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Title

Comparison of prokaryotic communities between the fields showing different disinfestation effects by anaerobic soil disinfestation

Authors

Chol Gyu Lee[1,2], Toshiya Iida[1], Eriko Matsuda[3], Kayo Yoshida[3], Masato Kawabe[4], Masayuki Maeda[5], Yasunori Muramoto[6], Hideki Watanabe[6], Yoko Otani[7], Kazhiro Nakaho[8], Moriya Ohkuma[1]

Affiliations

[1]*Japan Collection of Microorganisms, RIKEN BioResource Center, Tsukuba, Ibaraki 305-0074, Japan.*

[2]*Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Koganei, Tokyo, 184-8588, Japan*

[3]*Ishikawa Agriculture and Forestry Research Center, Kanazawa, Ishikawa 920-3198, Japan.*

[4]*Horticultural Research Institute, Toyama Prefectural Agricultural, Forestry and Fisheries Research Center, Tonami, Toyama 939-1327, Japan.*

[5]*Niigata Agricultural Research Institute, Niigata, Nagaoka, Niigata 940-0826, Japan.*

[6]*Gifu Prefectural Agricultural Technology Center, Matamaru, Gifu 501-1152, Japan.*

[7]*Wakayama Agricultural Experiment Station, Kinokawa, Wakayama, 640-0423, Japan.*

[8]*Institute of Vegetable and Floriculture Science, National Agriculture and Food Research Organization, Tsu, Mie 514-2392, Japan.*

Abbreviations

ASD: Anaerobic soil disinfestation, OTU: Operation taxonomic units, PCR: Polymerase chain reaction,

Keywords

Bacterial wilt, Betaproteobacteria, Clostridia, Indicator species analysis, Multi field study

Abstract

Several soil managements are used for preventing soil-borne disease of tomato in greenhouse, but they didn't effective in deeper layer soil. Anaerobic soil disinfestation (ASD) is a non-chemical fumigation method and can reduce the soil-borne pathogens under 40 cm soil depth. However, their effect is not stable under the field condition. Soil microbes have important roles for ASD. Microbial community reflects the soil environment and some unique microbes are be detected under the different condition. If common microbes are detected from well-disinfested soil among several fields, they will be a good indicator for succession of ASD. For this purpose, we conducted same ASD treatment with 17 tomato greenhouses in the geographically different regions. In this study, we compared soil prokaryotic communities in the field which indicate different disinfestation effects after ASD treatment among several fields. Totally, 52 soil samples were analyzed using high throughput next-generation sequencing. Weighted-UniFrac analysis showed that prokaryotic communities in the fields showing different disinfestation effect were not significantly different and sampling fields more strongly effect to microbial community. The relative abundance of *Betaproteobacteria*

and *Clostridia* were significantly increased in well- disinfested fields. We successfully detected specific bacteria common to various ASD treated fields in operational taxonomic unit (OTU) level. Indicator species analysis revealed that Twenty-five OTUs were specifically detected from well-disinfested soil and 18 OTUs belonged to phylogenetically diversified *Clostridia*. Other OTUs belonged to aerobic bacteria and were not previously detected from ASD treated fields. Our results showed the prokaryotic communities were not largely differed among ASD efficiency but small changing will be an important for ASD.

58 **1. Introduction**

59 Soil-borne pathogens cause various plant diseases, including take-all and damping-off,
60 crown rots, and wilting. Bacterial wilt caused by *Ralstonia solanacearum* has a host
61 range exceeding 200 species and over 50 families (Kelman 1998). Disinfestation of this
62 pathogen is difficult because the pathogen is distributed evenly in a deep layer soil below
63 40 cm (Graham and Lloyd 1979). Several approaches have been attempted for bacterial
64 wilt control, including soil amendments, crop rotation, and field sanitation (Wang and
65 Lin 2005). Soil fumigation with chemical pesticides is an effective method for killing
66 the bacterial wilt pathogens but its efficacy tends to be unstable in deep soil and it is
67 escaped for food safety and environmental pollution.

68 Anaerobic soil disinfestation (ASD) is an effective method to reduce soil-borne
69 pathogens (Shinmura 2000). This method consists of incorporation of labile organic
70 matter in the soil, irrigation, and covering soil surface with polyethylene film. Organic
71 matter increases microbial respiration and irrigation purges soil air, polyethylene film
72 prevents an inflow of oxygen from the atmosphere, then reductive soil condition is
73 induced (Block et al. 2000; Momma et al. 2006). It is effective for the soil in the lower

74 layer under 40 cm, which uses water-soluble organic such as low concentration ethanol
75 or molasses as a carbon source (Momma et al. 2013). Therefore, ASD using
76 water-soluble carbon sources is useful for the disinfection of *R. solanacearum* in
77 deep-layer soils and is environmentally-friendly. However, the disinfestation effects by
78 ASD is unstable under field condition (Shrestha et al. 2016). Enough soil temperature,
79 incubation period, and amount of carbon amendments are needed to success of
80 disinfestation.

81 Soil microbes have important roles for ASD. Several microbes increase after the ASD
82 treatment and they may be involved in suppression of pathogens (Momma et al. 2007;
83 Mowlick et al. 2013, Huang et al. 2015, 2016; Strauss et al. 2017; Mazzola et al. 2018).
84 Soil microbes reflect soil environment and they are considered as one of the indicators
85 for soil healthiness (van Burggen and Semenov 2000; Mendes et al. 2011). Therefore,
86 different disinfestation efficiency fields can have the different soil microbial
87 communities. Recently, Mazzola et al. (2018) suggested that microbial community
88 changing by ASD is different among sampling fields. If the microbes which is increased
89 in well-disinfested soil are commonly detected among several fields, they will be a good

90 indicator for ASD treatment near the future. For this purpose, we conducted the ASD

91 treatment with same substrate in 17 geographically different greenhouses and they

92 showed different disinfestation efficiency. We compared the prokaryotic communities

93 among the fields showing different disinfestation effect. This multi-fields study will

94 show that 1) microbial community may differ depending on disinfestation efficiency

95 and 2) the well-disinfested soil may have unique microbes compare with

96 not-well-disinfested soil among several fields.

97

98 **2. Materials and Methods**

99

100 **2.1. Sampling field and ASD treatment**

101 Field experiments were established in 17 greenhouses in 8 fields in Japan in which

102 occurrences of bacterial wilt were observed (Table 1). Sugar-containing diatoms,

103 defined as a sugar-rich byproduct, are a waste discharged from the food-processing

104 industry that 40 % sugar in weight. The sugar-containing diatoms were mixed into the

105 soil at a ratio of 15 t ha^{-1} with the rototiller in 30 cm soil layer. The field was covered by

106 polyethylene and flooded with water more about 150 L m^{-2}. Disinfestation was

107 conducted 21 days excepted for Ha field (17 days), because soil temperature in this field

108 were reached more than 35 °C during disinfestation period. ASD were treated in the

109 greenhouse (15 m × 6 m) and replicates were conducted among different greenhouses.

110 There were three replications in the Ha, Ni, Ts, and To fields, two replications in the Sa

111 field, and no replication in the IS, Gi, and Wa fields. The samples were collected from

112 each greenhouse before and after ASD treatment from two different depths (20-30 cm

113 and 40-50 cm) in each greenhouse using a Gauge Auger DIK-106B core sampler (Daiki

114 Rika Kogyo Co., LTD, Saitama, Japan) in Is, Ha, To, Gi, and Wa fields (9 greenhouse ×

115 4 soil samples, Totally 36 soil samples). In Ni, Ts and Sa field, only two soil samples

116 (before and after ASD treatment in the upper layer soil) were collected from each

117 greenhouse in these fields (8 greenhouse × 2 soil samples, Totally 16 soil samples). A

118 total of 52 soil samples were collected for analysis. Soil samples were collected from 5

119 randomly chosen points in each greenhouse and mixed well.

