1 **Title**

2 Comparison of prokaryotic communities between the fields showing different

3 disinfestation effects by anaerobic soil disinfestation

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Abbreviations

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

Keywords

Bacterial wilt, Betaproteobacteria, Clostridia, Indicator species analysis, Multiple fields

Abstract

Soil-borne pathogens are serious problems for crop production. Chemical-dependent soil fumigation method has been an effective method for controlling soil-borne pathogens, but it is escaped for food safety and environmental pollution. Anaerobic soil disinfestation (ASD) is a chemical-independent fumigation method and can reduce 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 different condition. If common microbes are shown in well-disinfested multiple fields, they will be a good indicator for the success of ASD. For this purpose, we conducted the same ASD treatment with 17 tomato greenhouses where tomato bacterial wilt occurred in the geographically different areas. In this study, we compared soil prokaryotic communities in the field which indicate different disinfestation effects after ASD treatment among several fields using the next-generation sequencer. Weighted-UniFrac analysis showed that prokaryotic communities in the fields showing different disinfestation effect were not different and they were roughly separated to sampling fields. The relative abundance

of *Betaproteobacteria* and *Clostridia* were significantly increased in well- disinfested fields. Indicator species analysis revealed that twenty-five operational taxonomic units (OTUs) were specifically detected from various 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 that the prokaryotic communities were not largely differed by ASD efficiency but small changing will be important for ASD.

1. Introduction

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

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

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

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

indicator for ASD treatment. For this purpose, we conducted the ASD treatment with same substrate in 17 geographically different greenhouses and they showed different disinfestation efficiency. We compared the prokaryotic communities among the fields showing different disinfestation effect. This multi-fields study will show that 1) microbial community may differ depending on disinfestation efficiency and 2) the well-disinfested soil may have unique microbes compare with not-well-disinfested soil among various fields.

2. Materials and Methods

2.1. Sampling field and ASD treatment

Field experiments were established in 17 greenhouses in 8 fields in Japan in which occurrences of bacterial wilt were observed (Table 1). Sugar-containing diatoms, defined as a sugar-rich byproduct, are a waste discharged from the food-processing industry that 40 % sugar in weight. The sugar-containing diatoms were mixed into the soil at a ratio of 15 t ha^{-1} with the rototiller in 30 cm soil layer. The field was covered by

polyethylene and flooded with water more about 150 L m^{-2}. Disinfestation was conducted 21 days excepted for Ha field (17 days), because soil temperature in this field were reached more than 35 °C during disinfestation period. ASD were treated in the greenhouse (15 m × 6 m) and replicates were conducted among different greenhouses. There were three replications in the Ha, Ni, Ts, and To fields, two replications in the Sa field, and no replication in the IS, Gi, and Wa fields. The samples were collected from each greenhouse before and after ASD treatment from two different depths (20-30 cm and 40-50 cm) in each greenhouse using a Gauge Auger DIK-106B core sampler (Daiki Rika Kogyo Co., LTD, Saitama, Japan) in Is, Ha, To, Gi, and Wa fields (9 greenhouse × 4 soil samples, Totally 36 soil samples). In Ni, Ts and Sa field, only two soil samples (before and after ASD treatment in the upper layer soil) were collected from each greenhouse in these fields (8 greenhouse × 2 soil samples, Totally 16 soil samples). A total of 52 soil samples were collected for analysis. Soil samples were collected from 5 randomly chosen points in each greenhouse and mixed well.

2.2 Quantification of *R. solanacearum* in the field

120 The most probable number (MPN)-PCR method, which is semi-quantitive *R*.
121 *solanacearum* counting method, was conducted as described by Inoue and Nakaho
122 (2014). Briefly, 10 g of soil was eluted in cultivation buffer and the soil extract was
123 diluted with buffer 10, 100, and 1000 times. Each aliquot was incubated at 35 °C for
124 approximately 24 h, and nested-PCR targeting *phcA* gene, which is a key gene for the
125 appearance of wilt disease, was conducted on the culture sample using the primer pairs
126 phcA2981f (5′-TGGATATCGGGCTGGCAA-3′) and phcA4741r
127 (5′-CGCTTTTGCGCAAAGGGA-3′) for the first step and phcA3538f
128 (5′-GTGCCACAGCATGTTCAGG-3′) and phcA4209r
129 (5′-CCTAAAGCGCTTGAGCTCG-3′) for the second step. The number of samples that
130 appeared in the PCR amplification around 700 bp length was collectively checked
131 against the MPN table. The detection limit of *R.solanacearum* by this method is 3 to
132 2400 colony forming units (cfu) g^{-1} dry soil and a detection indicates the risk of
133 outbreaking of bacterial wilt in the field.
134

135 **2.3 Tag-encoded amplicon sequencing targeted by 16S rRNA gene for prokaryotes**

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

fragments, and was quantified using a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA). After successful amplification, the PCR products were adjusted to equimolar concentrations and subjected to unidirectional pyrosequencing at Bioengineering Lab. Co., Ltd. (Kanagawa, Japan) on a MiSeq instrument (Illumina). A total of 3,114,333 sequences in the 51 samples were obtained after sequencing (Supplemental Table 1). Sequencing data were deposited in the DNA Data Base of Japan (DDBJ) Sequence Read Archive under accession number DRA006673.

