archaea*, were detected in the control samples without soil. However, they
383 reached no more than 4% of the number of bacterial rRNA genes copies in the smallest soil
384 samples, and thus, the influence of contamination on our results is negligible. The bacterial
385 community found in the control samples was clearly distinct from the soil communities suggesting
386 that the contamination originated from the reagents of the DNA extraction and sequencing library
387 preparation rather than cross-contamination between samples (Glassing, Dowd, Galandiuk,
388 Davis, & Chiodini, 2016; Salter et al., 2014). Another concern of working with small samples is that
389 molecular methods applied to such low amounts of template may perform inconsistently leading
390 to artificial variation in the results. The 2nd experiment showed that the DNA extraction, PCR, and
391 sequencing did not artificially generate more variation in the results from 5 mg samples than the

392 variation present among the 250 mg samples and the 1 mg samples showed only a slightly higher
393 variation. Therefore, the large heterogeneity in prokaryotic community composition and
394 structure among the 1 mg and 5 mg soil samples from the 1st experiment was not caused by
395 stochastic effects or PCR bias.

396 The samples of 25 mg up to 250 mg of soil were close to identical in prokaryotic community
397 composition, thus they provide a good representation of the overall prokaryotic diversity of our
398 soil. This is also indicated by the fact that increasing the amount of soil extracted up to 25 mg
399 increased the number of SVs detected in the samples, but larger soil samples did not yield more
400 SVs. Thus, the 25 mg samples already had a good coverage of the total prokaryotic community.
401 In contrast, the 1 mg and 5 mg samples and single aggregates were heterogeneous in community
402 structure. We found that while small soil samples could recover some SVs not necessarily
403 detected with the conventionally used 250 mg samples; these SVs were typically low in
404 abundance. Very few exceeded the relative abundance threshold we applied to control the
405 sparsity of the data in our analysis of community structure. Therefore, the large heterogeneity of
406 prokaryotic community structure we observed among the small samples was not because they
407 would have enabled the detection of more SVs. Instead, it appears that they contained different
408 subsets of the total community present in the 25 to 250 mg samples. This could be explained by
409 the fact that the smaller samples contain fewer micro-habitats, each of which harbors a local
410 community of fewer species (Leibold et al., 2004). Interestingly, the 1 mg and 5 mg samples did
411 not significantly differ in the abundance of *Bacteria, Archaea*, and *Fungi* compared to the 250 mg
412 samples. Similarly, the variation in bacterial abundance found with the individual aggregates was
413 not different from the 250 mg samples. Microbial abundance in soil has a patchy distribution at

414 the scale of a few micrometers (Nunan, Wu, Young, Crawford, & Ritz, 2003) but, for the soil of
415 this study, apparently not at the scale of macroaggregates or 1 to 5 mg samples.

416 Network analyses based on microbial co-occurrence have been applied to soil samples as large as
417 10 grams (Khan et al., 2019) and is typically used with 250 mg – 1 g samples (Barberan et al., 2012;
418 Karimi et al., 2020). In this study, however, we could not detect stable and significant associations
419 between SVs from 35 samples of 250 mg soil. These samples were taken from the same well-
420 mixed batch of soil and were very similar in their prokaryotic community composition. It is likely,
421 that each 250 mg sample gave a good representation of the overall prokaryotic diversity in our
422 soil, in which case, the variation of the relative abundance of SVs in these samples were mostly
423 random. Random patterns should not yield stable and significant associations in network analysis.
424 The value of utilizing much smaller samples is shown by our result that the 1 and 5 mg samples
425 contained subsets of the total soil microbial diversity captured by the 250 mg samples, and with
426 increasing spatial resolution the heterogeneity in the bacterial and archaeal community structure
427 increased among the samples. This results in detectable co-occurrence patterns. Furthermore,
428 the smaller spatial scale increases the likelihood that the observed co-occurrences actually
429 indicate interactions (Cordero & Datta, 2016).

