Structure and function of the methanogenic microbial communities in soils from flooded rice and upland soybean fields from Sanjiang Plain, NE China

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Abstract

About 50 years ago, most of the natural wetlands in northeast China, the Sanjiang plain, were converted to either flooded rice fields or to upland soybean fields. After the conversion, natural wetland soils were either managed as artificial wetland or as drained upland resulting in soil microbial community changes. The purpose of our study was to understand how methanogenic microbial communities and their functions had changed in the two different soils upon conversion, and whether these communities now exhibit different resistance/resilience to drying and rewetting. Therefore, we determined function, abundance and composition of the methanogenic archaeal and bacterial communities in two soils reclaimed from a *Carex* wetland 25 years ago. We incubated the soils under anoxic conditions and measured the rates and pathways of CH_4 production by analyzing concentration and $\delta^{13}C$ of CH_4 and acetate in the presence and absence of methyl fluoride, an inhibitor of acetoclastic methanogenesis. We also analyzed the abundance of bacterial and archaeal 16S rRNA genes, and of *mcrA* (coding for a subunit of the methyl coenzyme M reductase) using qPCR. The composition of the archaeal and bacterial 16S rRNA genes was determined by using MiSeq illumina sequencing. Our results showed clear differences in structure and function of methanogenic archaeal communities in rice field soil versus upland soil. Furthermore, in both soils composition of bacteria and archaea changed after artificial drying and became less diverse. The archaeal and bacterial signature species in the two soils were also different. However, functional changes were similar, with rates of CH_4 production and contribution of aceticlastic methanogenesis decreasing upon drying and rewetting in both soils.

Keywords: methanogenic microbial community; bacterial community; rice and soybean fields; Isotopic fractionation; 16S rRNA and mcrA genes; MiSeq Illumina sequencing

48

49 **1. Introduction**

50 Wetland soils are the largest single source in the global budget of methane (CH_4), an
51 important greenhouse gas in the atmosphere (Conrad, 2009). Methane is produced as an end
52 product of the degradation of organic matter under anoxic conditions, once oxidants like
53 nitrate, sulfate or ferric iron are all reduced (Conrad, 2007; Megonigal et al., 2004). The
54 degradation process is catalyzed by a complex microbial community consisting of bacteria
55 and archaea. Bacteria hydrolyze polymeric organic matter and ferment the resulting
56 monomers to mainly CO_2, H_2, and acetate, which are then used by methanogenic archaea
57 producing CH_4 (Conrad, 2007; Zinder, 1993). Methane is produced hydrogenotrophically
58 (from H_2 and CO_2) or aceticlastically (by disproportionation of acetate to CO_2 and CH_4).
59 Both the bacteria and the archaea operate in the absence of O_2 and are more or less sensitive
60 to O_2, which is toxic to them (Conrad, 2007; Zinder, 1993). The potential diversity of
61 methanogenic archaeal species in soil is large, usually encompassing five different
62 families/orders of methanogenic Euryarchaeota: *Methanobacteriaceae*,
63 *Methanomicrobiales*, *Methanocellaceae*, *Methanosarcinaceae* and *Methanosaetaceae*
64 (Bridgham et al., 2013; Conrad, 2007). Understanding how structure (i.e., the composition
65 and abundance) and function (rate and pathway of methanogenesis) of the methanogenic
66 bacterial and archaeal communities are affected by soil history and management remains an
67 important question.

68 Recently, we studied how activity and structure of methanogenic microbial
69 communities in dry upland soils change upon flooding (Angel et al., 2011; 2012; Angel &
70 Conrad, 2013). The results showed that upland soils contain only few genera (usually
71 *Methanocella* or *Methanosarcina*) at very low abundance which, however, proliferate upon
72 flooding and eventually produce CH_4. Similar results were obtained with upland pasture soil
73 turned into a pasture-rice field rotation (Fernandez Scavino et al., 2013). Although there

were substantial differences in the composition of bacterial and archaeal communities of upland versus flooded soil, subsequent drainage and drying had a similar effect on structure and function of the methanogenic communities in these two soil categories once they had become methanogenic (Ji et al., 2015). The methanogenic archaeal and bacterial communities were also affected when fields were every year rotated between flooded rice and upland crops (Ahn et al., 2014; Breidenbach & Conrad, 2015; Eusufzaia et al., 2010; Liu et al., 2015; Lopes et al., 2014; Watanabe et al., 2006; Zhao et al., 2014). However, there is no knowledge on how the methanogenic microbial community is affected, when natural wetlands are converted to either flooded rice soil or upland soil.

The Sanjiang Plain located in the northeast China may serve in this respect as an interesting model. As the name indicates, the plain in NE China is geographically defined by three large rivers which historically formed a large (10.9 Mha) natural wetland on permafrost soil. Since 1950, more than 84% of the wetlands were eventually converted to farmland, especially flooded rice fields, but also for growing dry upland crops. This conversion resulted in changes in greenhouse gas emission (Huang et al., 2010) and composition of soil bacterial communities (Liu et al., 2014). However, it is not known how structure and function of the methanogenic microbial community differ between flooded rice field soil and upland soil.

We hypothesized (1) that the two soils would differ in their potential to initiate methanogenic degradation of organic matter, presumably also differ in the path of methanogenesis, and that this difference would be paralleled by differences in the microbial community structures; (2) that the two soils would exhibit different resistance/resilience to drying and rewetting, again mirrored in different changes in the microbial community structure. To answer this, we used soil samples from regularly flooded rice fields and permanently drained upland fields from the Sanjiang area, which we sampled in fall when the rice fields were drained and thus in a similar physical state as the upland soils.

2. Methods

2.1. Sampling

Rice field soil and soybean upland soil were collected in October 2011 from Sanjiang plain, Heilongjiang province, China (47°35′N, 133°30′E) (Fig. S1). At this time, the rice fields were harvested and drained. Therefore, soil conditions were aerobic in both rice and soybean fields. This area is a seasonal frozen zone due to its high latitude with an annual mean temperature of 2°C and annual precipitation of 350~770 mm. These two soils were reclaimed from the Carex wetland 25 years ago, and according to Song et al. (2012), soils were classified as Albaquic Paleudalfs with a silty clay texture. Rice and soybean were planted with one harvest per year. In general, rice seedlings were transplanted into the flooded fields in late May, matured in early September and harvested in early October. The rice field was continuously flooded by 5-10 cm of water until rice maturity in early September. Soybean was planted in early May, matured in early September and harvested in early October. There was no man-made irrigation during the soybean growing season. The sampling fields were very close to the marsh wetland. The rice field was about 300 m × 500 m and soybean field was about 600 m × 500 m. In each field site, five or six soil cores (diameter 4 cm, depth 25 cm) were sampled randomly, and then mixed as one sample. Soil was dried, crushed, sieved (2-mm mesh size), mixed and stored at room temperature. Chemical analyses (Table 1) were done as described before (Conrad and Klose, 2006).

2.2 Incubation conditions

The incubation procedure was as described by Ji and colleagues (2015). Sieved soils were used for anoxic soil slurries. To mimic flooding periods, 5 g soil was placed into a 26-ml glass pressure tube and 5 ml anoxic sterile water added. Slurries were incubated at 25°C for 2-4 weeks, afterwards slurries were dried for one week at 35°C (drainage period). To

mimic reflooding, the dried soil was rewetted and reincubated at 25°C for another 2-4 weeks. Tubes were closed with a butyl rubber stopper and incubated (without shaking) under a N_2 atmosphere until CH_4 production was constant. Methylfluoride, an inhibitor for aceticlastic methanogenesis, was added (3%) as a control treatment (Janssen and Frenzel, 1997). All incubations were performed in triplicate.