2.4 Data analyses

The analysis of the sequencing data was conducted according to Lee et al. (2017). Raw fasta files were pre-processed in the Quantitative Insights into Microbial Ecology (QIIME) bioinformatic pipeline (Caporaso et al. 2010). Data from the read sequences, quality, flows, and ancillary metadata were analyzed using the QIIME pipeline. Quality filtering consisted of discarding reads < 200 bp or > 1000 bp in length, excluding homopolymer runs of > 6 bp and continuous ambiguous bases of > 6, and accepting one barcode correction and two primer mismatches. Moreover, a mean quality score < 25

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

verify the effects of sampling fields and ASD treatment on prokaryotic community. Finally, indicator value analysis was done using the indicspecies R package (De Cáceres 2009) with the aim of identifying OTUs associated with suppressive soil, rather than the conducive soil or vice versa ($p < 0.05$). We created two patterns of soil groups. One group was divided into samples from the soil before or after ASD treatment. The other group was divided based on the efficiency of disinfestation. The datasets were merged and the abundant OTUs that were associated with the pathogen non-detected soil after ASD treatment were evaluated in each layer ($p < 0.001$). The number of random permutation tests for the calculation of the indicator values was 999.

2.5 Construction of phylogenetic tree

The phylogenetic tree of clostridial members was constructed by a 1000-fold bootstrap analysis using neighbor-joining methods with the Clustal W program (version 2.0 Developments) software (Larkin et al. 2007) and the NJ plot software (Perrière and Gouy 1996). The phylogenetic tree contained 16S rRNA sequences of several clusters of clostridial strains.

200

201 ## 3. Results

202

203 ### 3.1. Effectiveness of ASD treatment with sugar-containing diatoms

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

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

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

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

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

209 ASD: not-well-disinfested (disinfestation failure) soil as F-soil and well-disinfested

210 (disinfestation success) soil as S-soil.

211

212 ### 3.2 Changes of prokaryotic communities after ASD treatment

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

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

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

indices and Chao1 were decreased after ASD in the upper. OTU numbers were significantly decreased in F-soil regardless of soil layers.

PCoAs based on weighted-UniFrac analysis showed that prokaryotic communities were roughly classified by sampling fields (Figure 2). In the upper layer, Ha fields contained one cluster regardless of ASD treatment and disinfestation efficiency (Figure 2A). IS, Wa and Ts fields were separated by before and after ASD treatment but not affected by disinfestation efficiency. In the lower layer soil, IS, Ha, and Gi were also different among sampling fields and ASD treatment, but not different by S- and F-soil (Figure 2B). The microbes collected from same fields comprised same clusters before and after ASD treatment in spite of ASD efficiency was different.

After ASD treatment, the relative abundance of *Firmicutes* was increased by more than 2-times in 8 greenhouses (Supplemental Table 3). In the upper layer soil, *Firmicutes* was significantly increased, while *Crenarchaeota* was decreased after ASD treatement (Figure 3). *Acidobacteria* and *Verrcomicrobia* in the upper layer of the S-soil, *Acidobacteria*, and *Crenarchaeota* in the lower layer of the F-soil were significantly decreased. In the class level, *Clostridia* that belonged to the *Firmicutes*

phylum were increased more than 2-times after ASD in 15 of 16 and 8 of 9 greenhouses in the upper and lower layers, respectively (Supplemental Table 4). Moreover, *Betaproteobacteria* and *Clostridia* were significantly increased after ASD treatment in the upper layer of the S-soil (Figure 4). In the S-soil, *Sphingobacteria* and *Cytophagia* were significantly decreased in the upper layer, and *Thermoleophilia* and *Actinobacteria* were decreased in the lower layer.

