Dynamics of bacterial communities in rice field soils as affected by different long-term fertilization practices[§]

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Fertilization and the response of the soil microbial community to the process significantly affect crop yield and the environment. In this study, the seasonal variation in the bacterial communities in rice field soil subjected to different fertilization treatments for more than 50 years was investigated using 16S rRNA sequencing. The simultaneous application of inorganic fertilizers and rice straw compost (CAPK) maintained the species richness of the bacterial communities at levels higher than that in the case of non-fertilization (NF) and application of inorganic fertilizers only (APK) in the initial period of rice growth. The seasonal variation in the bacterial community structure in the NF and APK plots showed cyclic behavior, suggesting that the effect of season was important; however, no such trend was observed in the CAPK plot. In the CAPK plot, the relative abundances of putative copiotrophs such as *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were higher and those of putative oligotrophs such as *Acidobacteria* and *Plactomycetes* were lower than those in the other plots. The relative abundances of organotrophs with respiratory metabolism, such as *Actinobacteria*, were lower and those of chemoautotrophs that oxidize reduced iron and sulfur compounds were higher in the CAPK plot, suggesting greater carbon storage in this plot. Increased methane emission and nitrogen deficiency, which were inferred from the higher abundances of *Methylocystis* and *Bradyrhizobium* in the CAPK plot, may be a negative effect of rice straw application; thus, a solution for these should be considered to increase the use of renewable resources in agricultural lands.

Keywords: rice, soil, bacterial community, 16S rRNA, fertilization

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Introduction

Climate change induced by the use of fossil fuels and the toxic effects of synthetic chemicals on human health and the environment are forcing all areas of industry to become more sustainable and eco-friendly, including the agricultural industry. One of the means for reducing the dependence on fossil fuels and synthetic chemicals in agriculture is to utilize soil microorganisms.

Microorganisms in agricultural soils are involved in nutrient supply, water use efficiency, degradation of harmful pollutants, control of pathogens, and promotion of plant stress tolerance (Brussaard *et al.*, 2007). They are also closely associated with atmospheric concentrations of greenhouse gases including carbon dioxide, methane, and nitrous oxide (Singh *et al.*, 2010).

However, land-use intensification can change soil biodiversity, leading to the mitigation of soil functions in agricultural systems (Acosta-Martínez *et al.*, 2008; Tardy *et al.*, 2015; Tsiafouli *et al.*, 2015). Therefore, it is important to develop strategies for maintaining beneficial soil organisms and enhancing their functions in agricultural lands. Reduced tillage, crop rotation, increased level of soil organic carbon, and biocontrol of pests and pathogens have been suggested as management options to maintain soil biodiversity and were shown to have beneficial effects on agricultural lands (Brussaard *et al.*, 2007; Wall *et al.*, 2015).

Rice is the main food crop in Asia including Korea, and thus, the sustainable cultivation of rice is very important in this region. Several studies investigated the effects of different fertilization regimes on the microbial communities in rice field soils and found that the application of rice straw generally increased the abundance, activity, and diversity of microorganisms in rice field soils (Bossio and Scow, 1995; Islam *et al.*, 2011; Wu *et al.*, 2011; Ahn *et al.*, 2012a, 2013; Yuan *et al.*, 2012, 2013).

In the present study, we investigated the seasonal variation in bacterial communities, based on their 16S rRNAs, in an experimental rice field subjected to different fertilizations for more than 50 years. Because most previous studies were based on the bacterial 16S rRNA genes with just one-time sampling, we sought to provide a more accurate description of the dynamics of active bacterial communities in rice field soils. Our results may facilitate the utilization of soil microorganisms for sustainable rice cultivation, where the use of synthetic chemicals and its negative effects on the environment are minimized and crop yield is maintained or enhanced.