120

121 **2.2 Quantification of *R. solanacearum* in the field**

122 The most probable number (MPN)-PCR method, which is semi-quantitive *R*.

123 *solanacearum* counting method, was conducted as described by Inoue and Nakaho

124 (2014). Briefly, 10 g of soil was eluted in cultivation buffer and the soil extract was

125 diluted with buffer 10, 100, and 1000 times. Each aliquot was incubated at 35 °C for

126 approximately 24 h, and nested-PCR targeting *phcA* gene, which is a key gene for the

127 appearance of wilt disease, was conducted on the culture sample using the primer pairs

128 phcA2981f (5′-TGGATATCGGGCTGGCAA-3′) and phcA4741r

129 (5′-CGCTTTTGCGCAAAGGGA-3′) for the first step and phcA3538f

130 (5′-GTGCCACAGCATGTTCAGG-3′) and phcA4209r

131 (5′-CCTAAAGCGCTTGAGCTCG-3′) for the second step. The number of samples that

132 appeared in the PCR amplification around 700 bp length was collectively checked

133 against the MPN table. The detection limit of *R.solanacearum* by this method is 3 to

134 2400 colony forming units (cfu) g^{-1} dry soil and a detection indicates the risk of

135 outbreaking of bacterial wilt in the field.

136

137 **2.3 Tag-encoded amplicon sequencing targeted by 16S rRNA gene for prokaryotes**

138 DNA was extracted from 0.5 g soil with an ISOIL kit for bead beating (Nippongene,
139 Tokyo, Japan) following the manufacturer's instructions. DNA quantification and
140 integrity were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific,
141 Waltham, MA, USA) and by gel visualization (0.8% agarose in TAE). PCR of each
142 sample was performed to amplify the V4 variable region of the 16S rRNA gene using
143 the bacterial and archaeal universal primers, 515F
144 (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R
145 (5'-GGAC-TACVSGGGTATCTAA-3') (Camporaso et al. 2011). The library was
146 prepared by adaptor ligation and PCR primer pairs using the TruSeq Nano DNA Library
147 Prep Kit (Illumina, San Diego, CA, USA). When two or more bands were detected by
148 1.5%-agarose gel electrophoresis, PCR products of approximately 300 bp in length were
149 excised from the gel and purified using a MonoFas DNA purification kit (GL Sciences,
150 Tokyo, Japan) for prokaryotes. One soil sample (the upper layer soil in To3 after ASD
151 treatment) was not amplified after PCR amplification and was excluded from the analyses.
152 Each PCR amplicon was cleaned twice using an Agencourt AMPure XP system
153 (Beckman Coulter, Inc, Palo Alto, CA, USA) to remove primers and short DNA

154 fragments, and was quantified using a Qubit Fluorometer (Invitrogen, Carlsbad, CA,

155 USA). After successful amplification, the PCR products were adjusted to equimolar

156 concentrations and subjected to unidirectional pyrosequencing at Bioengineering Lab.

157 Co., Ltd. (Kanagawa, Japan) on a MiSeq instrument (Illumina). A total of 3,114,333

158 sequences in the 51 samples were obtained after sequencing (Supplemental Table 1).

159 Sequencing data were deposited in the DNA Data Base of Japan (DDBJ) Sequence

160 Read Archive under accession number DRA006673.

161

162 **2.4 Data analyses**

163 The analysis of the sequencing data was conducted according to Lee et al. (2017). Raw

164 fasta files were pre-processed in the Quantitative Insights into Microbial Ecology

165 (QIIME) bioinformatic pipeline (Caporaso et al. 2010). Data from the read sequences,

166 quality, flows, and ancillary metadata were analyzed using the QIIME pipeline. Quality

167 filtering consisted of discarding reads < 200 bp or > 1000 bp in length, excluding

168 homopolymer runs of > 6 bp and continuous ambiguous bases of > 6, and accepting one

169 barcode correction and two primer mismatches. Moreover, a mean quality score < 25

170 was also removed. Finally, singleton operational taxonomic units (OTUs) and chimeric
171 sequences were removed for statistical analyses. Denoising was performed using the
172 built-in Denoiser algorithm, and chimera removal and OTU picking were accomplished
173 with USEARCH 61 considering a pairwise identity percentage of 0.97. Taxonomy
174 assignment was performed using the Ribosomal Database Project (RDP) naïve Bayesian
175 classifier with a minimum confidence of 0.8 against the Greengenes database, October
176 2012 release and Basic Local Alignment Search Tool (BLAST). OTU-based analysis
177 was performed on pyrotag-based datasets to calculate the richness and diversity using
178 the phyloseq R package, version 1.7.24 (McMurdie and Holmes 2013). The diversity
179 within each individual sample was estimated using the non-parametric Shannon
180 diversity index. The Chao1 estimator was calculated to estimate the richness of each
181 sample. Multivariate analysis of community structure and diversity was performed on
182 pyrotag-based datasets using a weighted Unifrac dissimilarity matrix calculated in
183 QIIME, jackknifing (1000 reiterations) read abundance data at the deepest level
184 possible (41,012 reads), and using unconstrained ordination by a Principal Coordinate
185 Analysis (PCoA) for prokaryotes in each soil layer. *K*-means clustering was used to

186 verify the effects of sampling fields and ASD treatment on prokaryotic community.

187 Finally, indicator value analysis was done using the indicspecies R package (De Cáceres

188 2009) with the aim of identifying OTUs associated with suppressive soil, rather than the

189 conducive soil or vice versa ($p < 0.05$). We created two patterns of soil groups. One

190 group was divided into samples from the soil before or after ASD treatment. The other

191 group was divided based on the efficiency of disinfestation. The datasets were merged

192 and the abundant OTUs that were associated with the pathogen non-detected soil after

193 ASD treatment were evaluated in each layer ($p < 0.001$). The number of random

194 permutation tests for the calculation of the indicator values was 999.

195

196 **2.5 Construction of phylogenetic tree**

197 The phylogenetic tree of clostridial members was constructed by a 1000-fold bootstrap

198 analysis using neighbor-joining methods with the Clustal W program (version 2.0

199 Developments) software (Larkin et al. 2007) and the NJ plot software (Perrière and

200 Gouy 1996). The phylogenetic tree contained 16S rRNA sequences of several clusters

201 of clostridial strains.

202

203 **3. Results**

204

205 **3.1. Effectiveness of ASD treatment with sugar-containing diatoms**

206 *R. solanacearum* in soil was not detected after treated ASD with sugar-contained

207 diatoms in 14 of the 17 greenhouses and 7 of the 9 greenhouses in the upper and lower

208 layers, respectively, (Table 2). Therefore, the ASD treatments were appropriate in each

209 fields. Some fields (in IS, Ha1, and Ha2 field) were not-well-disinfested by ASD. We

210 classified the soil samples from greenhouses into two groups based on the effect of

211 ASD: not well-disinfested soil as F-soil and well-disinfested soil as S-soil.

212

213 **3.2 Changes of prokaryotic communities after ASD treatment**

214 The sequences were clustered into 74,539 OTUs at 97% similarity. Among S- and F-

215 soil, the DNA concentrations were significantly decreased in the upper layer of the

216 S-soil and the lower layer of the F-soil (Figure 1, Supplemental Table 2). Shannon

217 indices and Chao1 were decreased after ASD in the upper. OTU numbers were

218 significantly decreased in F-soil regardless of soil layers.

219 PCoAs based on weighted-UniFrac analysis showed that prokaryotic communities

220 were roughly classified by sampling fields (Figure 2). In the upper layer, Ha fields

221 contained one cluster regardless of ASD treatment and disinfestation efficiency (Figure

222 2A). IS, Wa and Ts fields were separated by before and after ASD treatment but not

223 affected by disinfestation efficiency. In the lower layer soil, IS, Ha, and Gi were also

224 different among sampling fields and ASD treatment, but not different by S- and F-soil

225 (Figure 2B). The microbes collected from same fields comprised same clusters before

226 and after ASD treatment in spite of ASD efficiency was different.