2.3 Gas measurements

The chemical analysis of gas and liquid samples was done as described before (Conrad et al., 2014). Briefly, CH_4 and CO_2 were analyzed by gas chromatography (GC), total acetate by high-pressure liquid chromatography (HPLC) and the $\delta^{13}C$ by either GC combustion isotope ratio mass spectrometry (GC-C-IRMS) or HPLC-C-IRMS. The $\delta^{13}C$ of the methyl group of acetate was assumed to be 8‰ more negative than that of total acetate (Conrad et al., 2014). The $\delta^{13}C$ of organic matter was analyzed by the Centre for Stable Isotope Research and Analysis (KOSI) at the University of Göttingen using an elemental analyzer coupled to an IRMS. The fraction (f_{H2}) of CH_4 production by hydrogenotrophic methanogenesis was calculated by mass balance as described before (Conrad et al., 2010) using

$$f_{H2} = (\delta^{13}C_{CH4} - \delta^{13}C_{CH4\text{-}ma})/(\delta^{13}C_{CH4\text{-}mc} - \delta^{13}C_{CH4\text{-}ma}) \quad (1)$$

with $\delta^{13}C_{CH4} = \delta^{13}C$ of total CH_4 produced, $\delta^{13}C_{CH4\text{-}mc} = \delta^{13}C$ of CH_4 produced from hydrogenotrophic methanogenesis, which is equivalent to the CH_4 produced in the presence of CH_3F, and $\delta^{13}C_{CH4\text{-}ma} = \delta^{13}C$ of CH_4 produced from aceticlastic methanogenesis. The $\delta^{13}C_{CH4\text{-}ma}$ was assumed to be in the range of two values, one being equal to $\delta^{13}C_{ac\text{-}methyl}$ if there is no fractionation during the reduction of acetate-methyl to CH_4, the other being 8‰ lower than $\delta^{13}C_{ac\text{-}methyl}$ if fractionation occurs by *Methanosaeta* species as aceticlastic methanogens (Penning et al., 2006). The $\delta^{13}C$ of total acetate was measured by the end of incubations in the presence of CH_3F.

152

153 *2.4. DNA extraction and qPCR*

154 Soil DNA was extracted using the NucleoSpin Soil Kit (Macherey-Nagel, Düren,
155 Germany). Lysis buffer SL2 and enhancer SX were used and DNA was eluted in 100µl of
156 Elution Buffer. Extracted DNA was used as template for qPCR and MiSeq Illumina
157 analyses. The abundance of archaeal 16S rRNA and of methanogenic *mcrA* gene was
158 determined by qPCR with primer sets Arch364-f/ 934b-r and mlas-mod-f/mcrA-rev-r
159 respectively (Angel et al., 2012; Kemnitz et al., 2005), and conditions were as follows: for
160 archaeal 16S rRNA gene: 6 min at 94°C, 40 cycles of 94°C for 35 s, 66°C for 30 s, 72°C for
161 45 s, 86.5°C for 10 s (snapshot) and for *mcrA* gene: 5 min 94°C, 40 cycle at 95°C for 30 s,
162 57°C for 45 s, 72°C for 30 s, 84°C for 10 s (snapshot). For archaeal 16S rRNA genes
163 efficiencies of 87.6 – 88.2% with R^2 values > 0.99 were obtained. For *mcrA* genes
164 efficiencies of 72.5 – 77.8% with R^2 values > 0.99 were obtained. Technical duplicates were
165 performed for each of the replicates. QPCR cycling conditions for bacterial 16S rRNA genes
166 are detailed in Appendix S1 in Supplementary information.

167

168 *2.5. Illumina library preparation and sequencing:*

169 PCR primers (515F, 5′-GTGCCAGCMGCCGCGGTAA-3′ and 806R, 5′-
170 GGACTACVSGGGTATCTAAT -3′) targeting the V4 region of the 16S rRNA gene
171 (approximately 250 nucleotides) for both archaea and bacteria were used (Bates et al., 2011).
172 Individual PCRs contained a 6-bp molecular barcode integrated in the forward primer. PCR
173 conditions consisted of an initial denaturation at 94°C for 5 min, followed by 28 cycles of
174 94°C for 30 s, 50°C for 30 s, and 68°C for 30 s and a final extension at 68°C for 10 min
175 (Hernández et al., 2015). Amplicons were purified using a PCR cleanup kit (Sigma) and
176 quantified using a Qubit 2.0 fluorometer (Invitrogen). Finally, samples were pooled in an
177 equimolar concentration and sequenced on separate runs for MiSeq using a 2 x 300 bp

178 paired end protocol. Library preparation and sequencing was performed at the Max Planck
179 Genome Centre (MPGC), Cologne, Germany. Table S1 in the supplemental material shows
180 the sequences of barcodes and primers.
181
182 *2.6. Bioinformatic analyses*
183 The first step was to merge forward and reverse reads using the usearch fastq_mergepairs
184 command (Edgar, 2013). Trimming forward and reverse adaptors from the merged
185 sequences was done using the mothur software platform (Schloss et al., 2009). The fasta and
186 qual files from mothur were converted to fastq with USEARCH. Downstream processing
187 and operational taxonomy unit (OTU) picking was performed with UPARSE (Edgar, 2013)
188 as follows: barcodes and primers from the merged sequences were removed, quality-filtering
189 reads (sequences shorter than 250 bp were discarded), dereplication, abundance sort keeping
190 singletons, OTU clustering (97%) and de novo chimera filtering with UCHIME (Edgar et
191 al., 2011).
192
193 *2.7. Statistical analyses and OTU classification*
194 All statistical analyses were performed using the vegan package (Oksanen et al., 2013) in R
195 software version 3.0.2 (http://www.r-project.org). Tests with $P \leq 0.05$ were considered to be
196 statistically significant. Shapiro–Wilk normality test was performed for each analysis. Gene
197 abundances within the soils were compared by one-way analysis of variance (ANOVA)
198 followed by a Tukey post hoc test. Gene abundances between soils (rice *v/s* upland) of the
199 bacterial and archaeal 16S rRNA and *mcrA* were compared separately. ANOVA was
200 performed when qPCR data were normally distributed. A non-parametric Kruskal-Wallis
201 one-way analysis of variance was performed when the qPCR data were not normally
202 distributed. ANOVA was also performed for CH_4 production between the soils for each of
203 the treatments, and between of the treatments for each of the soils. For all OTU-based

statistical analyses, the data set was normalized by a Hellinger transformation (Legendre and Gallagher, 2001) using the *decostand* function. The rarefaction curve was performed using the *rarecurve* function (step: 500). For alpha-diversity, Shannon index (H) and Species evenness (J) were carried out using vegan package in R, and were calculated based on the lowest number of sequences available from each site, *i.e.*, 201,883 for bacterial- and 53,680 for archaeal-16S rRNA gene reads (subsample using the *rrarefy* function). This procedure standardizes the measures needed for comparison. For beta-diversity, principal coordinates analysis (PCoA) was carried out using the *cmdscale* function of Hellinger distances. Differences in population structure between both soils were analyzed using the statistic ANOSIM (based on Bray-Curtis dissimilarities) (Clarke, 1993). A heatmap was constructed with the gplots (Warnes et al., 2015) and vegan packages for the OTUs explaining most of the differences between samples. Principal components analysis (PCA) of the Hellinger transformed data was performed using the *prcomp* function in vegan. The 50 OTUs explaining most of the differences between samples were defined as the OTUs contributing the largest absolute loadings in the first and second dimensions of the PCA (Breidenbach et al., 2016), obtained from the rotation output file. To construct the map, a total of 78 unique OTUs were obtained for bacterial 16S rRNA genes and 65 OTUs were obtained for archaeal 16S rRNA genes (since some of the OTUs were selected from more than one PC). The OTU abundances were converted to percentage of reads from each sample, and Hellinger distances were calculated as described above. Hierarchical clustering of the distance matrix was carried out with the "ward" method using *hclust* function. The heatmap was constructed using the *heatmap.2* function in gplots package. The taxonomy of the selected OTUs was added separately.

2.8. Taxonomy analysis

A representative sequence from each of the OTUs was classified with the mothur software platform (Schloss et al., 2009). Sequences were aligned against the SILVA 16S rRNA gene database using the naïve Bayesian classifier with a bootstrap confidence threshold of 80%. The taxonomy of OTUs in the heatmaps was determined using the Sina classifier (Pruesse et al., 2012) based on both the Silva and RDP taxonomies. A consensus of both classifications is shown in the heatmap.