Table 1. Soil chemical properties in long-term fertilization rice fields in 2013

Treatment	pH	Organic matter (g/kg)	Av. P_2O_5 (mg/kg)	Ex. cations ($cmol_c$/kg)			Av. SiO_2 (mg/kg)	Crop yield (ton/ha)
				K	Ca	Mg		
NF	6.7	16	20	0.05	4.7	0.9	99	3.6
APK	6.0	21	142	0.04	4.2	0.8	47	6.7
CAPK	6.0	28	116	0.04	5.0	0.9	55	7.5

Materials and Methods

Experimental site and soil sampling

The sampling site was located at the experimental rice field of the National Institute of Crop Science, Suwon, Korea (37°16′ N, 126°59′ E). Long-term fertilization experiment began in 1954 and involved 32 different fertilization treatments for the cultivation of Samgwang rice (*Oryza sativa* L. var. *japonica*) as a single crop at the time of the sampling (Suh *et al.*, 2009; Islam *et al.*, 2011). Among the treatments, non-fertilization (NF plot), combined application of ammonium sulfate (symbolized as A, 110 kg as N/ha/year), fused and superphosphate (symbolized as P, 70 kg as P_2O_5 ha/year), and potassium chloride (symbolized as K, 80 kg as K_2O ha/year) (APK plot), and combined application of APK and a rice straw compost (symbolized as C, 7.5 Mg ha/year) (CAPK plot) were selected for the present study. Soil samples were collected during the drained and non-planted period (April 16), flooded and rice-growing period (May 26, July 5, and August 14), and drained and rice-ripening period (September 23) in 2013. Three soil cores (1.8 cm diameter, 15 cm height) were collected from each plot and stored at -70°C until molecular analyses was performed. Analysis of soil chemical properties was performed as previously described (Ahn *et al.*, 2012a) with the soils sampled in April, and data are presented in Table 1.

RNA extraction and pyrosequencing of bacterial 16S rRNA

Soil RNA was extracted using the RNA PowerSoil Total RNA Isolation Kit (MO BIO Laboratories), according to the manufacturer's instructions. Aliquots of RNA extracts were treated with RQ1 RNase-Free DNase (Promega) and purified using the RNeasy MinElute Cleanup kit (Qiagen). cDNA was synthesized using the GoScript reverse transcription system and random hexamers (Promega) according to the manufacturer's instructions. Bacterial 16S rRNA genes were PCR-amplified as previously described (Ahn *et al.*, 2012a) and pyrosequencing of the PCR products was performed by ChunLab, Inc. using a 454 GS FLX Titanium Sequencing System (Roche), according to the manufacturer's instructions.

Processing of pyrosequencing data

Operational taxonomic unit (OTU) clustering of the sequence outputs was carried out using the UPARSE pipeline with some modifications (Edgar, 2013). First, bar-code and primer sequences were removed using the trim.fastq script and the sequences shorter or longer than 300 bp were removed or properly trimmed. Sequences were clustered to OTUs at a cut-off of 0.03 using the average-neighbor algorithm, and chimera were removed in *de novo* mode and reference mode using UCHIME and USEARCH. The resulting otu.table was transformed to a shared file using a modified Perl script. The remaining analysis was performed using the Mothur software package (version 1.29.1) (Schloss *et al.*, 2009) unless otherwise stated. Qualified sequences were classified to the genus level using the Bayesian method based on the Ribosomal Database Project (RDP) taxonomy (version 14). Richness estimators and diversity indices were calculated using the
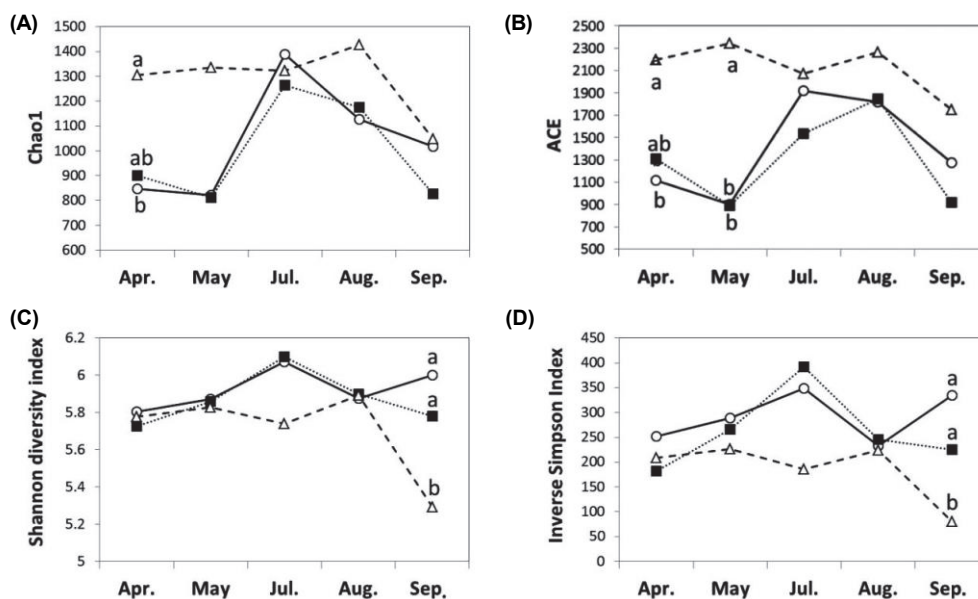