227 After ASD treatment, the relative abundance of *Firmicutes* was increased by more

228 than 2-times in 8 greenhouses (Supplemental Table 3). In the upper layer soil,

229 *Firmicutes* was significantly increased, while *Crenarchaeota* was decreased after ASD

230 treatement (Figure 3). *Acidobacteria* and *Verrcomicrobia* in the upper layer of the

231 S-soil, *Acidobacteria*, and *Crenarchaeota* in the lower layer of the F-soil were

232 significantly decreased. In the class level, *Clostridia* that belonged to the *Firmicutes*

233 phylum were increased more than 2-times after ASD in 15 of 16 and 8 of 9 greenhouses

234 in the upper and lower layers, respectively (Supplemental Table 4). Moreover,

235 *Betaproteobacteria* and *Clostridia* were significantly increased after ASD treatment in

236 the upper layer of the S-soil (Figure 4). In the S-soil, *Sphingobacteria* and *Cytophagia*

237 were significantly decreased in the upper layer, and *Thermoleophilia* and *Actinobacteria*

238 were decreased in the lower layer.

239

240 **3.4 Specifically increased OTUs in S-soil**

241 We successfully detected 25 OTUs specifically increased in the S-soil by ASD from

242 more than 8 of the 16 sites or 4 of the 6 sites from the upper and lower layers,

243 respectively (Table 3). They belonged to several classes, including *Clostridia*, *Bacillus*,

244 *Betaproteobacteria*, *Gammaproteobacteria*, and *Mollicutes*. Eighteen out of the 25

245 OTUs belonged to *Clostridia*. The phylogenetic trees consisting of OTUs related to

246 clostridial members showed that diversified *Clostridia* were specifically increased after

247 ASD treatment in the S-soil (Figure 5). Three OTUs belonged to the *Caloramator* group,

248 *Clostridium* cluster III and two OTUs belonged to the *Christensenella* group. OTU 50

249 (*Caloramator*) was commonly detected in 11 upper layer and five lower layer soil

250 samples. OTU311169 (*Bacillus*), OTU199301 and OTU624373 (*Christensenella*), OTU

251 112 (*Clostridium*), and OTU56 (*Rhodothermaceae*) were also increased in 10 of the 14

252 S-soils, while OTU934369 and OTU304 (*Caloramator*) and OTU212333

253 (*Symbiobacterium*) were commonly detected in more than five of the six fields of S-soil.

254 Prokaryotes decreased by ASD treatment common to the various fields in this criterion

255 were not detected (data not shown).

256

257 **4. Discussion**

258 In this study, we showed that microbial communities were not different even if the

259 fields indicated different disinfestation effect by ASD. Especially, in Ha field, we used

260 three contiguous greenhouses and they treated the same field managements for more

261 than 10 years. However, they showed a different disinfestation effect among the field.

262 Our previous study also showed that prokaryotic community structures were affected

263 more by indigenous communities than the disease suppression effect (Lee et al. 2017).

264 These results suggested that the difference of indigenous soil communities are not

265 affected to controlling of soil-borne pathogen. Runia et al. (2014) showed Firmicutes
266 are increased by ASD treatment among 5 soil types using the real-time PCR method.
267 We also showed that Firmicutes have increased after ASD treatment regardless of
268 disinfestation efficiency. In phylum level, *Clostridia* and *Betaproteobacteria* were
269 significantly increased by ASD in S-soil. *Clostridia*, which belonged to Firmicutes, are
270 gram-positive anaerobic bacteria. During ASD, decreases in oxygen availability along
271 with increases in pH occur, which promotes the increased prevalence of anaerobic
272 microbes (Momma et al. 2006; Mowlick et al. 2013, 2014). Several studies indicated
273 that *Clostridia* increase in number and become dominant bacteria following ASD
274 treatment using plant material or low concentration ethanol as a substrate (Huang et al.
275 2015, 2016; Mowlick et al. 2012; Strauss et al. 2017). The prior and present results
276 indicate that *Clostridia* increased after ASD treatment regardless of soil types, field
277 locations, and substrate types and they may be key microbes for disinfestation by ASD.
278 *Caloramator*-like OTUs were the most commonly found OTUs in the upper and lower
279 layers of various fields. They are rod-shaped, obligate anaerobic, thermophilic
280 endospore producers that can ferment several sugars and produce acetate, isobutyrate,

281 lactate, and other VFAs, while they do not degrade cellulose (Collins et al. 1994; Ogg
282 and Patel 2011). Mowlick et al. (2012) reported that 63% of clostridial clones that were
283 increased after ASD treatment belonged to clostridium cluster I, including the
284 *Oxobacter* and *Caloramator* groups. *Symbiobacterium* is rod-shaped thermophiles that
285 are syntrophic with *Bacillus* spp., which possess a glucose degradation pathway (Ohno
286 et al. 2000). *Christensenella* is a non-spore forming, short, straight rod with tapered
287 ends; it can use various sugars and produce VFAs during fermentation (Morotomi et al.
288 2012). *Christensenella* were detected in an anaerobic cellulolytic microbial consortium
289 from mangrove soil (Gao et al. 2014). These results suggest that phylogenetically
290 diverse clostridial members might be responsible for suppression of pathogens through
291 their production of VFAs during anaerobic decomposition of sugar-containing diatoms.
292 Strauss et al. (2017) reported the increased of *Betaproteobacteria* by ASD treatment
293 with organic bran at soil depths of 15.2 cm and 45.7 cm. We showed
294 *Noviherbaspirillum*, belonged to *Betaproteobacteria*, which were specifically detected
295 from S-soil. They are gram-negative, aerobic, non-spore forming rods capable of
296 utilizing several carbohydrates as carbon sources (Lin et al. 2013). Moreover, several

297 *Bacillus* species have been described as biological control agents for bacterial wilt and
298 have been detected from soil after ASD treatment (Mowlick et al. 2012; Yamamoto et al.
299 2014; Yuliar et al. 2015). The family *Rhodothermaceae* of the class Cytophagia
300 comprises six genera; most are isolated from extreme environments, such as hypersaline
301 crystallized ponds in the Dead Sea and submarine hot spring (Xia et al. 2015). They
302 exhibit extreme thermophilic or halophilic growth characteristics. Mazzola et al (2018)
303 showed that these *Cytophagaceae* bacteria were significantly increased after ASD
304 treatment with rice bran. However, their role for disinfestation does not appear.
305 *Symbiobacterium, Christensenella, Noviherbaspirillum,* and *Rhodothermaceae* were not
306 previously detected from ASD treated soil. They might have important roles for
307 disinfestation of *R. solanacearum*. *Noviherbaspirillum*, *Bacillus,* and *Rhodothermaceae*
308 are aerobic bacteria that were also increased by ASD, as with anaerobic bacteria. In the
309 soil environment, some *Bacillus* might contribute to the rapid decrease of soil redox
310 potential at the initial stage of ASD treatment by consuming oxygen (Mowlick et al.
311 2012). Therefore, they might have important roles in maintaining anoxic soil conditions
312 during ASD treatment.

313 In conclusion, we revealed prokaryotic communities were not strongly affected by

314 disinfestation efficiency compare with sampling fields, but *Clostridia* and

315 *Betaproteobacteria* were significantly increased in well-disinfested soil. Twenty-five

316 OTUs were specifically detected in S-soil and most of them were affiliated to *Clostridia*,

317 which was well-known bacteria for ASD effect. However, some microbes were not

318 previously detected from ASD treated fields. These microbes will be a good indicator

319 for well-disinfested soil and future study will appear the role of these microbes for

320 disinfestation.

321

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464
465 **Figure legend**
466
467 **Figure 1.** Comparison of DNA concentration, prokaryotic diversity and richness
468 between the F- and S-soil.
469 Asterisk represents a pair of means that show significant difference between the S- and
470 F- soil (t-test, $p < 0.05$).
471

472 **Figure 2.** A UniFrac-weighted principal component analysis of the soil prokaryotic

473 communities in the upper layer (A) and lower layer (B) of the field before and after

474 ASD treatment.

475 Closed circle: S-soil before ASD treatment, Open circle: S-soil after ASD treatment,

476 Closed triangle: F-soil before ASD treatment, Open triangle: F-soil after ASD

477 treatment.