2.9. Data accession

Sequence data were deposited in the NCBI Sequence Read Archive (SRA) in two separate Bio-projects. Rice field soil samples under accession number SRP074575 and upland soil samples under accession number SRP074610 (see Table S1).

3. Results

3.1. Functional characteristics

The carbon and nitrogen content of rice soil and upland soil were similar, but the $\delta^{13}C$ of organic carbon was slightly more negative and the content of iron and sulfate was higher in rice soils (Table 1). Nevertheless, production of CH_4 showed significant differences between the soils (ANOVA, $P< 0.001$) and started after a shorter lag phase in rice soil than in upland soil (Fig. 1,A) and also reached a higher rate (Table 2). The reason for the longer lag phase in upland soil versus rice soil was probably the relatively low number of methanogenic archaea (*mcrA* gene) (Fig. 2,A). Significant differences between both soils were found when analyzing *mcrA* gene abundance in all the treatments (ANOVA for initial soils and Kruskal-Wallis for incubated, dried and rewetted treatments, $P<0.001$, Fig. 2,A) as well as for almost all of the treatments for bacterial (Kruskal-Wallis, $P< 0.001$, Fig. S2) and archaeal 16S rRNA gene abundances (Kruskal-Wallis, $P< 0.001$, Fig. 2,B). The low numbers of *mcrA* gene abundance increased significantly (ANOVA, $P< 0.005$) upon

anaerobic incubation of the upland soil by almost two orders of magnitude, albeit still being 10 times lower than in the rice soil (Fig. 2,A). After drying and rewetting the soils, numbers of methanogens (*mcrA*) stayed constant in both soils (Fig. 2,A), while those of bacteria and archaea stayed constant only in the rice field soil but decreased in upland soil (bacteria 16S rRNA gene: ANOVA $P< 0.005$) by a factor of up to10 (Fig. S2; Fig. 2,B). The lag phase of CH_4 production in rice soil became almost as long as that for the upland soil, and the final CH_4 production rates were in both soils much lower (about 30%) than before drying.

The $\delta^{13}C$ values of the produced CH_4 were similar in all incubations (Fig. 1,B; Table 2). In the presence of CH_3F, an inhibitor of aceticlastic methanogenesis, the $\delta^{13}CH_4$ values were lower, with most negative $\delta^{13}C$ (about -90‰) in the rice soil after drying and rewetting (Fig. 1,C; Table 2). The $\delta^{13}C$ of acetate produced in the presence of CH_3F was not much different from that of the $\delta^{13}C$ of organic carbon (Table 2). In the absence of CH_3F, concentrations of acetate were too low to allow determination of $\delta^{13}C$. The isotopic data allowed the estimation of the fraction of hydrogenotrophic methanogenesis (f_{H2}). The values of f_{H2} were generally higher in upland soil versus rice soil, but in both cases increased upon drying and rewetting (Table 2). This tendency was independent of the assumption whether acetate consumption was instantaneous and complete, thus causing no isotope fractionation during the conversion to CH_4, or was incomplete and causing fractionation with the enrichment factor (ε_{ma} = -8‰) typical for *Methanosaeta species* (Penning et al., 2006) (Table 2).

3.2. Microbial community composition and overall diversity

The composition of the microbial communities in differently treated rice soil and upland soil was assessed by Illumina sequencing, resulting in about 1000-5000 archaeal and 200,000 to 500,000 bacterial sequences in each of the replicates (Fig. 3,A; Fig. S3,A; Table 3). The rice soil and upland soil differed in composition of both archaea and bacteria, and

281 also differed between the different treatments, i.e., Initial (O), incubated (I), dried (D),
282 rewetted (R) (Fig. 3,B; Fig. S3,B).
283 There were differences in the rarefaction curves obtained for both archaeal and
284 bacterial 16S rRNA gene sequence analysis (Fig. S4). Rarefaction curves for archaeal 16S
285 rRNA gene showed different trends, where rice field soil presented greater richness than
286 upland soil (Fig. S4,A). Rarefaction curves for bacterial 16S rRNA showed similar trends
287 for rice field soils and for upland soils (Fig. S4,B). OTU numbers in bacterial 16S rRNA
288 gene composition were higher in samples from rice field (especially in the initial soils) than
289 those of upland soil (Table 3). The estimated alpha diversity indices (Shannon index H, and
290 species eveness J) were similar in both soils, but were generally lowest after drying and
291 rewetting (Table 3). Shannon index (H) and Species evenness (J) were significantly different
292 between rice and upland soil and were also significantly different for bacterial and for
293 archaeal 16S rRNA genes in each of the soils (t test, $P < 0.005$) (Hutcheson, 1970). The
294 same behavior was observed for archaeal 16S rRNA gene sequence analysis, where the total
295 number of OTUs in rice field soils where higher than those of upland soils (Table 3).
296 Principal coordinate analysis (PCoA) of archaeal OTUs showed significant
297 differences in community composition for both soils (ANOSIM, R= 0.655, *P*,0.001), and
298 also differences after drying, rewetting, and again incubating under anaerobic conditions
299 (Fig. 4). PCoA also confirmed that the bacterial communities of rice soil and upland soil
300 were significantly different (ANOSIM, R= 0.608, *P*,0.001) and that the communities
301 changed when the initial soil was anaerobically incubated, dried and then rewetted and again
302 incubated under anaerobic conditions (Fig. S5).
303
304 *3.3. Composition of the dominant archaeal communities*
305 The rice soil was dominated by putatively methanogenic Archaea, i.e.,
306 *Methanosarcinaceae* (20-50%), *Methanobacteriaceae* (10-30%), *Methanocellaceae* (5-

307 15%), *Methanosaetaceae* (5-15%) and *Methanomicrobiales* (<5%) (Fig. 3,B). In addition,
308 there were *Thaumarchaeota* (5-15%) and *Themoplasmatales* (<5%).
309 The initial upland soil was dominated by non-methanogenic *Thaumarchaeota* (80%).
310 Upon anaerobic incubation, however, the archaeal community composition changed
311 drastically (Fig. 3,B) and was then dominated by methanogenic *Methanosarcinaceae* (30-
312 55%), *Methanocellaceae* (15-30%) and *Methanobacteriaceae* (2-4%). In contrast to rice
313 soil, upland soil in general contained only small relative amounts (<1%) of
314 *Methanosaetaceae* and *Methanomicrobiales*. However, *Thaumarchaeota* (20-25%) and
315 *Thermoplasmatales* (5-10%) were always present.
316 Incubation, drying and rewetting of rice soil had no systematic effect on which
317 archaeal orders or families (there were always five different ones) were detected. In the
318 upland soil, however, incubation resulted in an increase of the methanogenic archaea relative
319 to the non-methanogenic ones. In contrast to rice soil, however, the methanogenic
320 community mainly consisted of only three groups, i.e., *Methanosarcinaceae,*
321 *Methanocellaceae* and *Methanobacteriaceae* (Fig. 3,B).
322 The composition of the *Methanosarcinaceae*, as one of the most abundant
323 methanogenic archaea, was also different in rice soil and upland soil. In rice soil the
324 *Methanosarcinaceae* consisted of seven different OTUs (each >1% of the total archaeal
325 sequences), OTU-95 and OTU-14518 being the most dominant ones (Fig. 5,A). In upland
326 soil, by contrast, members of the *Methanosarcinaceae* became relatively more abundant
327 when the initial soil was incubated, and stayed relatively abundant when the soil was dried
328 and rewetted (Fig. 5,A). In this soil, the *Methanosarcinaceae* consisted mainly of OTU-326
329 and OTU-14896. OTU-14518 was only abundant in the rewetted soil, while OTU-16169
330 was not thus abundant (Fig. 5,A). By contrast, the family *Methanocellaceae* almost
331 exclusively consisted of OTU-99 in both soils (Fig. 5,B). This OTU was also the dominant
332 *Methanocellaceae* in upland soil. However, in upland soil the relative abundance of