Fig. 1. Seasonal variability of Chao1 richness estimate (A), ACE richness estimate (B), Shannon diversity index (C), and inverse Simpson index (D) of bacterial communities in rice field soils based on bacterial 16S rRNAs according to fertilization scheme. Symbols: ○, no fertilization (NF); ■, fertilized with ammonium sulfate, fused and superphosphate, and potassium chloride (APK); △, fertilized with ammonium sulfate, fused and superphosphate, potassium chloride, and rice straw compost (CAPK). Values are the means of triplicate measurements. Different lowercase letters indicate significant difference among the fertilization treatments for a given season.

summary.seqs command in the Mothur package. Weighted Fast UniFrac analysis (Hamady *et al.*, 2009) was performed using a phylogenetic tree constructed with the FastTree program (http://meta.microbesonline.org/fasttree) (Price *et al.*, 2009) and the pcoa command in the Mothur package .

Statistical analysis

Significant differences were analyzed by Student's *t*-test or one-way analysis of variance followed by Tukey's pairwise comparison. *P* values < 0.05 were considered significant. All analyses were performed using the R software (version 3.2.4) (The R Core Team, 2016).

DNA sequencing data

The raw pyrosequencing data produced from this study were deposited in the National Agricultural Biotechnology Information Center (http://nabic.rda.go.kr/) under the accession numbers NN-2681-000001 ~ NN-2681-000045.

Results and Discussion

Bacterial community diversity

The qualified pyrosequencing reads for each sample ranged from 1,098 to 12,156 and were normalized to the smallest sample size of 1,098. Using this sample size, Good's coverage values ranged from 0.59 to 0.81 (data not shown), indicating that the sample size selected in this study was not sufficient to accommodate all bacterial diversity in the rice field soils.

  The values representing community richness (Chao1 richness estimate and ACE richness estimate) and community diversity (Shannon index and inverse Simpson index) generally increased from April to July and then decreased in the NF and APK plots, while they remained relatively stable from

April to August and then decreased in September in the CAPK plot (Fig. 1).

  The community richness estimates indicated that the species number was higher in the CAPK plot than that in the NF and APK plots in April and May, but there were generally no significant differences from July to September among treatments (Fig. 1A and B). In contrast, the diversity indices showed no significant differences among treatments from April to August, but were in the order of NF > APK > CAPK in September.

  We predicted that in the NF and APK plots, soil organic matter increased as rice grew because of plant residues, and this increased the species richness and diversity of the active bacterial communities while this effect may not have been as large in the CAPK plot because of the rice straw compost applied to this plot.

  Increased diversity during ecosystem succession is thought to be related to the increase in potential niches, stratification, and other biological organization (Odum, 1969). Dilly *et al.* (2004) showed that the diversity indices of bacterial communities based on denaturing gradient gel electrophoresis analysis increased during litter decomposition and suggested that this was due to bacterial adaptation to more heterogeneous environmental conditions, complex composition of the remaining organic matter, and increased interactions within the community.