478 The clustering was conducted by *k*-means analysis.

479

480 **Figure 3.** Comparison of the prokaryotic phyla which observed significant difference

481 between the S- and F-soil

482 Asterisk represents a pair of means that show a significant difference between the S-

483 and C- soil (Tukey's test, $p < 0.05$).

484

485 **Figure 4.** Comparison of the prokaryotic class that observed a significant difference

486 between the S- and F-soil

487 Asterisk represents a pair of means that show a significant difference between the S-

488 and C- soil (Tukey's test, $p < 0.05$).

489

490 **Figure 5.** Neighbor-joining tree showing the phylogenetic relationships of the

491 clostridial OTUs based on 16S rRNA gene sequencing (According to the clostridial

492 cluster analysis by Collins et al. 1994). The OTUs obtained from this study are shown

493 in gothic letters. *Bacillus subtilis* was used as the outgroup. Bold letters indicate OTUs

494 that specifically appeared in the S-soil.

Figure1

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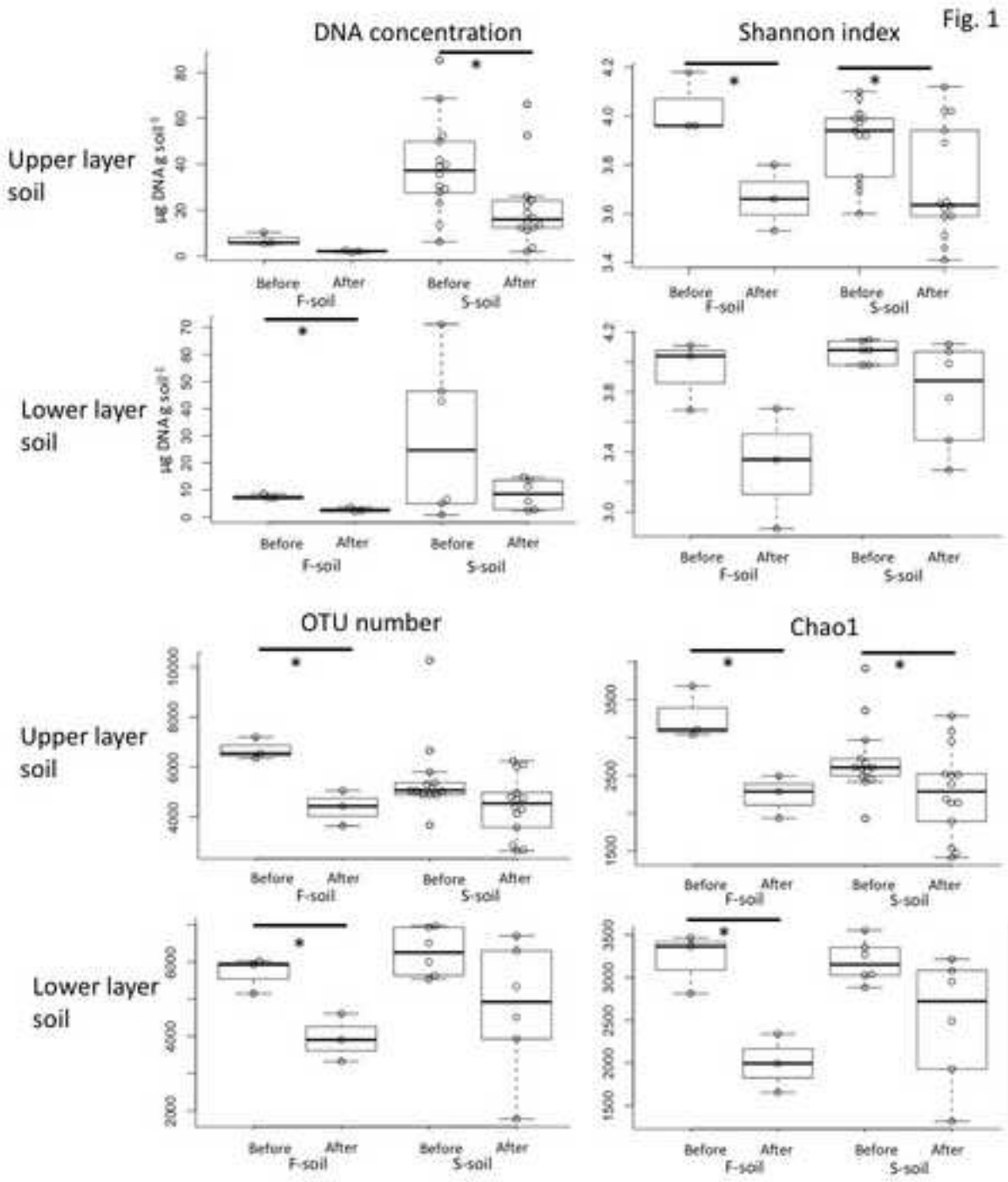