3.4 Specifically increased OTUs in S-soil

We successfully detected 25 OTUs specifically increased in the S-soil by ASD from more than 8 of the 16 sites or 4 of the 6 sites from the upper and lower layers, respectively (Table 3). They belonged to several classes, including *Clostridia*, *Bacillus*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Mollicutes*. Eighteen out of the 25 OTUs belonged to *Clostridia*. The phylogenetic trees consisting of OTUs related to clostridial members showed that diversified *Clostridia* were specifically increased after ASD treatment in the S-soil (Figure 5). Three OTUs belonged to the *Caloramator* group, *Clostridium* cluster III and two OTUs belonged to the *Christensenella* group. OTU 50

248 (*Caloramator*) was commonly detected in 11 upper layer and five lower layer soil
249 samples. OTU311169 (*Bacillus*), OTU199301 and OTU624373 (*Christensenella*), OTU
250 112 (*Clostridium*), and OTU56 (*Rhodothermaceae*) were also increased in 10 of the 14
251 S-soils, while OTU934369 and OTU304 (*Caloramator*) and OTU212333
252 (*Symbiobacterium*) were commonly detected in more than five of the six fields of S-soil.
253 Prokaryotes decreased by ASD treatment common to the various fields in this criterion
254 were not detected (data not shown).
255

256 **4. Discussion**

257 In this study, we showed that microbial communities were not different even if the
258 fields indicated different disinfestation effect by ASD. Especially, in Ha field, we used
259 three contiguous greenhouses and they treated the same field managements for more
260 than 10 years. They showed a different disinfestation effect but were included in the
261 same cluster. Our previous study also showed that prokaryotic community structures
262 were affected more by indigenous communities than the disease suppression effect (Lee
263 et al. 2017). These results suggested that the difference of indigenous soil communities

264 are not affected to controlling of soil-borne pathogen. Runia et al. (2014) showed
265 Firmicutes are increased by ASD treatment among 5 soil types using the real-time PCR
266 method. We also showed that *Clostridia* belonged to Firmimutes have increased in
267 S-soil. *Clostridia*, are gram-positive anaerobic bacteria. During ASD, decreases in
268 oxygen availability along with increases in pH occur, which promotes the increased
269 prevalence of anaerobic microbes (Momma et al. 2006; Mowlick et al. 2013, 2014).
270 Several studies indicated that *Clostridia* increase in number and become dominant
271 bacteria following ASD treatment using plant material or low concentration ethanol as a
272 substrate (Huang et al. 2015, 2016; Mowlick et al. 2012; Strauss et al. 2017). The prior
273 and present results indicate that *Clostridia* increased after ASD treatment regardless of
274 soil types, field locations, and substrate types and they may be key microbes for
275 disinfestation by ASD. *Caloramator*-like OTUs were the most commonly found OTUs
276 in the upper and lower layers of various fields. They are rod-shaped, obligate anaerobic,
277 thermophilic endospore producers that can ferment several sugars and produce acetate,
278 isobutyrate, lactate, and other VFAs, while they do not degrade cellulose (Collins et al.
279 1994; Ogg and Patel 2011). Mowlick et al. (2012) reported that 63% of clostridial

clones that were increased after ASD treatment belonged to clostridium cluster I, including the *Oxobacter* and *Caloramator* groups. *Symbiobacterium* is rod-shaped thermophiles that are syntrophic with *Bacillus* spp., which possess a glucose degradation pathway (Ohno et al. 2000). *Christensenella* is a non-spore forming, short, straight rod with tapered ends; it can use various sugars and produce VFAs during fermentation (Morotomi et al. 2012). *Christensenella* were detected in an anaerobic cellulolytic microbial consortium from mangrove soil (Gao et al. 2014). These results suggest that phylogenetically diverse clostridial members might be responsible for suppression of pathogens through their production of VFAs during anaerobic decomposition of sugar-containing diatoms.

Strauss et al. (2017) reported the increased of *Betaproteobacteria* by ASD treatment with organic bran at soil depths of 15.2 cm and 45.7 cm. We showed *Noviherbaspirillum*, belonged to *Betaproteobacteria*, which were specifically detected from S-soil. They are gram-negative, aerobic, non-spore forming rods capable of utilizing several carbohydrates as carbon sources (Lin et al. 2013). Moreover, several *Bacillus* species have been described as biological control agents for bacterial wilt and

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

311 In conclusion, we revealed prokaryotic communities were not strongly affected by
312 disinfestation efficiency compare with sampling fields, but *Clostridia* and
313 *Betaproteobacteria* were significantly increased in well-disinfested soil. Twenty-five
314 OTUs were specifically detected in S-soil and most of them were affiliated to *Clostridia*,
315 which was well-known bacteria for ASD effect. However, some microbes were not
316 previously detected from ASD treated fields. These microbes will be a good indicator
317 for well-disinfested soil and future study will appear the role of these microbes for
318 disinfestation.

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462 **Figure legend**

463

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

468

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

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

471 ASD treatment.

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

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

474 treatment.

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

476

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

478 between the S- and F-soil

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

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

481

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

483 between the S- and F-soil

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

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

486

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

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

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

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

491 that specifically appeared in the S-soil.