333 *Methanocellaceae* increased strongly when the initial soil was incubated, and stayed
334 relatively abundant when the soil was dried and rewetted (Fig. 3,B).
335 The 50 most abundant OTUs defining each PCoA axis were selected and used to
336 create a heatmap (Fig. 6). The heatmap showed that several OTUs were present in all stages
337 of incubation for both soils and belonged to the genera *Methanobacterium* (OTU-192, OTU-
338 4724), *Methanocella* (OTU-99), *Methanosarcina* (OTU-95, OTU-14518) and the
339 Miscellaneous Chrenarchaeotic group (OTU-221, OTU-14845). The heatmap also showed
340 that the *Methanobacterium* OTUs were predominantly found in rice soil (OTU-299 almost
341 exclusively). OTUs belonging to the *Woesearchaeota* phylum (formerly Euryarchaeota
342 DHVEG-6) were only found in rice soils after rewetting (OTU-525, OTU-527, OTU-1048)
343 (Fig. 6). The OTUs belonging to the *Thaumarcheota* phylum, on the other hand, were
344 exclusively found in the initial upland soil (OTU-2046, OTU-16713), some also in
345 incubated upland soil (OTU-554, OTU-623). Consistent with Fig. 5, several
346 *Methanosarcina* OTUs (OTU-326, OTU-8700, OTU-14896) were characteristic for upland
347 soil incubations, but were not found in the initial upland soil (Fig. 6).
348
349 *3.4. Composition of the dominant bacterial communities*
350 The major (>5%) classes of the bacteria were in both soils and under all treatments the
351 following: *Actinobacteria* (up to 31%), *Firmicutes* (up to 27%), *Acidobacteria* (up to 21%),
352 *Planctomycetes* (up to 21%), *Cloroflexi* (up to 19%), *Proteobacteria* (up to 19%), and
353 *Verrucomicrobia* (up to 9%) (Fig. S3,B).
354 The heatmap confirmed that the selected bacteria were different between rice soil and
355 upland soil, and also different between the individual treatments (see Fig. S6). None of the
356 OTUs was equally found in all soils and incubations. However, *Acidobacteria* OTUs were
357 almost exclusively found in incubated upland soil and only few were also found in rewetted
358 rice field soil. *Actinobacteria* OTUs were predominant in initial upland soil. *Bacteroidetes*

OTUs were found only in incubated rice field soils. Many different *Chloroflexi* OTUs were almost mainly found in incubated rice field soil. Among *Firmicutes*, incubated rice field soils contained several *Clostridium* OTUs, while upland soils instead contained a *Paenibacillaceae*. Differential presence of OTUs in rice field versus upland soil was also observed among *Planctomycetes*, *Proteobacteria* and *Verrucomicrobia* (Fig. S6).

4. Discussion

We found that rice field soil and upland soil exhibited different compositions of bacterial and archaeal communities albeit both soils originated from the same wetland about 25 years ago. Differences were also apparent in the numbers of gene copies, especially those of the *mcrA* gene. The *mcrA* gene codes for the key enzyme of methanogens, and was virtually absent in the initial upland soil, and was probably the reason for the delayed methanogenic activity in the upland soil. Hence, the two soils also showed different functionality. These differences were not surprising given the fact that the two soils had completely different histories in management. While the rice field soil was regularly flooded, thus causing anoxic conditions and permitting methanogenesis, this was not the case in the soybean upland soil, which was always more or less well aerated.

In contrast to the rice field soil, which contained large and diverse populations of methanogenic archaea, the upland soil was dominated by *Thaumarchaeota* (Fig. 3,B), which presumably were aerobic ammonia oxidizers (Pester et al., 2011), and contained only relatively small populations (about 5% of total archaea) as compared with the anaerobic methanogens, *Methanobacteriaceae*, *Methanosarcinacea* and *Methanocellaceae* in particular (Fig. 3,B). *Thaumarchaeota* have also been found to become dominant when rice fields were converted to crop rotation with maize, apparently a reaction upon the now aerated soil conditions (Breidenbach et al., 2015). Vice versa, relative abundance of *Thaumarchaeota* has been found to decrease, and that of *Methanosarcinaceae* and

385 *Methanobacteriaceae* to increase, when pasture soil was incubated under anoxic conditions
386 (Ji et al., 2015). Change in abundance and community composition of methanogens has also
387 been observed in crop rotations between rice and soybean fields (Liu et al., 2015). To our
388 knowledge, this is the first study addressing differences in methanogenic microbial
389 communities in soils having undergone 25 years of different management. However, there
390 are reports on effects of crop rotation between flooded rice and upland crops (Breidenbach et
391 al., 2016; Lopes et al., 2014; Zhao et al., 2014). Similarly as in these studies we also
392 observed differences in the bacterial community structures (see supplemental material),
393 which however could not be interpreted with respect to functionality (see below).
394 Incubation of the soils under anaerobic conditions, followed by drying and reincubation,
395 resulted in systematic change in the structure and function of the microbial communities.
396 These changes were different in rice field and upland soil, and are discussed in more detail
397 below. However, both soils had the capacity to convert soil organic matter to CH_4. Rates of
398 CH_4 production were different in the two soils, and the lag phase in the upland soil was
399 longer than in the paddy soil. Therefore, resilience of the methanogenic communities upon
400 desiccation was apparently lower in the upland soil than the rice field soil, but functionality
401 was eventually the same. Abundances of *mcrA* genes were generally lower in upland than in
402 rice field soils. 16S rRNA gene abundances stayed constant in rice field soils, while they
403 tended to decrease in upland soil upon treatments (incubation, drying, reincubation) (Fig. 2).
404 The lower resilience of upland versus rice field soils thus may be due to the different
405 abundances of methanogenic archaea. However, it may also be due to the different microbial
406 community compositions.
407 The treatments resulted in systematic changes in the composition of archaeal and
408 bacterial communities in both soils. In both soils diversity indices of bacteria and archaea
409 tended to decrease (Table 3). However, the actual composition of the microbial communities
410 in rice field soil versus upland soil changed in different ways, as seen by PCoA analysis

411 (Fig. 4; Fig. S5) and the most representative OTUs, but also by the relative sequence
412 abundance on the phylum level (Fig. 3,B; Fig. S3,B). The different trends in bacterial
413 community composition upon anaerobic incubation, desiccation and rewetting, such as the
414 relative increase of *Acidobacteria* OTUs in upland soil and *Bacteroidetes* OTUs in rice field
415 soil, can presently not be interpreted with respect to functionality. However, the trends in
416 archaeal community are more telling. In upland soil, for example, the methanogenic archaeal
417 community became increasingly dominated by sequences of *Methanosarcinaceae*, and
418 *Methanocella*. Both genera are known to survive well in dry soils, where they are usually the
419 only methanogens present (Angel et al., 2011; 2012). However, *Methanosaetaceae* were
420 always hardly detectable in upland soil (Fig. 5,B; Fig. 6). While *Methanosarcina* species can
421 utilize acetate only at milimolar concentrations, *Methanosaeta* species are able to form CH_4
422 from micromolar acetate concentrations (Jetten et al., 1992). However, all the different
423 methanogenic species can hydrogenotrophically produce CH_4. Hence, the data are consistent
424 with the observation that hydrogenotrophic methanogenesis was relatively large in upland
425 soil and increased upon desiccation (Table 2). Interestingly, upland soil also became
426 increasingly dominated by two OTUs from the genus *Methanomassiliicocus*, which can
427 reduce methanol with H_2 to CH_4 (Lang et al., 2015), raising the possibility that methanol
428 production from pectin or lignin may happen in upland soil. The rice field soil, on the other
429 hand, did contain *Methanosaetaceae*, also contained OTUs of *Methanosarcinaceae* that
430 were different from those in upland soils, and also contained several OTUs of the genus
431 *Methanobacterium* (Fig. 6). We believe that the presence of *Methanosaetaceae* may explain
432 why aceticlastic methanogenesis contributed relatively more to CH_4 production in rice field
433 than in upland soil. However, there is no clue why desiccation resulted in a relative increase
434 of hydrogenotrophic methanogenesis.
435 It is remarkable, that all these different changes in community composition still
436 guaranteed methanogenic function in both rice field soil and upland soil. Notably, in both

soils rates of CH_4 production decreased and contributions of hydrogenotrophic methanogenesis increased after desiccation (Table 2). Similar functional change has been observed in pasture soil and rice rotational soil from Uruguay, where archaeal and bacterial community compositions were also affected similarly as in the present experiments (Ji et al., 2015). By contrast, desiccation of lake sediments from the Amazon region resulted in enhanced CH_4 production and decreased contribution of hydrogenotrophic methanogenesis, albeit the effects on archaeal and bacterial community composition were similar to the present experiments (Conrad et al., 2014). In all examples, relative abundance of methanogenic archaeal populations belonging to *Methanocellaceae* (sometimes also *Methanobacteriaceae*) and *Methanosarcinaceae* were enhanced, whereas those of *Methanosaetaceae* and *Methanomicrobiales* were decreased.