Figure2A
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Fig. 2A

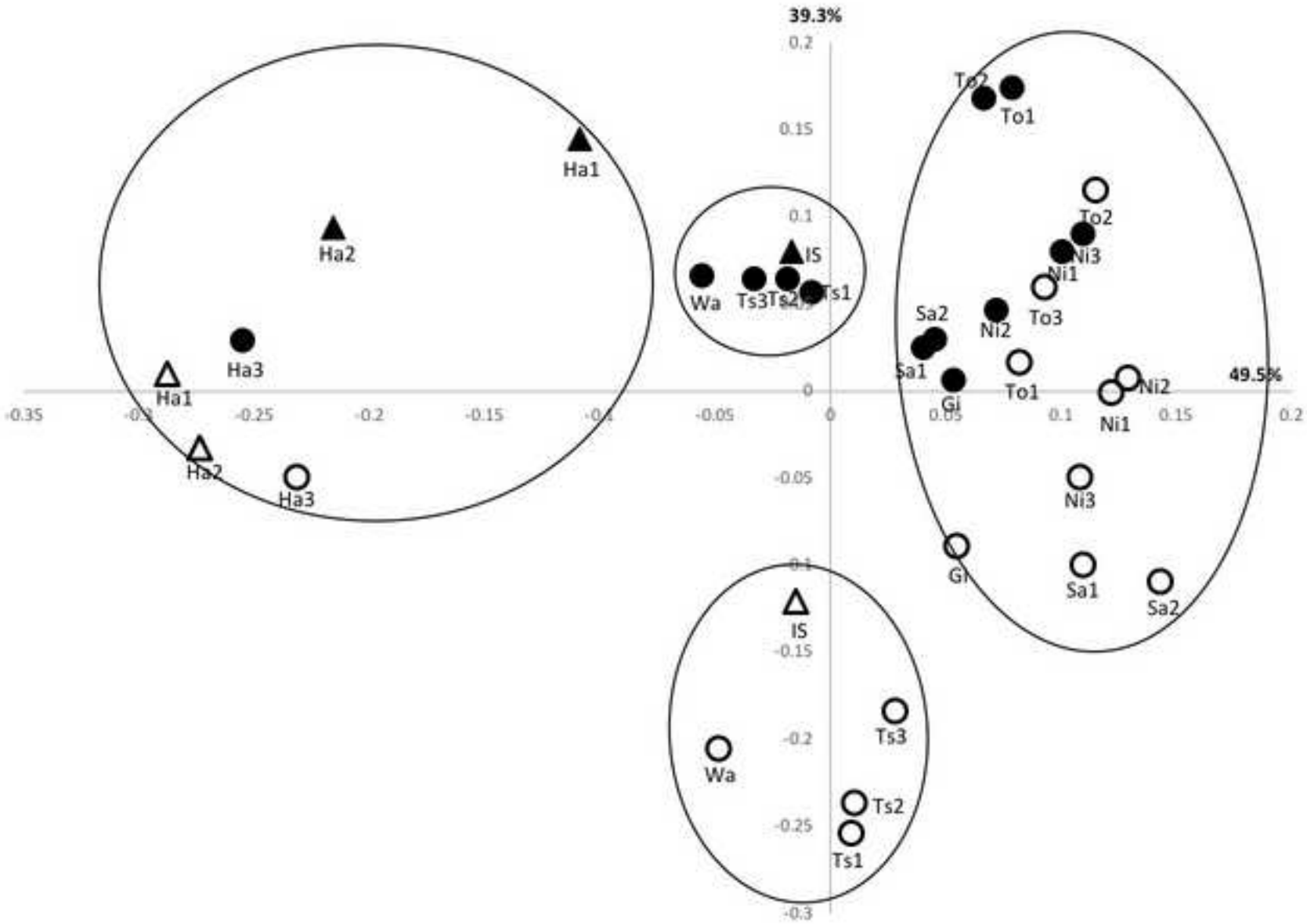

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Fig. 2B

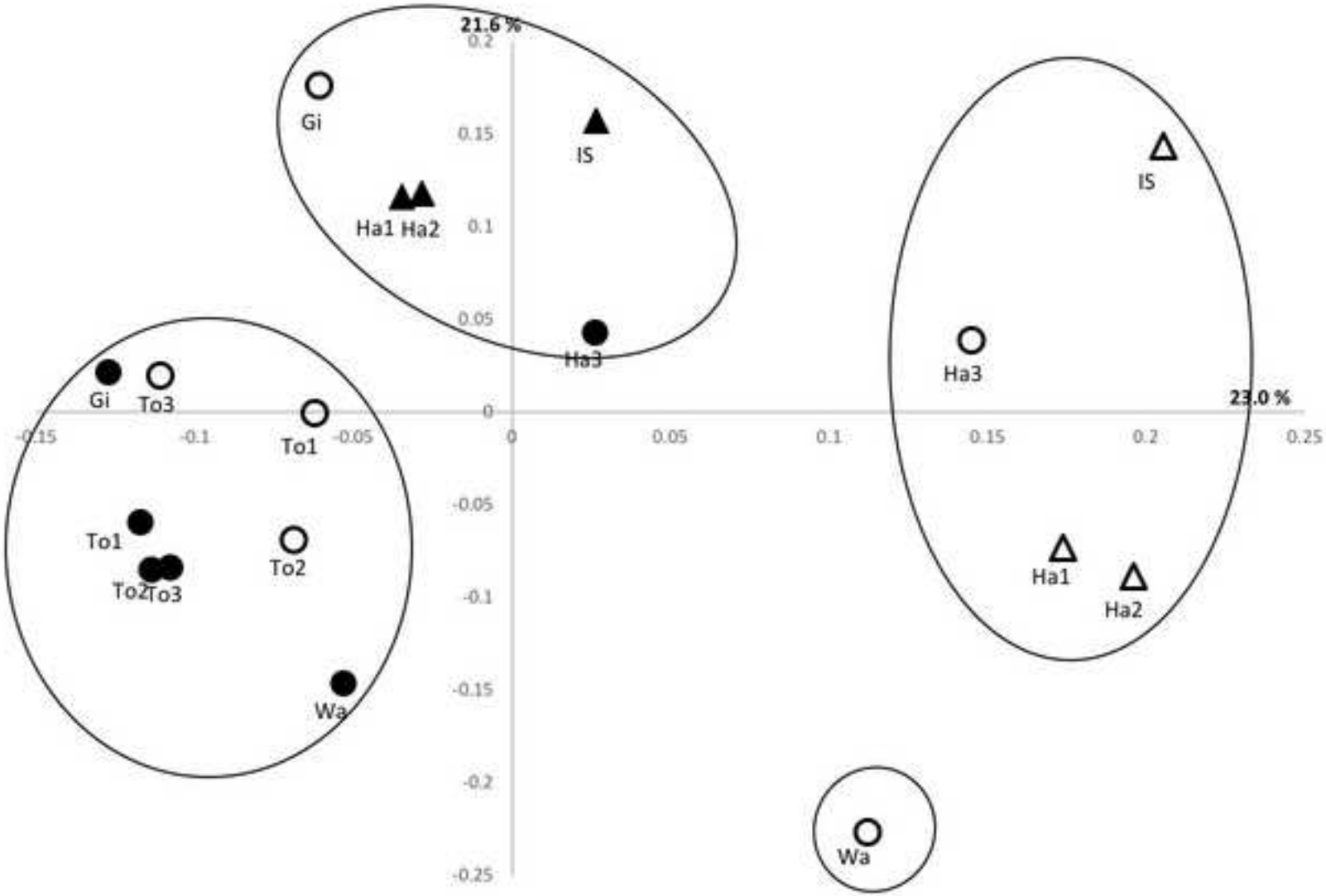

Figure3

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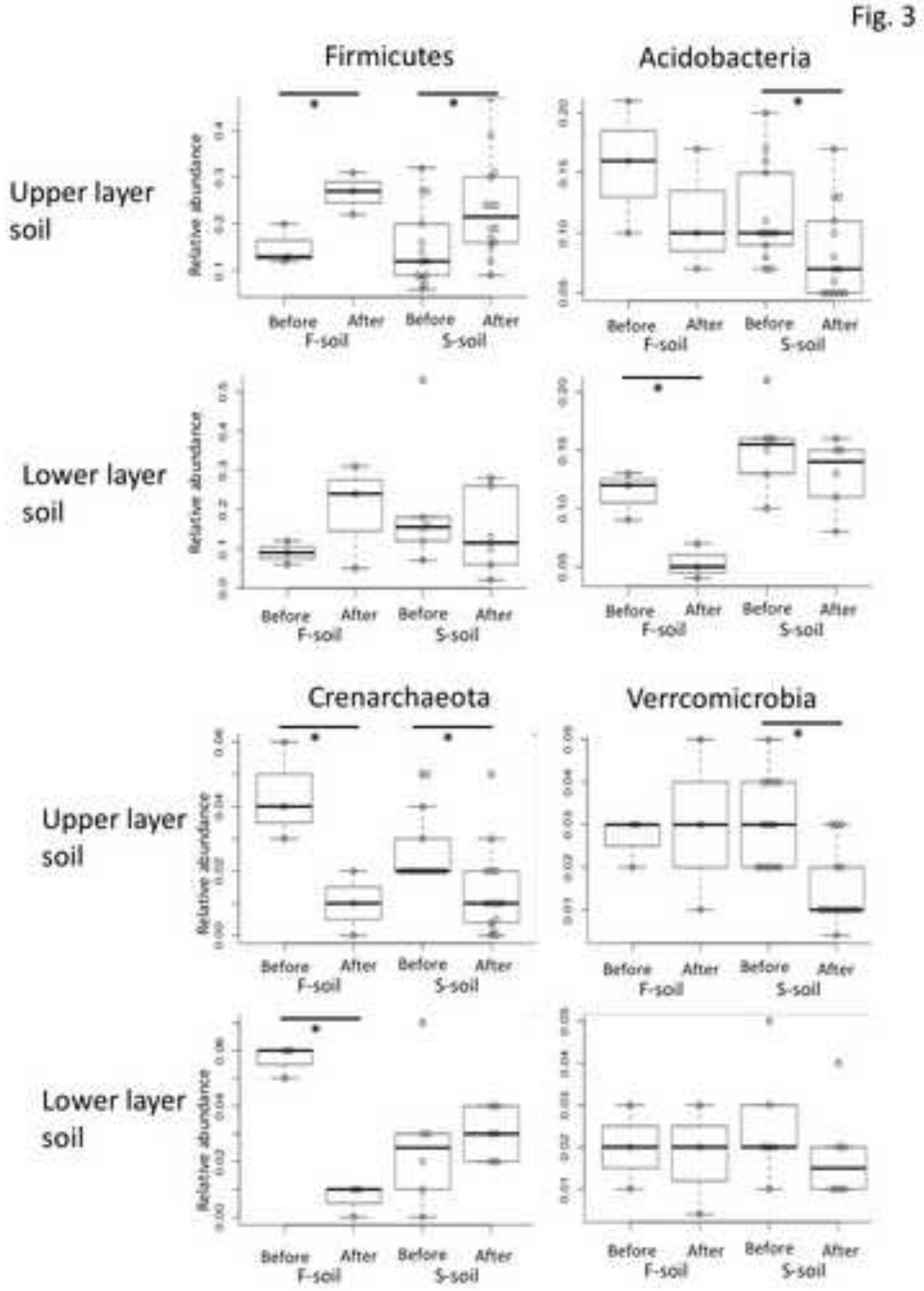