430 From 37 soil aggregates, we obtained a complex network of bacterial and archaeal co-occurrence
431 that contained two clusters, one with several *Thaumarchaeota*, *Verrucomicrobia* and
432 *Actinobacteria* SVs, the other mainly with *Acidobacteria* subgroup 6 SVs. Three SVs, which could
433 represent keystone taxa, were found to connect these clusters. If these putative keystone SVs are
434 abundant in an aggregate, we can expect that members of both clusters are present there. The
435 two major clusters present in the network may provide complementary functions in the soil

436 ecosystem. There are indications that *Acidobacteria* subgroup 6, dominating one of the clusters,
437 prefer agricultural soils with low nitrogen input where it could be involved in the slower turnover
438 of soil organic carbon (SOC) originating from microbial necromass or plant material (Hester et al.,
439 2018; D. D. Li et al., 2018; Navarrete et al., 2013). The soil of this study originated from a long-
440 term nitrogen-depleted agricultural soil, thus supporting the preference to low nitrogen
441 concentrations and SOC turnover. The other cluster included several abundant SVs from phylum
442 *Thaumarchaeota*, which is known to be a strong contributor to ammonium oxidation in
443 agricultural soils (Leininger et al., 2006). Compared to ammonium-oxidizing bacteria,
444 *Thaumarcheaota* are thought to be adapted to lower nitrogen concentrations (Pester, Schleper,
445 & Wagner, 2011), thus the nitrogen-depleted soil of this study is likely a favorable environment
446 for them. The two clusters in our aggregate co-occurrence network could represent two distinct
447 types of metabolism adapted to a nitrogen depleted soil: a chemoorganotroph that oxidizes SOC,
448 and a chemolithotroph that oxidizes ammonia produced for example by ammonification from
449 crop residues. The presence of the less abundant *Verrucomicrobia* and *Actinobacteria* SVs within
450 the *Thaumarchaota* dominated cluster is possibly linked to an oligotrophic lifestyle (Bergmann et
451 al., 2011; Fierer, Bradford, & Jackson, 2007), but considering the limited information that 16S
452 rRNA gene analyses can provide for these phyla, this remains yet only a hypothesis. Shotgun
453 sequencing and metagenomic analysis of DNA extracted from individual soil aggregates could
454 shed more light on the nature of the associations we detected in the co-occurrence network. In
455 general, such aggregate-level analyses of the soil microbiota, which we call “aggregatomics”,
456 could inspire new ways of linking structure to function in soil microbial communities.

457 While the spatial scale that we reached in this study is not yet fine enough to reveal most
458 microbial interactions as they may occur in microaggregates (Raynaud & Nunan, 2014), it should
459 be able to support the development of hypotheses and experiments to understand the patterns
460 and processes shaping the assembly of soil microbial communities and modelling their behavior
461 (Faust & Raes, 2012; Tecon & Or, 2017). Developing DNA extraction protocols from even smaller
462 soils samples, approaching the microaggregate-level, should be a way forward to fuel soil
463 aggregate-oriented research ("Aggregatomics"; https://www.thuenen.de/en/bd/fields-of-
464 activity/feld-und-laborstudien/microbiology-and-molecular-ecology/soil-aggregatomics/) for unveiling
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473 Conflict of interest

474 The authors declare no conflict of interest.

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697 ## Data accessibility statement

698 Sequencing data is available at the European Nucleotide Archive under the accession numbers
699 PRJEB36881 (1st experiment), PRJEB36883 (2nd experiment), and PRJEB36887 (3rd experiment).
700 The qPCR data is in Supplementary file 1.