Although these changes in archaeal methanogenic community structures are consistent with the different rates and pathways of CH_4 production, they may not be the only explanations. For example, changes in bacterial community composition may also be relevant, since the bacteria provide the methanogenic substrates (H_2, acetate) by degradation of organic matter. However, the rRNA sequences of the Bacteria give only few hints for their phenotypes and therefore can hardly be interpreted with respect to their function. Furthermore, the quality of organic matter may also affect the methanogenic functions. For example, it was found that aceticlastic methanogenesis dominated the anaerobic degradation of fresh organic matter, while hydrogenotrophic methanogenesis relatively increased for the residual one (Hodgkins et al., 2014; Liu et al., 2016). Desiccation (and aeration) may make aged organic matter better accessible to subsequent methanogenic degradation (Borken and Matzner, 2009). Therefore, we can presently not dismiss that the functions of methanogenic degradation processes (rates, pathways, resilience) are not only affected by the structure of the microbial community but also by the chemical nature of the organic matter.

5. Conclusions

In conclusion, our results proved our hypotheses that the different histories of the two soils that originated from a former wetland resulted in the development of different methanogenic microbial communities, which also exhibited different paths of methanogenesis and different resilience to desiccation (aeration) stress. However, the function of the two soils, i.e. the methanogenic degradation of organic matter, was not impaired. While one soil had been managed for 25 years as an annually flooded rice field, the other was managed as a non-flooded soybean field. The present study is consistent with the view that the management history of soils affects the structure and function of the methanogenic microbial communities (Conrad et al., 2014; Ji et al., 2015; Evans and Wallenstein, 2012).

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608
609

610 **Figure legends**

611 Figure 1: Time course of the production of CH_4 (A), $\delta^{13}CH_4$ (B) and $\delta^{13}CH_4$ in presence of
612 methyl fluoride CH_3F (C) in fresh and dried rice field and upland soils. Values of $\delta^{13}C$ are
613 given in permil (‰). The horizontal lines indicate the time period used for calculation of the
614 average values given in Table 2.

615

616 Figure 2: Abundance of methanogenic *mcrA* (A), and archaeal 16S rRNA (B) genes in rice
617 soil and upland soils. Sample description: initial soils (O), incubated soils (I), dried soils
618 (D), rewetted and incubated soils (R). Bars represent standard errors of six replicate samples
619 (except for some samples in initial soil: archaeal 16S rRNA genes (rice soil and upland soil):
620 4 replicates each and *mcrA* gene (upland soil): 3 replicates. Asterisks above the bars
621 represent statistically significant differences (P <0.05) between soils.

622

623 Figure 3: (A). Number of sequences representing total bacteria of rice (green) and upland
624 (red) soils and relative abundance of archaeal (B) sequences. The descriptions of sample
625 names are shown in legend of figure 2.

626

627 Figure 4: Principal coordinate analysis (PCoA) based on abundances of archaeal 16S rRNA
628 gene OTUs (97% sequence similarity). Samples name starting with "R" represent rice field
629 soils and "U" are those for upland soils and treatments: original soil (0a, 0b, 0c), incubated
630 soil (1, 2, 3), dried soils (7, 8, 9) and rewetted and incubated soils (10, 11, 12). The R- and
631 P- values from the ANOSIM analysis are shown in each plot.

632

633 Figure 5: Relative abundance of major Methanosarcinaceae (A) and Methanocellaceae (B)
634 OTUs. The descriptions of sample names are shown in legend of figure 2.

635

Figure 6: Heatmap showing the relative abundance of dominant archaeal OTUs. The samples and OTUs were clustered according to Euclidean distances between all Hellinger transformed data. The colors correspond to the relative abundance of each OTU in the samples (indicated by the color legend). The taxonomy of OTUs was determined using the Sina classifier. The descriptions of sample names are shown in legend of figure 4.

Table 1: Chemical characteristics of the rice soil and upland soil from Sanjiang (mean ± SE; n = 3)

	Rice soil	Upland soil
Org. C (%)	2.29 ± 0.02	1.88 ± 0.07
Total N (%)	0.218 ± 0.002	0.182 ± 0.007
$\delta^{13}C_{org}$ (‰)	-26.76 ± 0.05	-23.95 ± 0.53
Fe_{total} (μmol g^{-1})	152	104
Sulfate (μmol g^{-1})	0.33	0.12
pH	6.8	6.8

Table 2: Functional parameters of rice soil (R) and upland soil (U) in initial state (O) and after drying and rewetting (R). The values are means ± SE of the data ranges marked in Fig.1. are means ± SE at the end of triplicate incubations. Estimations of f_{H2} assumed (I) $\delta_{ma} = \delta^{13}C_{acetate\text{-}methyl}$ and (II) $\delta_{ma} = \delta^{13}C_{acetate\text{-}methyl}$ - 8‰; with $\delta^{13}C_{acetate\text{-}methyl} = \delta^{13}C_{acetate}$ - 8‰.

Parameter	Rice O	Rice R	Upland O	Upland R
CH_4 production (nmol d^{-1} gdw^{-1})	382 ± 7.3*	115 ± 3.4*	190 ± 10.2*	78 ± 2.4*
$\delta^{13}CH_4$ (‰)	-44.1 ± 0.6	-53.8 ± 0.1	-48.1 ± 0.5	-51.8 ± 0.7
$\delta^{13}CH_4$ (+CH_3F) (‰)	-71.7 ± 0.2	-90.5 ± 0.9	-79.5 ± 1.2	-78.2 ± 0.2
$\delta^{13}C_{acetate}$ (+CH_3F) (‰)	-25.1 ± 0.1	-28.4 ± 0.1	-22.5 ± 0.1	-25.7 ± 2.0
f_{H2} (I) (%)	29	32	36	41
f_{H2} (II) (%)	10	20	23	28

indicates significant differences between treatment (O v/s R) for each soil ($P<0.05$ for rice and $P<0.001$ for upland) and also between soils (R v/s U) for each treatment ($P<0.001$ in both treatments).

Table 3. Sample summary, operational taxonomic unit (OTU) and alpha diversity indices: Shannon index (H) and Species evenness (J) of bacterial- and archaeal- 16S rRNA gene sequences. Values represent average ± Standard deviation of triplicate samples per treatment

Soil treatment*	bacterial 16S rRNA gene				archaeal 16S rRNA gene			
	Nr. reads**	Nr. OTUs***	H	J	Nr. reads**	Nr. OTUs***	H	J
ricefield / O	322863.0 ±17773	10463.0 ±96	6.9 ±0.03	0.8 ±0.00	94358.7 ±26636	5559.0 ±490	7.3 ±0.07	0.9 ±±0.01
ricefield / I	314017.0 ±22113	9217.7 ±205	7.0 ±0.01	0.8 ±0.00	83052.7 ±184	5039.7 ±33	7.2 ±0.05	0.9 ±0.00
ricefield / D	435556.0 ±25435	8038.0 ±196	6.2 ±0.02	0.7 ±0.00	98619.3 ±7336	5282.3 ±86	7.4 ±0.07	0.9 ±0.01
ricefield / R	465847.0 ±25965	7928.7 ±187	5.7 ±0.12	0.7 ±0.01	116891.7 ±11356	5452.0 ±534	7.2 ±0.29	0.9 ±0.02
upland/ O	325628.3 ±158919	8082.3 ±402	6.6 ±0.17	0.7 ±0.01	92417.7 ±25014	4961.3 ±1000	7.1 ±0.21	0.9 ±0.01
upland/ I	221594.3 ±25643	7159.3 ±251	6.4 ±0.20	0.7 ±0.02	102486.3 ±19913	4439.7 ±385	6.9 ±0.18	0.8 ±0.01
upland/ D	337639.7 ±74935	6908.0 ±149	5.8 ±0.17	0.7 ±0.02	72911.0 ±11220	3060.7 ±238	6.2 ±0.12	0.8 ±0.01
upland/ R	263941.0 ±12958	6814.0 ±101	5.9 ±0.12	0.7 ±0.01	60351.3 ±5778	3088.7 ±106	6.3 ±0.03	0.8 ±0.00

* Soi treatment: O. initial soil; I. incubated soil; D. dried soil; R. rewetted and incubated soil. ** Qualified reads after filtering low quality reads and chimera. ***OTUs were defined at a sequence identity level of 97%.