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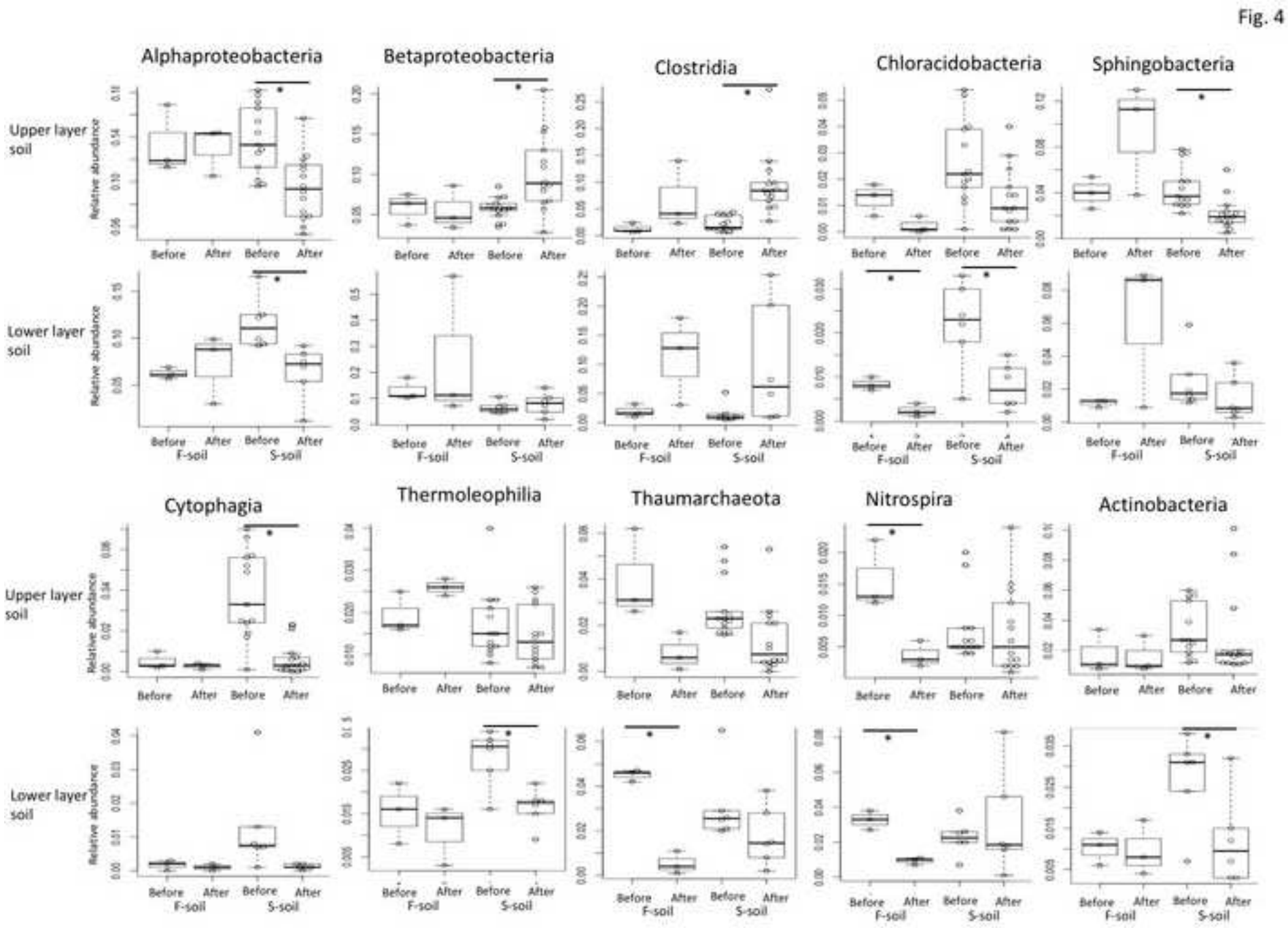

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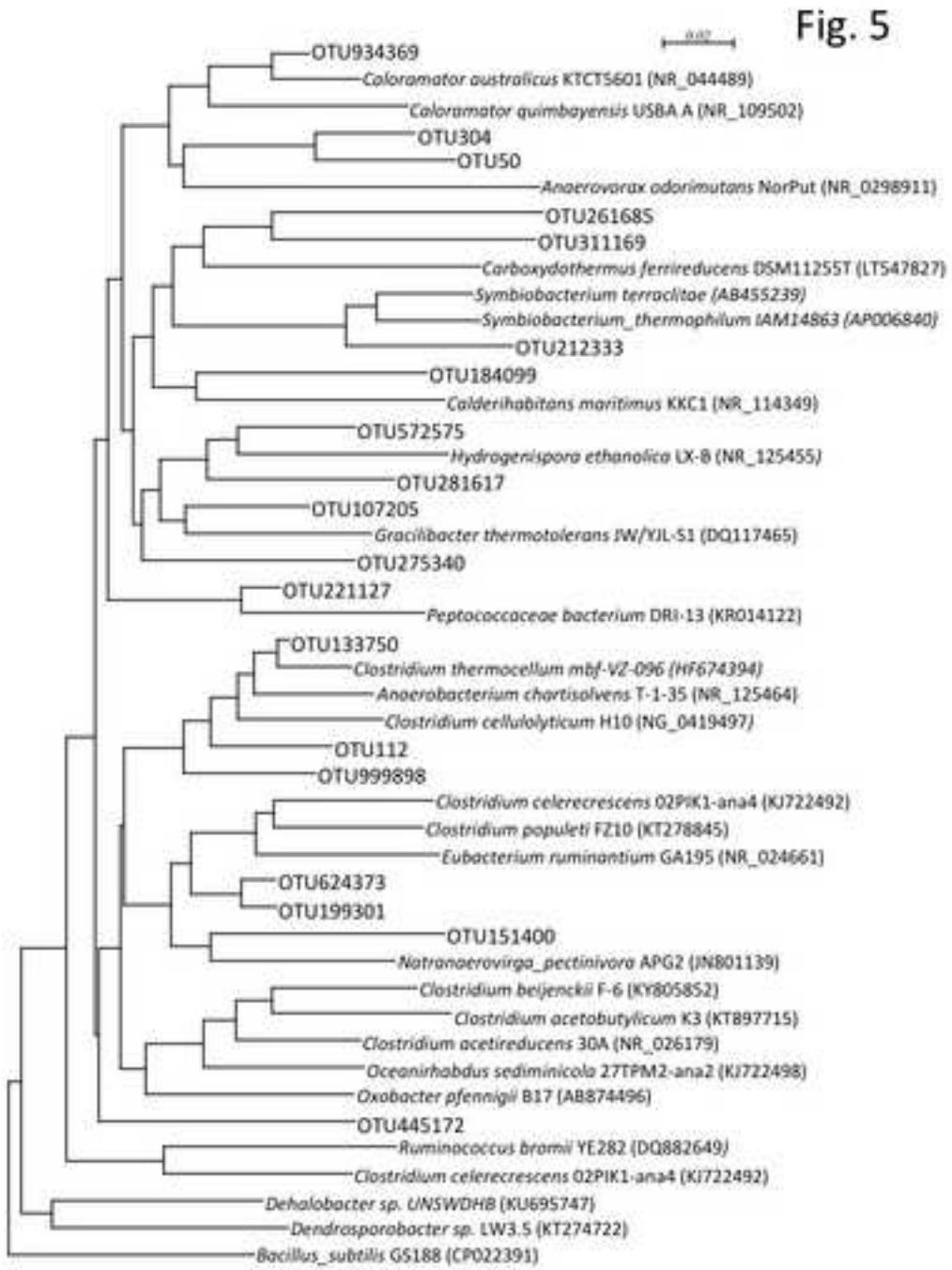