701 ## Author contributions

702 Both authors contributed to the design of the experiments, analysis of the results, and writing the
703 manuscript.

704 ## Tables and figures

705 **Table 1** Number of samples and soil weights within each sample category

Soil weight class or sample type	Sample weight [mg] ± SD	Number of soil samples
1st Experiment		
250 mg	251 ± 1	8
125 mg	125 ± 1	8
25 mg	25.1 ± 0.4	8
5 mg	4.9 ± 0.2	8
1 mg	1.1 ± 0.2	8
Control, no soil		6
2nd Experiment		
250 mg	252 ± 3	8
25 mg soil homogenate		8
5 mg soil homogenate		8
1 mg soil homogenate		8

Control, no soil		6
3rd Experiment		
250 mg	251 ± 0	35
Soil aggregate	5.3 ± 1.6	37
Control		9

706

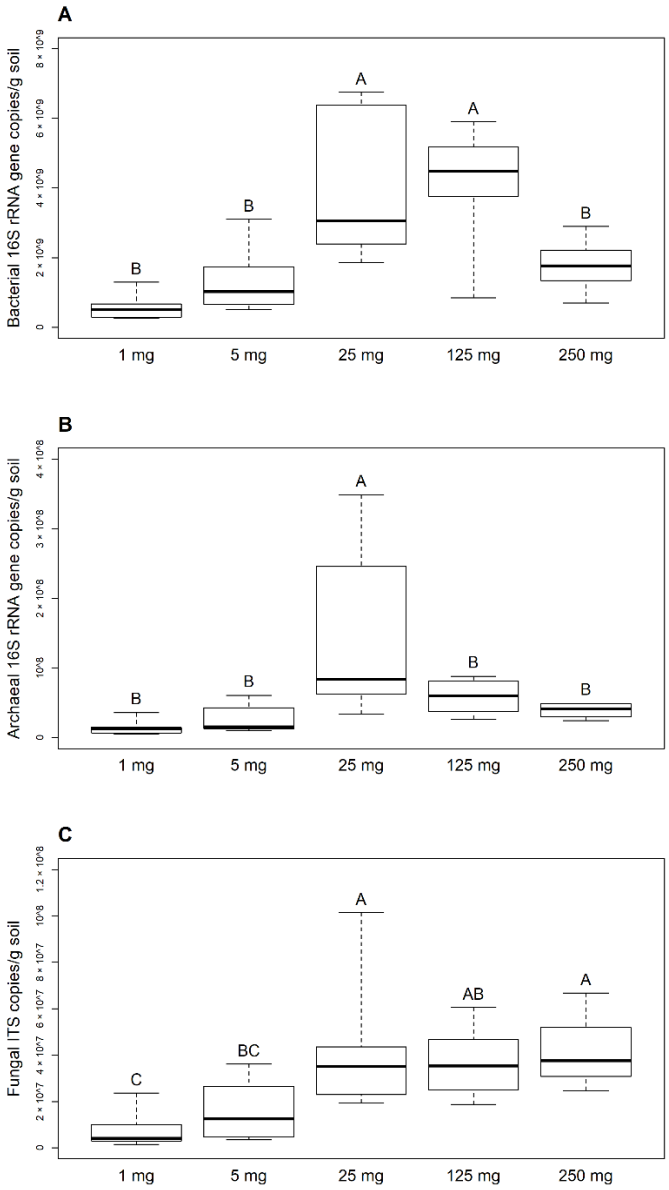
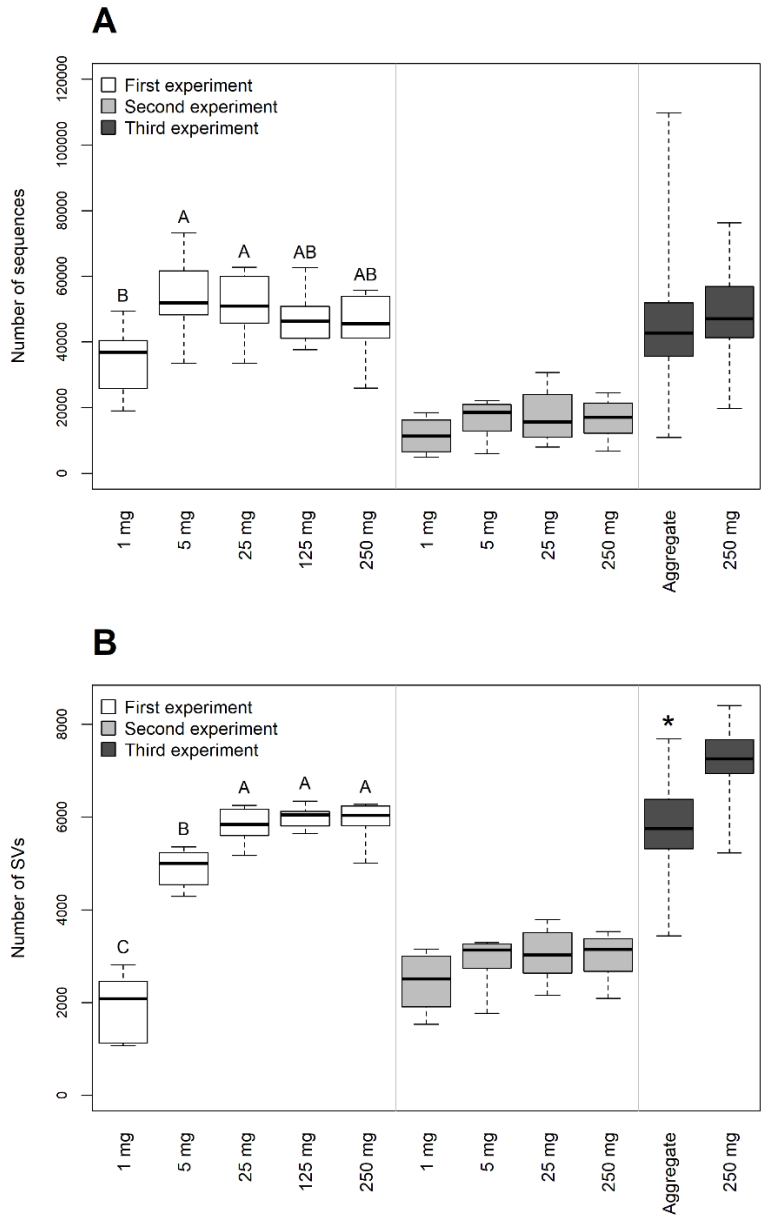
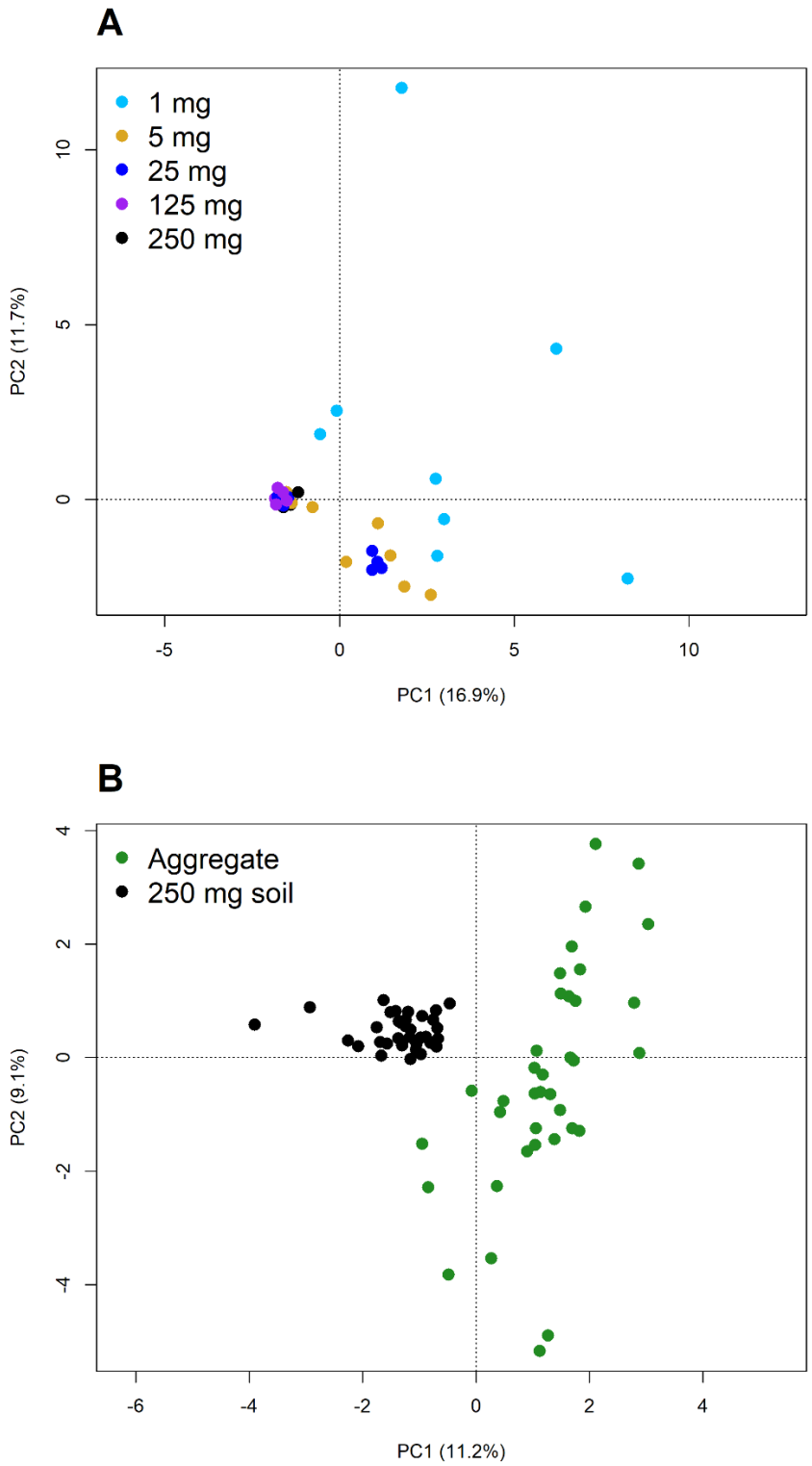