Figure

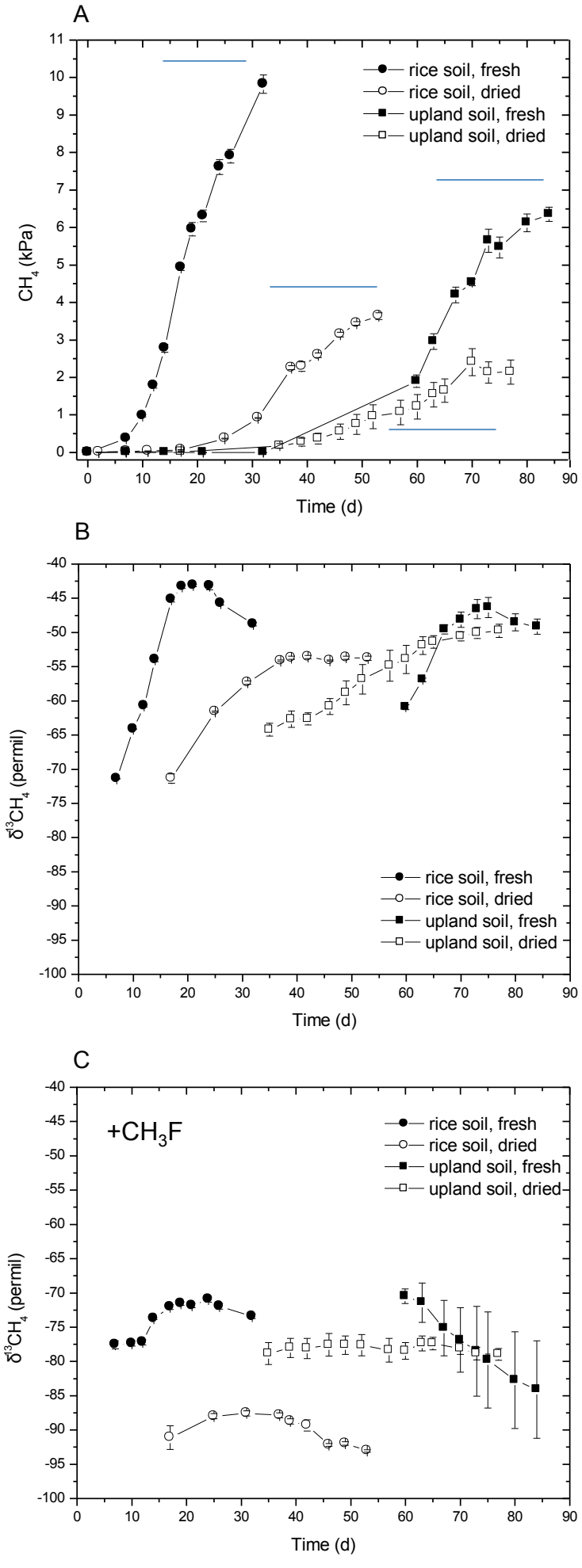
A
CH4 (kPa)
Time (d)
rice soil, fresh
rice soil, dried
upland soil, fresh
upland soil, dried
B
δ13CH4 (permil)
Time (d)
rice soil, fresh
rice soil, dried
upland soil, fresh
upland soil, dried
C
+CH3F
δ13CH4 (permil)
Time (d)
rice soil, fresh
rice soil, dried
upland soil, fresh
upland soil, dried

Fig. 1

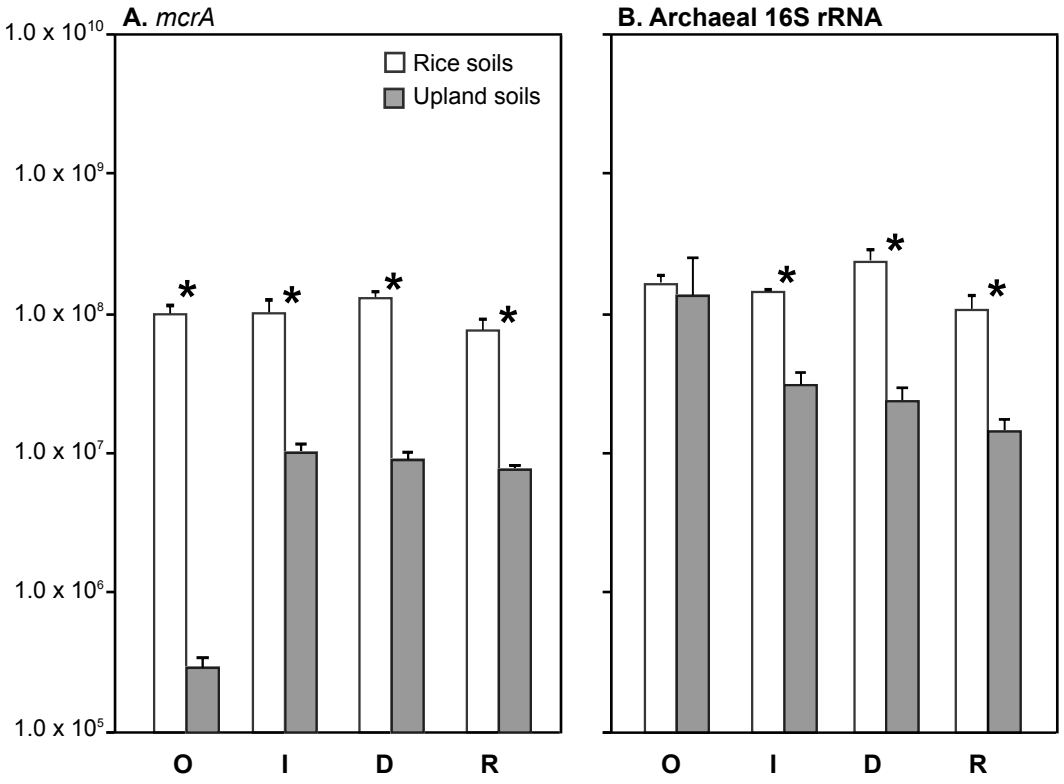
A. mcrA
B. Archaeal 16S rRNA
Rice soils
Upland soils
1.0 x 10^10
1.0 x 10^9
1.0 x 10^8
1.0 x 10^7
1.0 x 10^6
1.0 x 10^5
O
I
D
R
O
I
D
R