Figure1

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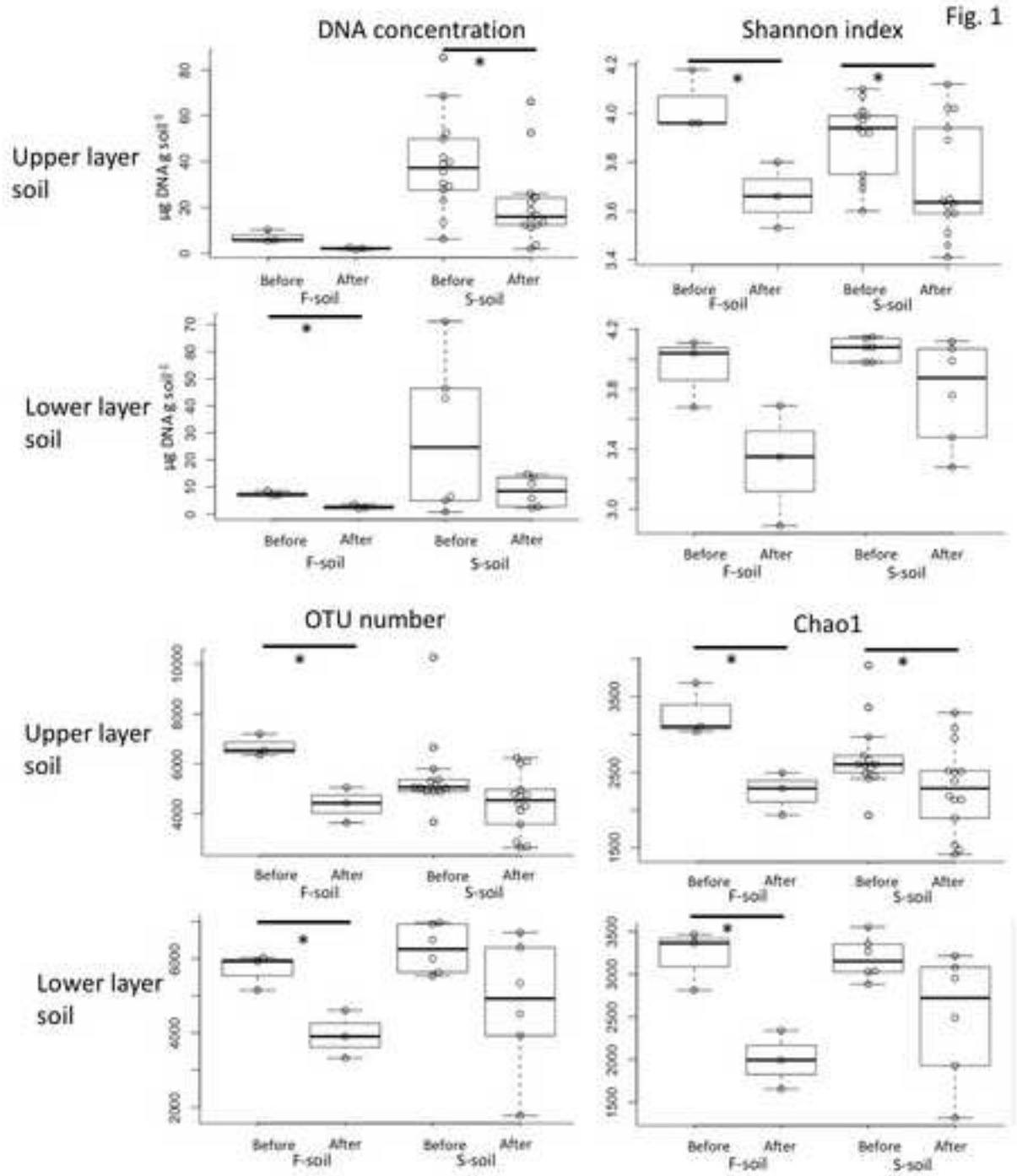