Figure 1 Estimates of (A) bacterial, (B) archaeal, and (C) fungal abundance in a gram of soil based on qPCR from the samples from the 1st experiment (gene copy numbers per g of soil wet weight). One of the 250 mg soil samples was an outlier in the fungal ITS qPCR results and is not included in the plot. Sample groups not labelled with the same letter were significantly different in Tukey's HSD tests. Thick lines indicate the median values, the upper and lower hinges the 75th and 25th percentile, whiskers extend to the data extremes.



714

715 **Figure 2** Number of (A) sequences and (B) sequence variants (SVs) in the samples after the
716 removal of potentially contaminant SVs. Thick lines indicate the median values, the upper and
717 lower hinges the 75th and 25th percentile, whiskers extend to the data extremes. Letters indicate
718 significant differences between sample groups of the 1st experiment according to Tukey's HSD
719 tests. * indicates significant difference based on Welch's t-test between the aggregate and the
720 250 mg soil samples of the 3rd experiment.



721

722 **Figure 3** Principle component analyses (PCA) plots from the 16S rRNA gene sequencing data from

723 the 1st (A) and 3rd (B) experiments.

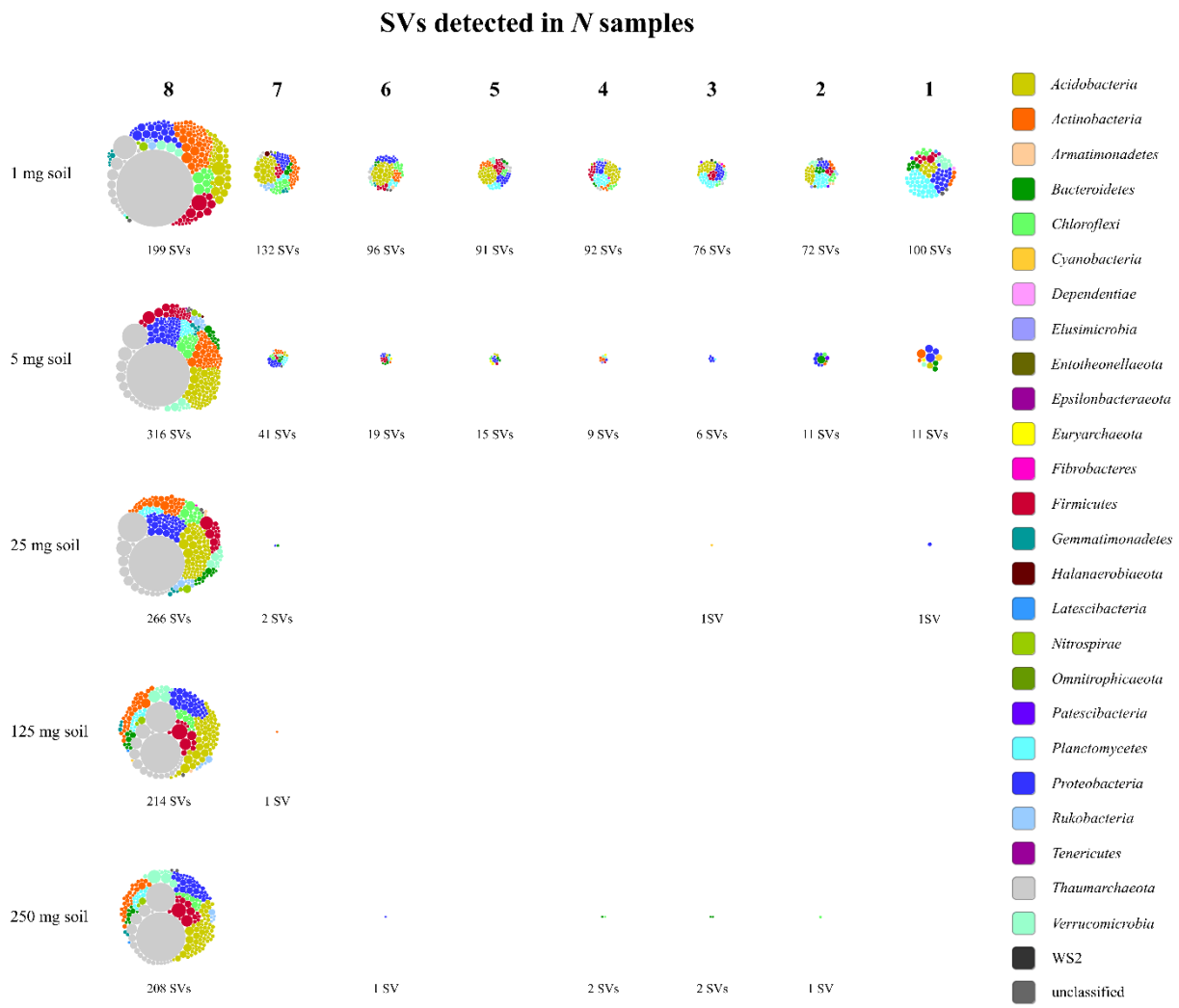
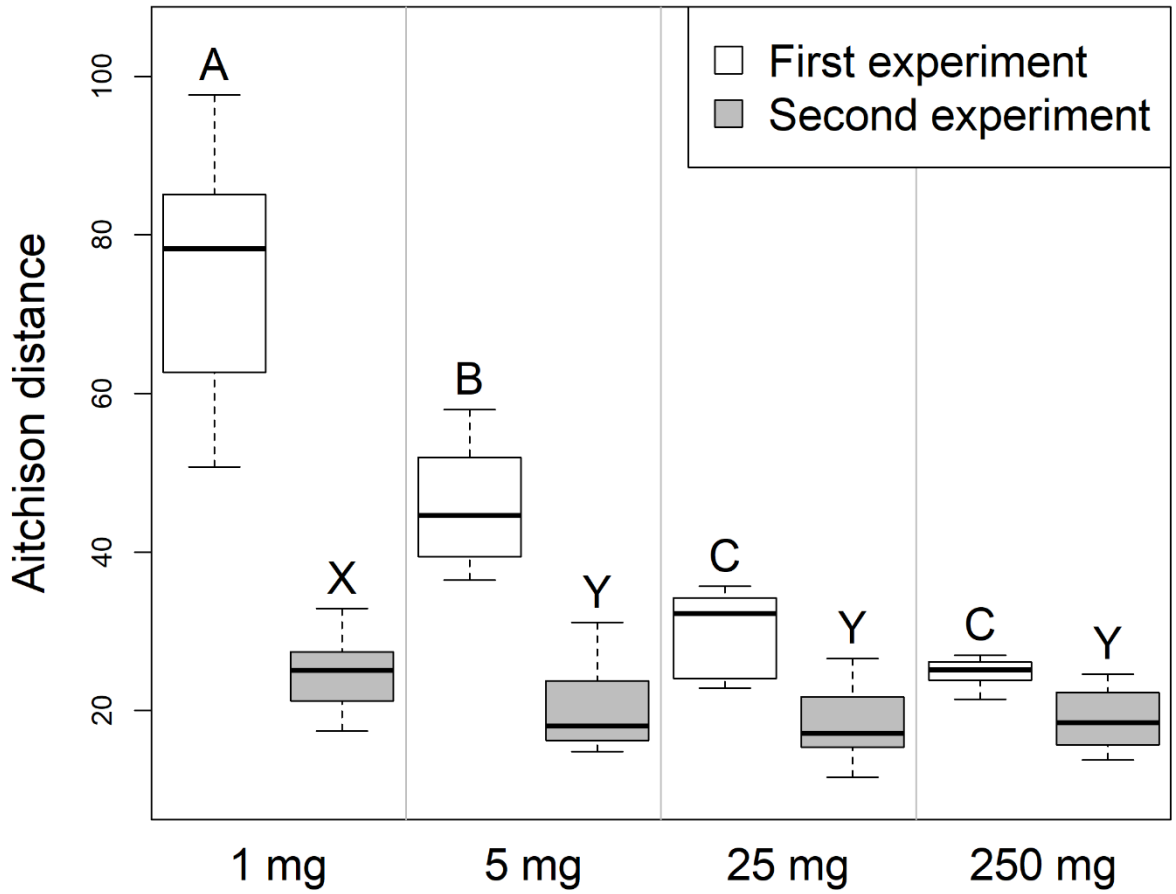
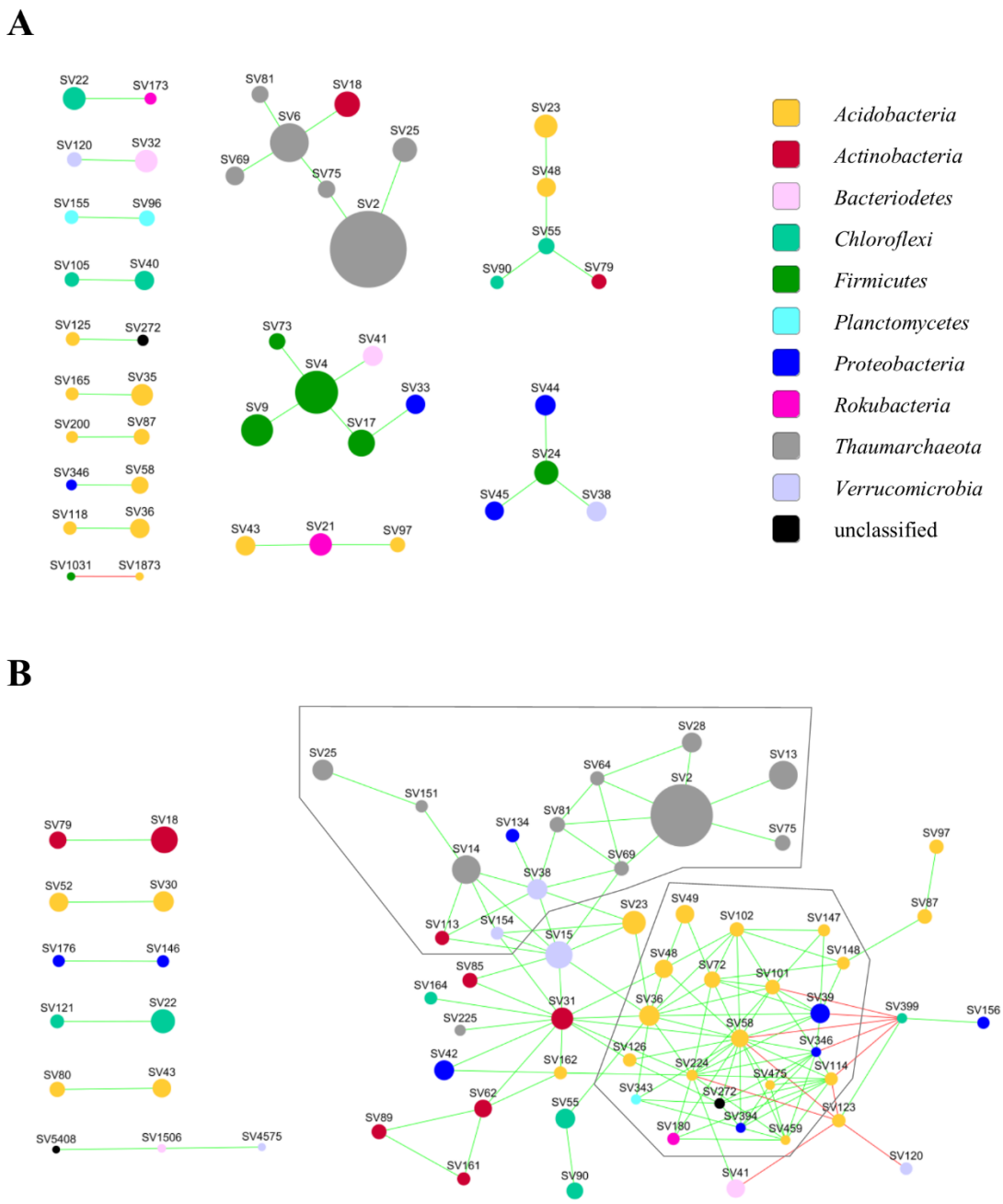


Figure 4 SVs arranged according to in how many of the 1 mg, 5 mg, 25 mg, 125 mg, or 250 mg samples from the 1st experiment they were detected. Only SVs that reached at least 0.1% relative abundance in any of the samples are included. Each node represents one SV colored based on its phylum-level classification and sized according to its average relative abundance across all samples excluding those in which it was not detected.



730

731 **Figure 5** Aitchison distances in the bacterial and archaeal community structure (16SrRNA gene
732 amplicons) within sample groups from the 1st and 2nd experiments. Thick lines indicate the median
733 values, the upper and lower hinges the 75th and 25th percentile, whiskers extend to the data
734 extremes. Sample groups from the same experiment not labelled with the same letter were
735 significantly different in Tukey's HSD tests.



736

737 **Figure 6** Co-occurrence networks from the (A) 250 mg samples and (B) the single aggregates from

738 the 3rd experiment. The frames mark the two clusters discussed in the text. It should be noted

739 that in (A) unstable edges were *not* removed and the Benjamini-Hochberg correction for multiple

740 comparisons was not applied.