Fig. 2

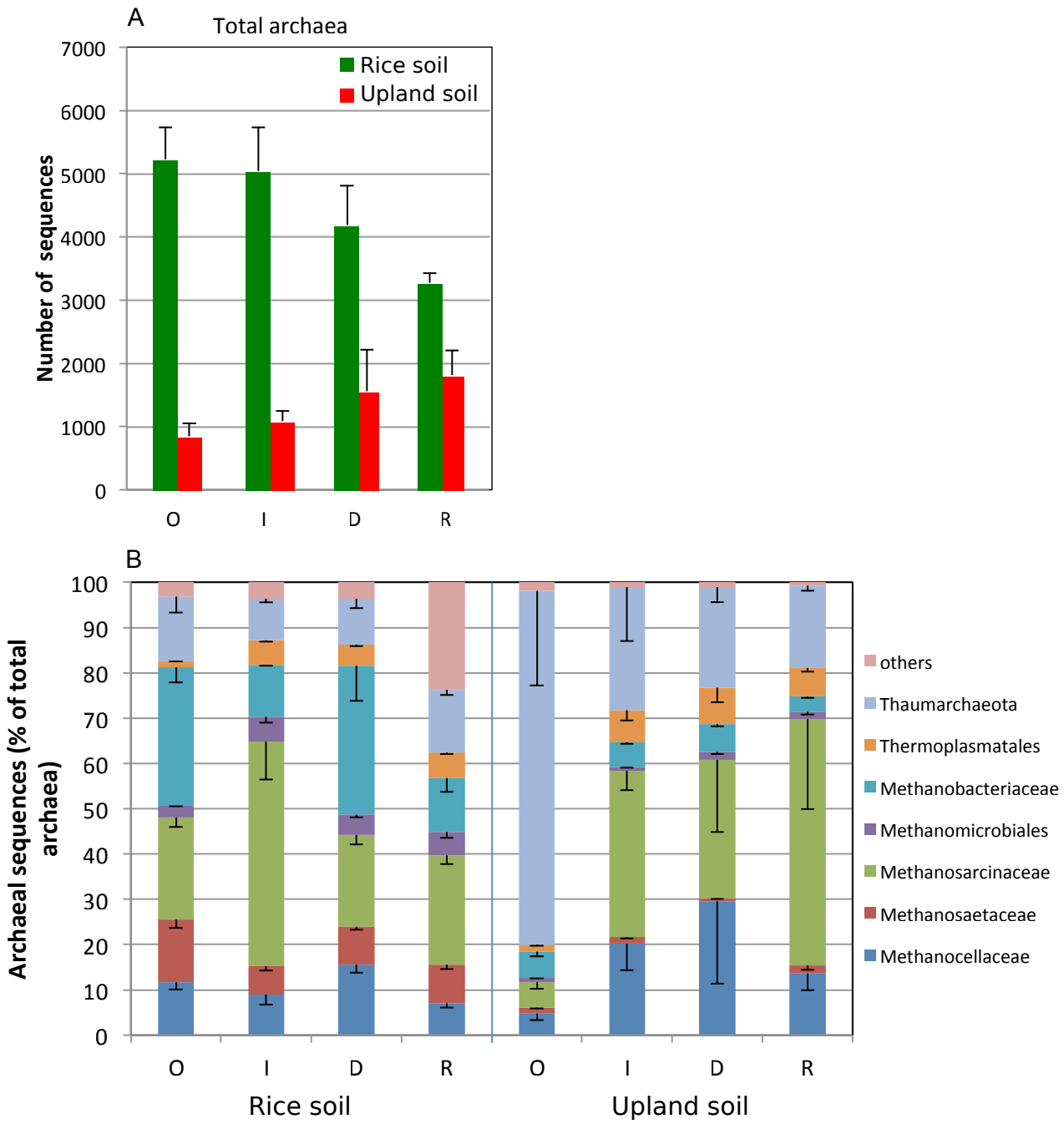
A
Total archaea
Rice soil
Upland soil
Number of sequences
7000
6000
5000
4000
3000
2000
1000
0
O
I
D
R
B
Archaeal sequences (% of total archaea)
100
90
80
70
60
50
40
30
20
10
0
O
I
D
R
O
I
D
R
Rice soil
Upland soil
others
Thaumarchaeota
Thermoplasmatales
Methanobacteriaceae
Methanomicrobiales
Methanosarcinaceae
Methanosaetaceae
Methanocellaceae

Fig. 3

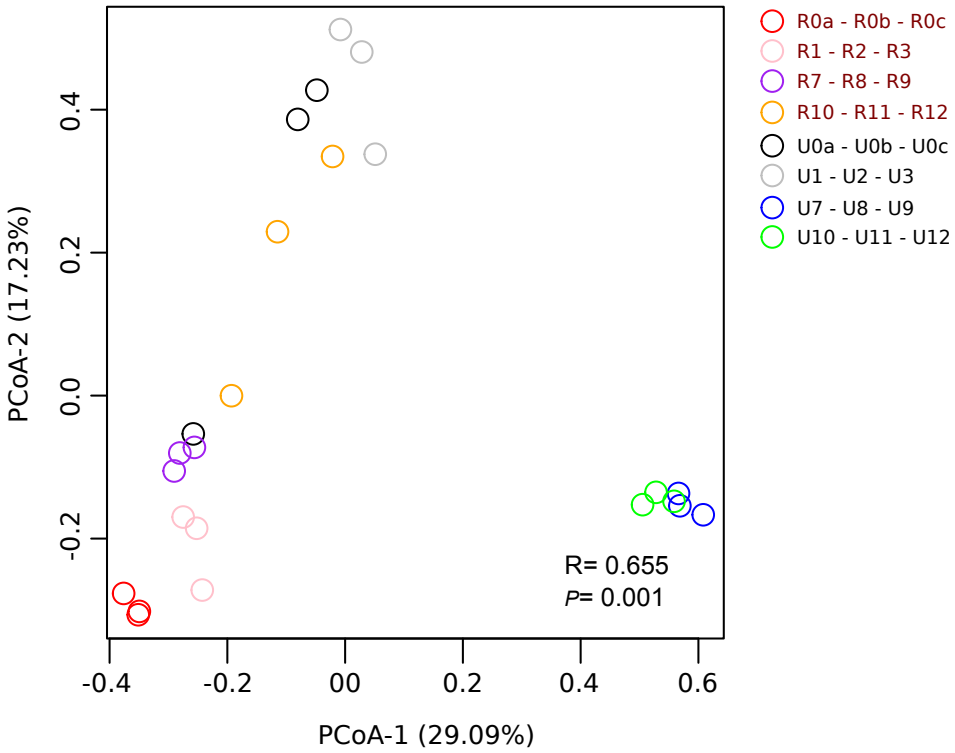
R0a - R0b - R0c
R1 - R2 - R3
R7 - R8 - R9
R10 - R11 - R12
U0a - U0b - U0c
U1 - U2 - U3
U7 - U8 - U9
U10 - U11 - U12
PCoA-2 (17.23%)
0.4
0.2
0.0
-0.2
-0.4
-0.2
00
0.2
0.4
0.6
PCoA-1 (29.09%)
R= 0.655
P= 0.001