Figure2A

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Fig. 2A

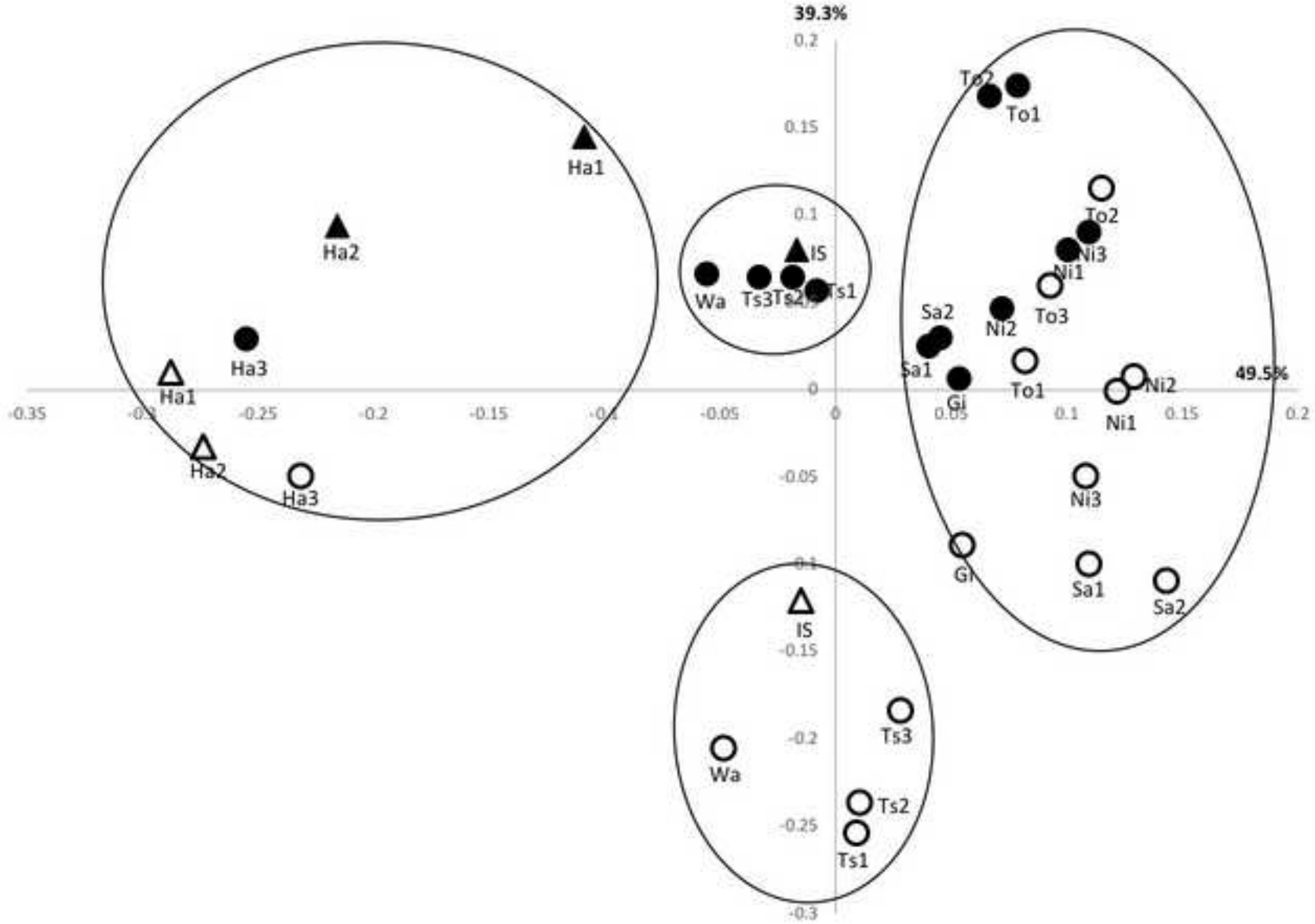

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Fig. 2B