Table1
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Table 1. Detailed information on the sampling fields

Field	Ishikawa	Hakusan	Niigata	Tsubame	Sanjo	Toyama	Gifu	Wakayama
Code	IS	Ha	Ni	Ts	Sa	To	Gi	Wa
latitude and longitude	36° 38′ N, 136° 42′ E	36° 29′ N, 136° 30′ E	37° 55′ N, 139° 9′ E	37° 38′ N, 138° 55′ E	37° 39′ N, 139° 57′ E	36° 61′ N, 136° 96′ E	35° 44′ N, 136° 70′ E	33° 27′ N, 135° 14′ E
Soil types	Gleysol	Gleysol	Fluvisol	Fluvisol	Fluvisol	Gleysol	Gleysol	Cambisols
Replication	1	3	3	3	2	3	1	1
pH	5.54	7.22	7.11	7.03	7.24	6.07	6.08	7.49
EC (mS cm-1)	0.03	0.31	0.32	0.24	0.20	0.29	0.12	0.88
ASD conducted schedule	7/27/2016 - 8/18/2016	7/11/2016 - 7/28/2016	8/18/2016 - 9/9/2016	7/31/2016 - 8/21/2016	9/1/2016 - 9/22/2016	8/2/2016 - 8/26/2016	8/11/2016 - 9/1/2016	7/29/2016 - 8/19/2016

Table2

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Table 2. The number of *R.solanacearum* in each field before and after ASD treatment

	Soil layer	ASD treatment	IS	Ha1	Ha2	Ha3	Ni1	Ni2	Ni3	Ts1	Ts2	Ts3	San1	San2	To1	To2	To3	Gi	Wa
Number of *R.solanacearum* in soil (cfu soil^{-1})	Upper	Before	> 2400	1100	46	23	95	210	460	95	75	120	160	43	64	43	23	> 2400	> 2400
		After	75	21	15	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Lower	Before	240	1100	39	13	-	-	-	-	-	-	-	-	11	53	12	1100	1100
		After	3	21	11	N.D.	-	-	-	-	-	-	-	-	N.D.	N.D.	N.D.	N.D.	N.D.

The number of each fields indicate replication number

N.D.; *R. solanacearum* were not detected with this method.

-; No samples

Supplemental Table1
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Supplemental Table 1. Read numbers of the 16S rRNA gene in each soil sample

			Before	After
IS	IS	Upper	48,338	56,750
		Lower	51,336	66,408
	Ha-1	Upper	50,451	41,012
		Lower	54,223	65,242
	Ha-2	Upper	50,052	50,403
		Lower	54,362	65,817
	Ha-3	Upper	44,053	158,609
		Lower	50,653	56,204
To	1	Upper	49,838	52,458
		Lower	42,379	44,913
	2	Upper	120,447	106,275
		Lower	46,493	50,754
	3	Upper	-	55,666
		Lower	47,673	56,354
Ni	Shi1	Upper	70,925	58,473
	Shi2	Upper	65,166	65,462
	Shi3	Upper	63,392	63,830
	Tsu1	Upper	67,593	69,027
	Tsu2	Upper	60,868	58,135
	Tsu3	Upper	59,287	60,065
	San1	Upper	79,424	55,336
	San2	Upper	64,800	60,657
Gi		Upper	54,337	62,183
		Lower	58,254	47,825
Wa		Upper	57,969	61,009
		Lower	55,401	57,752

Supplemental Table2

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Supplemental Table 2. The change rate of the soil chemical properties or prokaryotic diversity, richness and DNA concentration before to after ASD treatment.

		IS				To			Ni								Gi	Wa
		IS	Ha-1	Ha-2	Ha-3	1	2	3	Shi1	Shi2	Shi3	Tsu1	Tsu2	Tsu3	San1	San2		
DNA concentration ($\mu g\ g\ soil^{-1}$)	Upper	0.20	0.26	0.49	0.31	0.32	0.32	0.30	0.50	0.59	0.58	0.35	0.64	0.23	1.35	1.66	0.27	0.45
	Lower	0.34	0.23	0.53	0.44	0.32	0.21	0.24	-	-	-	-	-	-			3.5	0.89
Shannon index	Upper	0.96	0.92	0.84	1.00	0.99	1.00	-	0.99	1.01	0.92	0.86	0.88	0.87	0.97	0.97	0.97	0.90
	Lower	0.79	0.90	0.83	0.95	0.99	0.98	0.98	-	-	-	-	-	-			0.80	0.87
Observed OTU	Upper	0.77	0.62	0.58	0.98	0.9	0.59	-	0.98	0.96	0.82	0.52	0.51	0.53	0.94	0.88	0.99	0.76
	Lower	0.65	0.78	0.65	0.80	0.91	1.03	0.77	-	-	-	-	-	-			0.32	0.66
Chao 1 estimator	Upper	0.08	0.62	0.64	0.98	0.88	0.84	-	0.96	0.99	0.82	0.55	0.54	0.58	0.99	0.89	1.01	0.72
	Lower	0.59	0.68	0.59	0.82	0.91	0.94	0.88	-	-	-	-	-	-			0.44	0.67

The red and blue cells indicate increasing or decreasing factors after ASD treatment compared with the before treatment. The color intensities indicates the rate of each difference.

-; No samples

Supplemental Table3

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Supplemental Table 3. The change rate of the soil prokaryotic phylum before to after ASD treatment.

		IS				To			Ni								Gi	Wa
		IS	Ha-1	Ha-2	Ha-3	1	2	3	Shi1	Shi2	Shi3	Tsu1	Tsu2	Tsu3	San1	San2		
Proteobacteria	Upper	1.00	0.85	1.01	0.81	1.26	0.93	-	1.09	1.08	1.46	0.58	0.62	0.72	1.24	1.26	0.94	0.39
	Lower	1.79	1.07	1.52	1.07	1.28	1.30	1.18	-	-	-	-	-	-	-	-	1.18	0.79
Firmicutes	Upper	1.54	1.84	0.82	2.39	2.11	1.42	-	1.09	0.80	1.34	2.64	2.83	2.95	0.98	0.71	1.43	3.85
	Lower	0.87	3.45	2.02	1.93	0.59	0.73	0.28	-	-	-	-	-	-	-	-	0.54	0.12
Acidobacteria	Upper	0.66	1.06	1.66	0.64	0.68	1.03	-	0.65	0.71	0.35	0.59	0.65	0.70	0.53	0.49	1.17	1.22
	Lower	0.42	0.54	0.37	0.48	1.05	0.88	1.90	-	-	-	-	-	-	-	-	1.17	0.34
Bacteroidetes	Upper	0.56	3.51	0.87	2.07	1.17	0.60	-	0.86	0.79	1.05	0.10	0.17	0.07	1.13	1.86	0.23	0.40
	Lower	0.46	4.15	5.44	2.04	1.42	1.39	0.55	-	-	-	-	-	-	-	-	0.30	0.47
Chloroflexi	Upper	1.39	0.94	1.03	0.78	0.70	1.77	-	1.35	1.44	0.79	2.53	2.03	2.68	1.30	1.36	0.92	0.72
	Lower	0.45	0.73	0.64	1.16	0.75	1.05	1.23	-	-	-	-	-	-	-	-	0.90	0.52
Gemmatimonadetes	Upper	0.80	0.83	1.54	1.18	0.93	0.84	-	0.74	0.79	0.52	2.30	2.33	1.63	0.92	1.10	0.55	0.90
	Lower	0.45	0.44	0.33	0.62	1.07	0.89	1.02	-	-	-	-	-	-	-	-	1.36	0.59
Actinobacteria	Upper	1.05	1.30	1.03	1.08	1.30	0.98	-	0.68	0.76	0.64	1.52	1.09	0.88	0.70	0.71	0.41	0.42
	Lower	0.75	0.87	0.78	0.68	1.02	0.52	0.62	-	-	-	-	-	-	-	-	0.67	0.20
Crenarchaeota	Upper	0.29	0.19	9.36	0.00	0.50	1.24	-	0.53	1.21	0.27	0.23	0.20	0.96	0.17	0.27	0.50	0.14
	Lower	0.12	0.19	0.04	0.59	1.02	1.02	0.55	-	-	-	-	-	-	-	-	1.17	5.82
Verrucomicrobia	Upper	0.47	0.89	0.64	1.35	0.53	0.78	-	0.79	1.01	0.60	0.27	0.40	0.16	0.49	0.38	0.20	0.26
	Lower	0.32	1.29	0.82	0.65	1.58	0.61	1.84	-	-	-	-	-	-	-	-	0.36	0.85
Planctomycetes	Upper	0.81	0.59	1.10	0.78	0.44	1.15	-	0.78	0.79	0.59	0.58	0.49	0.98	0.66	0.83	0.74	0.31
	Lower	0.32	0.37	0.39	0.58	1.11	0.67	1.43	-	-	-	-	-	-	-	-	1.29	0.07

The red and blue cells indicate increasing or decreasing factors after ASD treatment compared with the before treatment. The color intensities indicates the rate of each difference.