Fig. 4

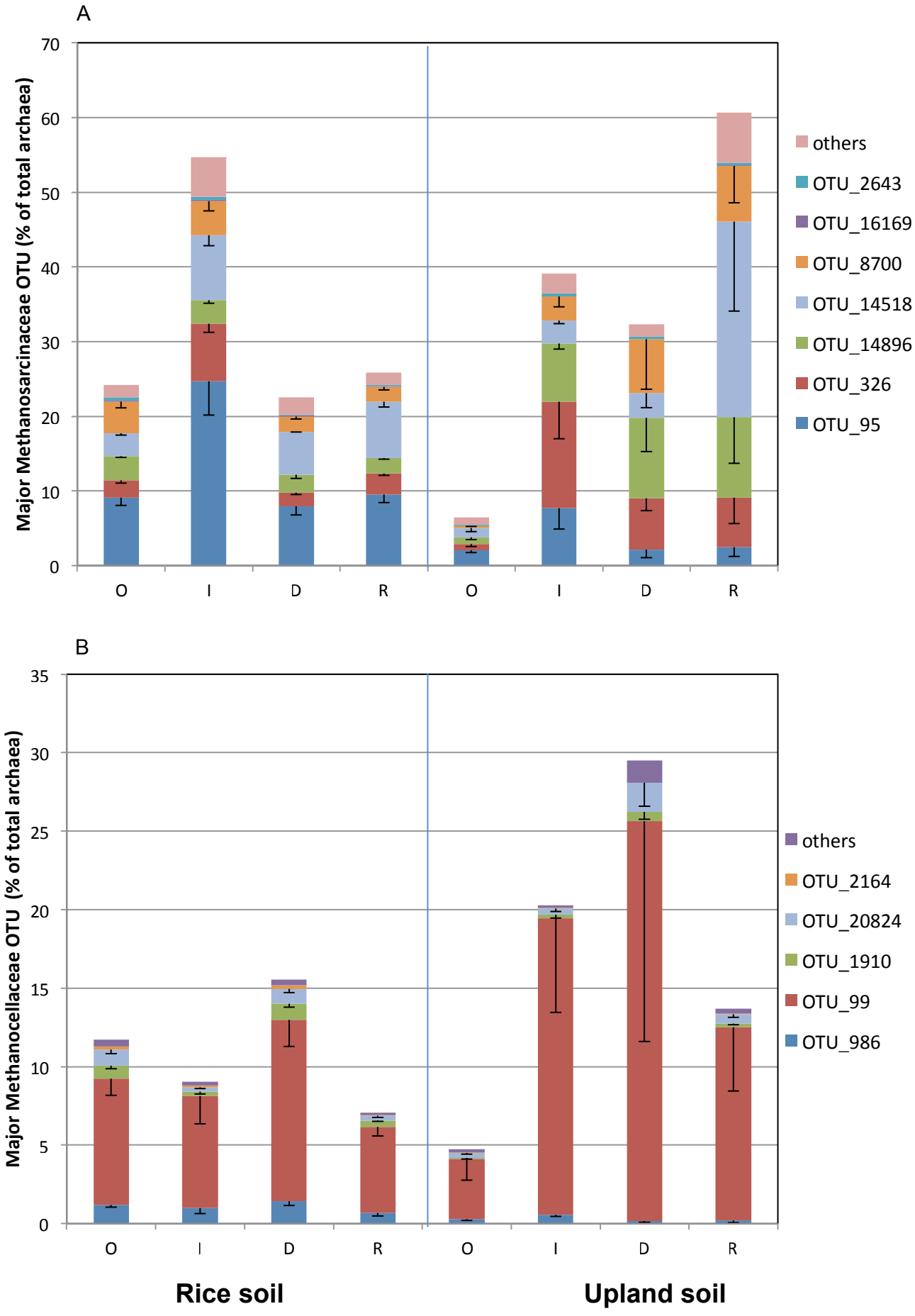
A
Major Methanosarcinaceae OTU (% of total archaea)
70
60
50
40
30
20
10
0
O
I
D
R
O
I
D
R
others
OTU_2643
OTU_16169
OTU_8700
OTU_14518
OTU_14896
OTU_326
OTU_95
B
Major Methanocellaceae OTU (% of total archaea)
35
30
25
20
15
10
5
0
O
I
D
R
O
I
D
R
others
OTU_2164
OTU_20824
OTU_1910
OTU_99
OTU_986
Rice soil
Upland soil

Fig. 5


R10 R11 R12 R1 R2 R3 R7 R8 R9 R0a R0b R0c U0a U0b U0c U3 U1 U2 U9 U7 U8 U12 U10 U11
0.0 2.0 4.0
Crenarchaeota, Thermoprotei (OTU-4962)
Euryarchaeota, Methanobacteria, *Methanobacterium* (OTU-192)
Euryarchaeota, Methanobacteria, *Methanobacterium* (OTU-299)
Euryarchaeota, Methanobacteria, *Methanobacterium* (OTU-3306)
Euryarchaeota, Methanobacteria, *Methanobacterium* (OTU-4724)
Euryarchaeota, Methanobacteria, *Methanobacterium* (OTU-13817)
Euryarchaeota, Methanobacteria, *Methanobacterium* (OTU-17332)
Euryarchaeota, Methanobacteria, *Methanobacterium* (OTU-19148)
Euryarchaeota, Methanomicrobia (OTU-2503)
Euryarchaeota, Methanomicrobia, *Methanocella* (OTU-99)
Euryarchaeota, Methanomicrobia, *Methanocella* (OTU-986)
Euryarchaeota, Methanomicrobia, *Methanocella* (OTU-1910)
Euryarchaeota, Methanomicrobia, *Methanocella* (OTU-2164)
Euryarchaeota, Methanomicrobia, *Methanocella* (OTU-11668)
Euryarchaeota, Methanomicrobia, *Methanocella* (OTU-15185)
Euryarchaeota, Methanomicrobia, *Methanocella* (OTU-15327)
Euryarchaeota, Methanomicrobia, *Methanocella* (OTU-20824)
Euryarchaeota, Methanomicrobia, Methanoregulaceae (OTU-502)
Euryarchaeota, Methanomicrobia, *Methanosarcina* (OTU-95)
Euryarchaeota, Methanomicrobia, *Methanosarcina* (OTU-326)
Euryarchaeota, Methanomicrobia, *Methanosarcina* (OTU-2643)
Euryarchaeota, Methanomicrobia, *Methanosarcina* (OTU-8700)
Euryarchaeota, Methanomicrobia, *Methanosarcina* (OTU-14518)
Euryarchaeota, Methanomicrobia, *Methanosarcina* (OTU-14896)
Euryarchaeota, Methanomicrobia, *Methanosarcina* (OTU-16169)
Euryarchaeota, Methanomicrobia, unclassified Methanosarcinales (OTU-1605)
Euryarchaeota, Methanomicrobia, *Methanothrix* (OTU-492)
Euryarchaeota, Methanomicrobia, *Methanothrix* (OTU-835)
Euryarchaeota, Methanomicrobia, *Methanothrix* (OTU-872)
Euryarchaeota, Methanomicrobia, *Methanothrix* (OTU-999)
Euryarchaeota, Methanomicrobia, *Methanothrix* (OTU-1406)
Euryarchaeota, Methanomicrobia, *Methanothrix* (OTU-1660)
Euryarchaeota, Methanomicrobia, *Methanothrix* (OTU-1731)
Euryarchaeota, Methanomicrobia, *Methanothrix* (OTU-9704)
Euryarchaeota, Methanomicrobia, *Methanothrix* (OTU-12837)
Euryarchaeota, Methanomicrobia, *Methanothrix* (OTU-13246)
Euryarchaeota, Methanomicrobia, *Methanothrix* (OTU-14367)
Euryarchaeota, Methanomicrobia, *Methanothrix* (OTU-15102)
Euryarchaeota, Methanomicrobia, *Methanothrix* (OTU-15330)
Euryarchaeota, Methanomicrobia, *Methanothrix* (OTU-17636)
Euryarchaeota, Thermoplasmata, *Methanomassiliicoccus* (OTU-246)
Euryarchaeota, Thermoplasmata, *Methanomassiliicoccus* (OTU-5752)
Miscellaneous Chrenarchaeotic group (OTU-221)
Miscellaneous Chrenarchaeotic group (OTU-2407)
Miscellaneous Chrenarchaeotic group (OTU-3776)
Miscellaneous Chrenarchaeotic group (OTU-13456)
Miscellaneous Chrenarchaeotic group (OTU-14845)
SM1K20 (OTU-2424)
Thaumarchaeota, Group C3 (OTU-3127)
Thaumarchaeota, *Nitrososphaera* (OTU-554)
Thaumarchaeota, *Nitrososphaera* (OTU-623)
Thaumarchaeota, *Nitrososphaera* (OTU-2046)
Thaumarchaeota, *Nitrososphaera* (OTU-3987)
Thaumarchaeota, *Nitrososphaera* (OTU-4048)
Thaumarchaeota, *Nitrososphaera* (OTU-7028)
Thaumarchaeota, *Nitrososphaera* (OTU-8734)
Thaumarchaeota, *Nitrososphaera* (OTU-16713)
Thaumarchaeota, Terrestrial group (OTU-5510)
Woesearchaeota (formerly Euryarchaeota DHVEG-6) (OTU-525)
Woesearchaeota (formerly Euryarchaeota DHVEG-6) (OTU-527)
Woesearchaeota (formerly Euryarchaeota DHVEG-6) (OTU-1048)
Woesearchaeota (formerly Euryarchaeota DHVEG-6) (OTU-1202)
Woesearchaeota (formerly Euryarchaeota DHVEG-6) (OTU-1241)
Woesearchaeota (formerly Euryarchaeota DHVEG-6) (OTU-2455)
Woesearchaeota (formerly Euryarchaeota DHVEG-6) (OTU-4097)

Fig. 6

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