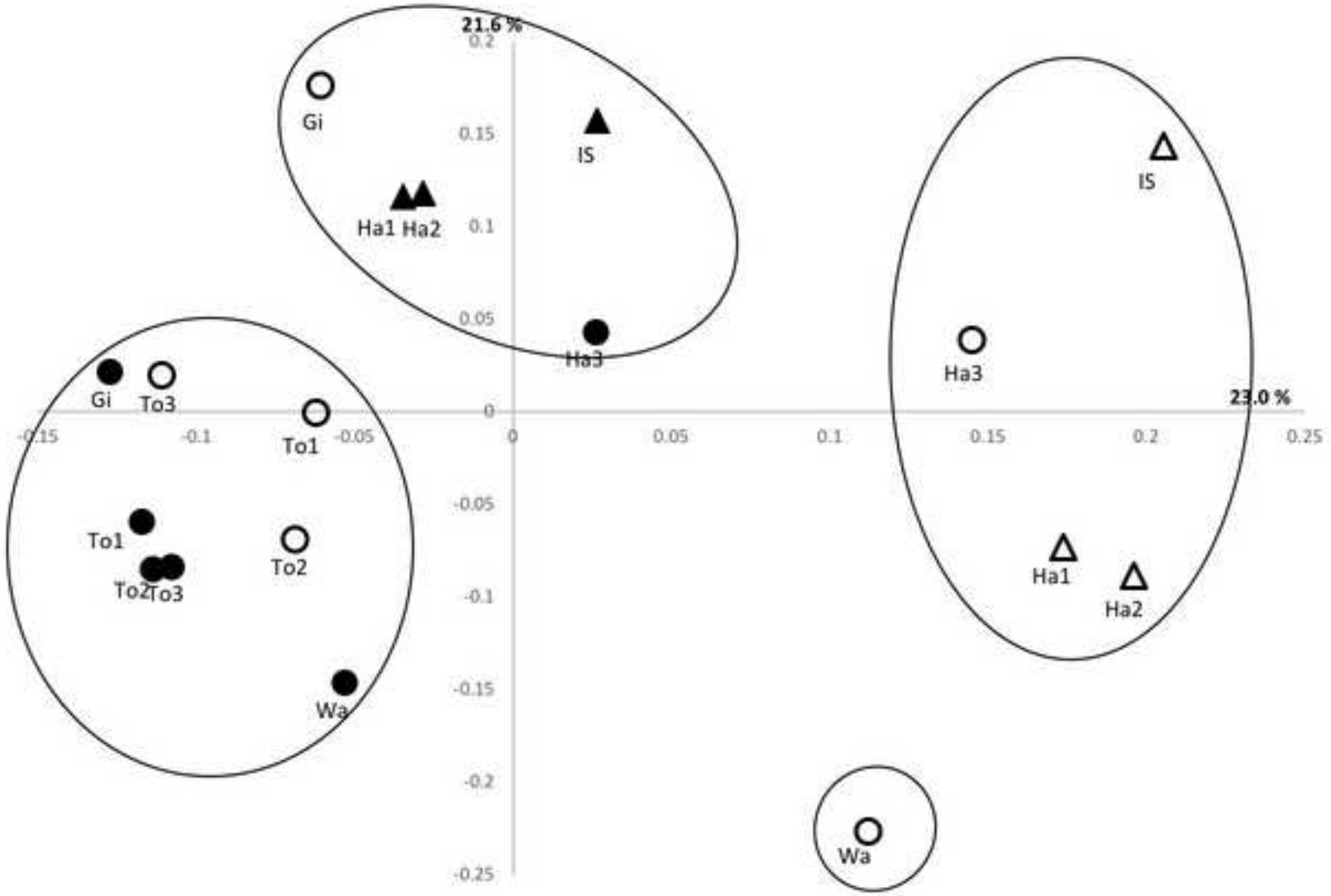

Figure3

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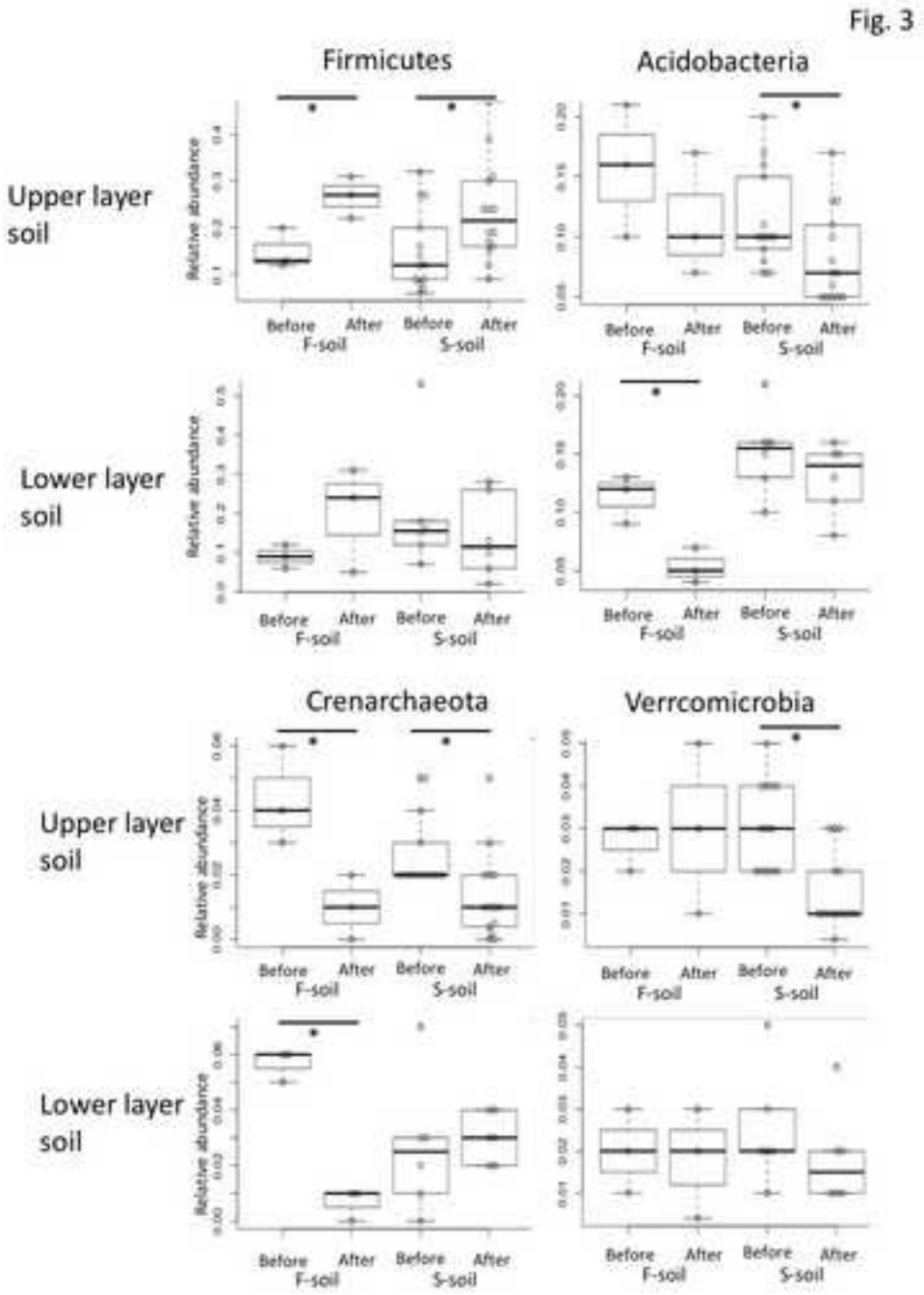