-; No samples

Supplemental Table4

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Supplemental Table 4. The change rate of the soil prokaryotic class before to after ASD treatment.

			IS				To			Ni								Gi	Wa
			IS	Ha-1	Ha-2	Ha-3	1	2	3	Shi1	Shi2	Shi3	Tsu1	Tsu2	Tsu3	San1	San2		
Proteobacteria	Alphaproteobacteria	Upper	0.88	1.273	0.85	0.945	1.242	0.928	-	0.803	0.833	0.955	0.346	0.376	0.408	0.844	0.761	0.518	0.405
		Lower	0.52	1.435	1.452	0.563	0.997	0.754	0.678	-	-	-	-	-	-	-	-	0.129	0.324
	Betaproteobacteria	Upper	1.34	0.459	1.229	1.329	2.182	1.295	-	1.491	1.309	3.709	0.89	1.205	1.822	3.358	3.412	1.009	0.491
		Lower	3.16	1.026	0.695	1.957	2.069	1.463	1.36	-	-	-	-	-	-	-	-	0.45	0.433
	Deltaproteobacteria	Upper	0.86	0.66	1.045	1.307	0.936	0.758	-	1.163	1.581	0.797	1.676	1.555	1.25	0.755	0.873	2.116	0.449
		Lower	1.02	0.953	1.036	2.279	1.335	0.924	1.117	-	-	-	-	-	-	-	-	1.383	0.329
	Gammaproteobacteria	Upper	1.00	0.772	0.596	0.375	0.755	0.78	-	1.242	1.067	1.071	0.245	0.393	0.345	0.808	1.172	0.56	0.205
		Lower	0.30	0.88	4.755	0.411	0.919	0.832	0.342	-	-	-	-	-	-	-	-	0.284	0.274
Firmicutes	Bacilli	Upper	0.96	1.798	1.865	1.934	1.048	1.077	-	0.718	0.663	0.646	1.826	1.981	2.518	0.821	0.458	1.096	1.773
		Lower	0.47	1.736	1.291	0.868	0.542	1.11	0.688	-	-	-	-	-	-	-	-	0.2	2.731
	Clostridia	Upper	5.79	2.375	6.151	5.619	11.921	4.588	-	2.266	1.354	3.242	7.663	7.569	5.503	2.804	3.574	3.132	21.455
		Lower	3.017	11.363	3.987	3.901	5.254	7.113	2.289	-	-	-	-	-	-	-	-	1.46	16.45
Acidobacteria	Acidobacteria-6	Upper	0.66	0.216	0.444	0.639	0.561	1.706	-	0.729	0.736	0.422	0.594	0.676	0.637	0.727	0.701	0.628	0.285
		Lower	0.251	0.246	0.257	0.589	0.531	0.515	0.818	-	-	-	-	-	-	-	-	0.382	0.216
	Acidobacteriia	Upper	0.466	1.995	0.637	0.826	0.834	0.612	-	0.726	0.798	0.373	0.299	0.494	0.877	5.479	3.111	1.75	0.293
		Lower	0.44	1.385	0.81	0.436	2.013	1.525	3.538	-	-	-	-	-	-	-	-	1.08	0.129
	Solibacteres	Upper	2.782	1.361	0.882	1.372	1.292	0.808	-	0.733	0.527	0.742	2.317	1.496	1.581	1.118	1.231	0.701	5.314
		Lower	1.068	0.841	0.778	0.587	0.974	1.05	1.949	-	-	-	-	-	-	-	-	0.895	3.996
	[Chloracidobacteria]	Upper	0.329	0.06	0.037	0.926	0.735	1.022	-	0.38	0.384	0.346	0.053	0.231	0.041	0.33	0.267	0.669	0.352
		Lower	0.204	0.259	0.401	0.74	0.868	0.349	0.366	-	-	-	-	-	-	-	-	0.069	0.185
Bacteroidetes	Sphingobacteria	Upper	0.698	4.267	3.244	1.958	0.613	0.57	-	0.991	0.606	0.282	0.183	0.324	0.063	0.298	0.364	0.521	0.82
		Lower	0.644	6.449	10.178	1.27	0.582	0.526	0.292	-	-	-	-	-	-	-	-	0.188	0.609
	Cytophagia	Upper	0.051	1.157	2.666	2.625	0.163	0.325	-	0.401	0.412	0.379	0.001	0.003	0.018	0.139	0.271	0.041	0.011
		Lower	0.139	1.044	2.852	0.691	0.287	0.212	0.212	-	-	-	-	-	-	-	-	0.05	0.012
Chloroflexi	Anaerolineae	Upper	2.26	0.072	0.064	1.752	0.476	2.129	-	1.884	1.859	0.919	0.851	1.12	3.981	1.56	2.082	1.365	0.302
		Lower	0.47	0.389	0.606	1.719	0.853	1.309	2.35	-	-	-	-	-	-	-	-	1.293	0.149
Gemmatimonadetes	Gemmatimonadetes	Upper	0.94	1.829	0.782	1.442	1.018	0.824	-	0.647	0.744	0.559	4.01	4.075	2.878	0.654	0.654	0.387	0.874
		Lower	0.89	1.118	0.524	0.629	1.134	0.856	0.487	-	-	-	-	-	-	-	-	0.075	0.627
	Gemm-1	Upper	0.596	0.198	0.051	0.424	0.728	0.761	-	0.83	0.683	0.427	1.102	0.96	0.754	1.325	1.807	0.654	0.791
		Lower	0.284	0.236	0.274	0.621	1.005	0.827	1.452	-	-	-	-	-	-	-	-	0.516	0.716
Actinobacteria	Actinobacteria	Upper	0.88	1.057	0.921	0.742	1.4	0.922	-	0.699	0.738	0.56	1.913	1.395	0.858	0.753	0.706	0.306	0.312
		Lower	1.279	1.334	0.407	0.414	0.362	1.05	0.208	-	-	-	-	-	-	-	-	0.145	0.406
	Thermoleophilia	Upper	1.484	1.776	0.943	1.359	1.343	0.916	-	0.805	0.89	0.958	1.077	0.652	1.096	0.737	0.779	0.466	0.565
		Lower	0.397	0.728	0.902	0.462	0.707	0.537	0.613	-	-	-	-	-	-	-	-	0.559	0.632
Crenarchaeota	Thaumarchaeota	Upper	0.284	0.209	0.027	0.003	0.5	1.237	-	0.529	1.226	0.253	0.194	0.193	0.902	0.13	0.233	0.451	0.139
		Lower	0.093	0.239	0.034	0.581	0.527	0.616	0.281	-	-	-	-	-	-	-	-	1.326	0.112
Nitrospirae	Nitrospira	Upper	0.417	0.127	0.19	0.241	0.609	1.283	-	1.422	1.748	0.747	0.438	0.926	0.873	0.53	0.377	1.773	0.288
		Lower	0.413	0.184	0.317	0.695	0.98	0.813	1.872	-	-	-	-	-	-	-	-	2.201	0.186