Figure4
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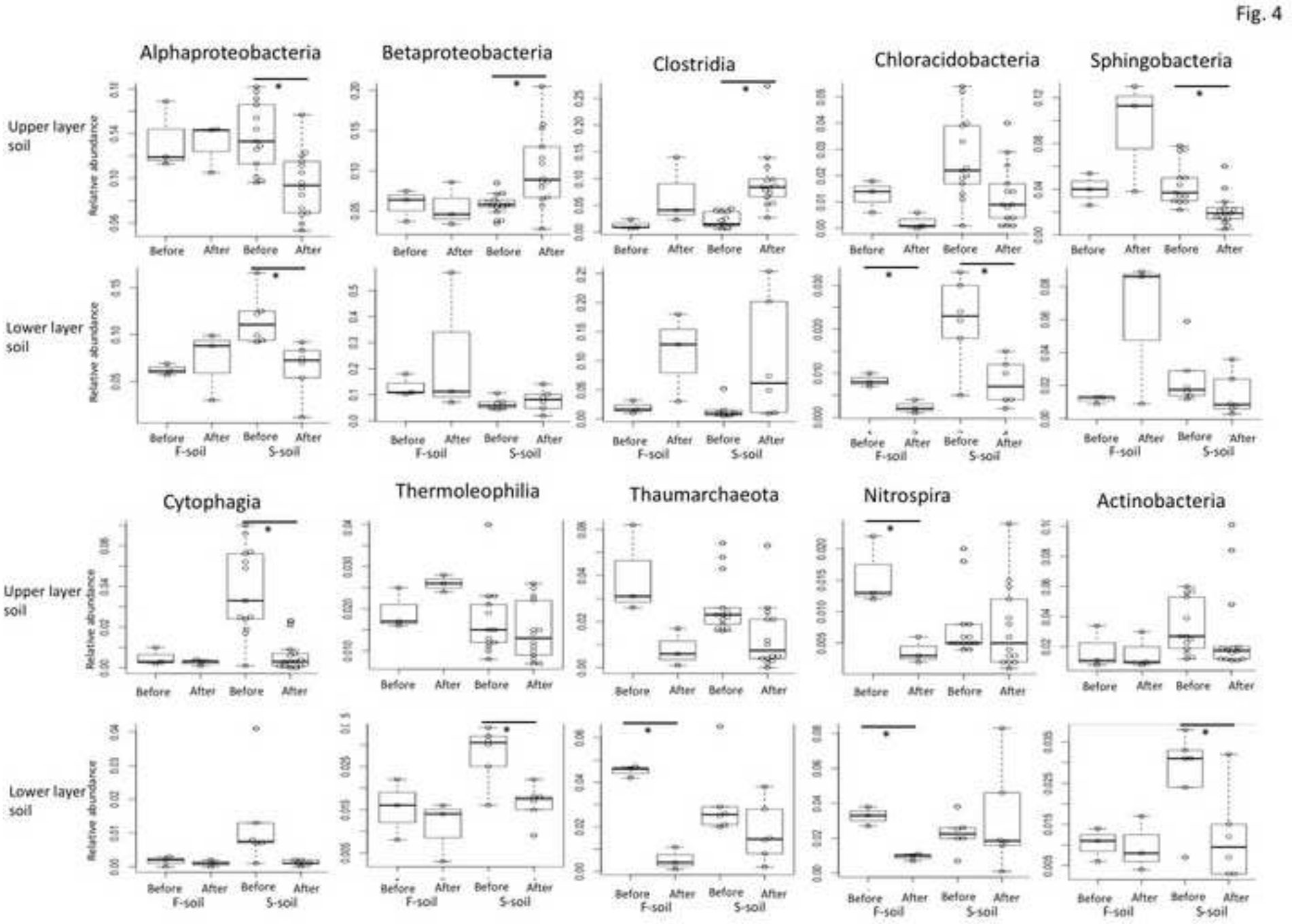

Figure5

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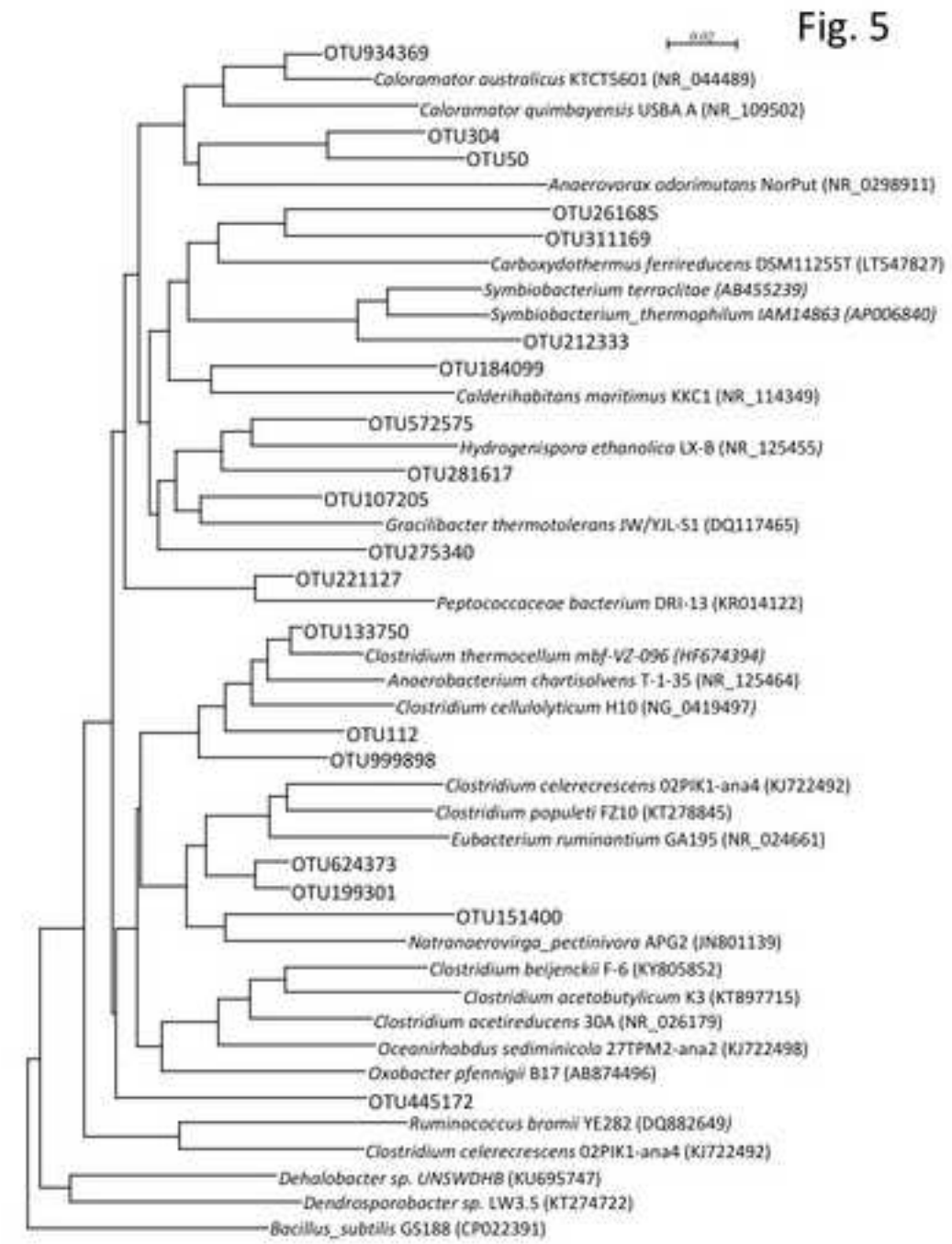

Table1

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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