Variation in overall community structure

Although the species richness of the bacterial communities gradually became similar among the treatments from April to August (Fig. 1A and B), the taxonomic composition was significantly different among treatments (Fig. 2). Principal coordinate 1 separated the bacterial communities in the NF and APK plots and that in the CAPK plot, indicating that the application of rice straw compost significantly changed the bacterial community structure. In the NF and APK plots,
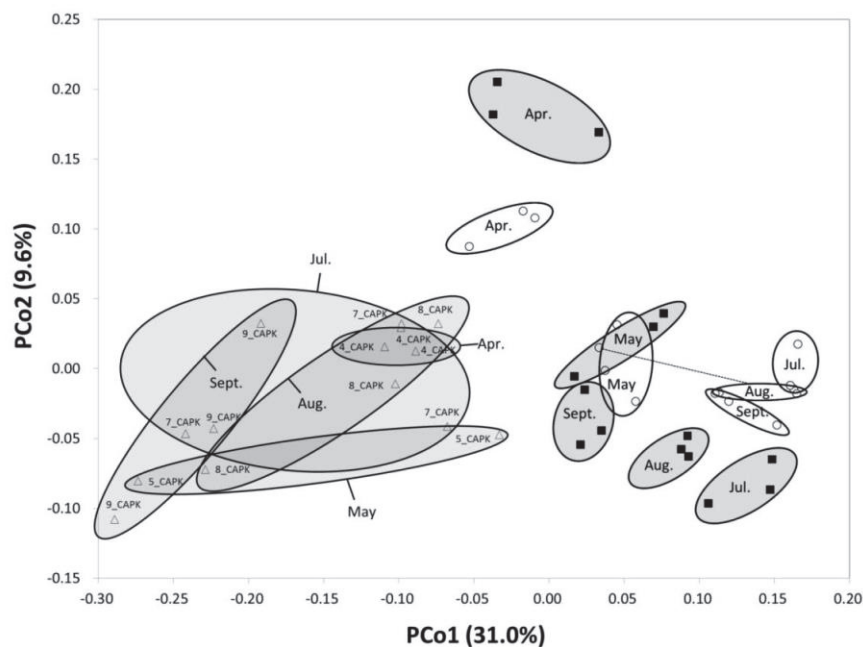

Fig. 2. Principal coordinate analysis of bacterial communities in the rice field soils based on their 16S rRNAs and the Weighted UniFrac metric. Refer to Fig. 1. for the symbols. For the CAPK plot, the sampling month is indicated at each point because of the high degree of dispersion.

the bacterial communities in April were separated from those in the remaining seasons, indicating that flooding and rice growth had a significant effect on the bacterial communities as shown in a previous study based on 16S rRNA genes extracted from the same site (Ahn *et al.*, 2012a). Seasonal variation in the bacterial community structure in the NF and APK plots after flooding showed cyclic behavior, in which the bacterial community structure changed greatly from May to July and again approached that in May during the remaining seasons, particularly in the APK plot. This result suggests that the bacterial community structure was closely associated with season, most likely the variation in the amounts of organic matters produced by the rice plant, such as root exudates and plant residues, which are related to the plant growth. In the CAPK plot, however, the bacterial community structure did not show such a trend and appeared to be unrelated to season. This might be due to the high amount of organic matters from the rice compost, which would exceed

those from the rice plant. Experimental proof will be needed to support this hypothesis.

Taxonomic distributions of bacterial communities in rice field soils

Regardless of fertilization and season, *Proteobacteria* was the most abundant bacterial phylum, accounting for 37–61% of the bacterial 16S rRNAs (Supplementary data Table S1). *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* were also present, in order. This is similar to the results found for the 16S rRNA genes (Ahn *et al.*, 2012b) and 16S rRNAs (Ahn *et al.*, 2013) extracted from the same site.

Acidobacteria: The abundances of *Acidobacteria* increased until August and then decreased in the NF and APK plots, while it generally decreased throughout the rice growing season in the CAPK plot (Fig. 3A). This suggests that rice growth stimulates the activities of *Acidobacteria*, but the
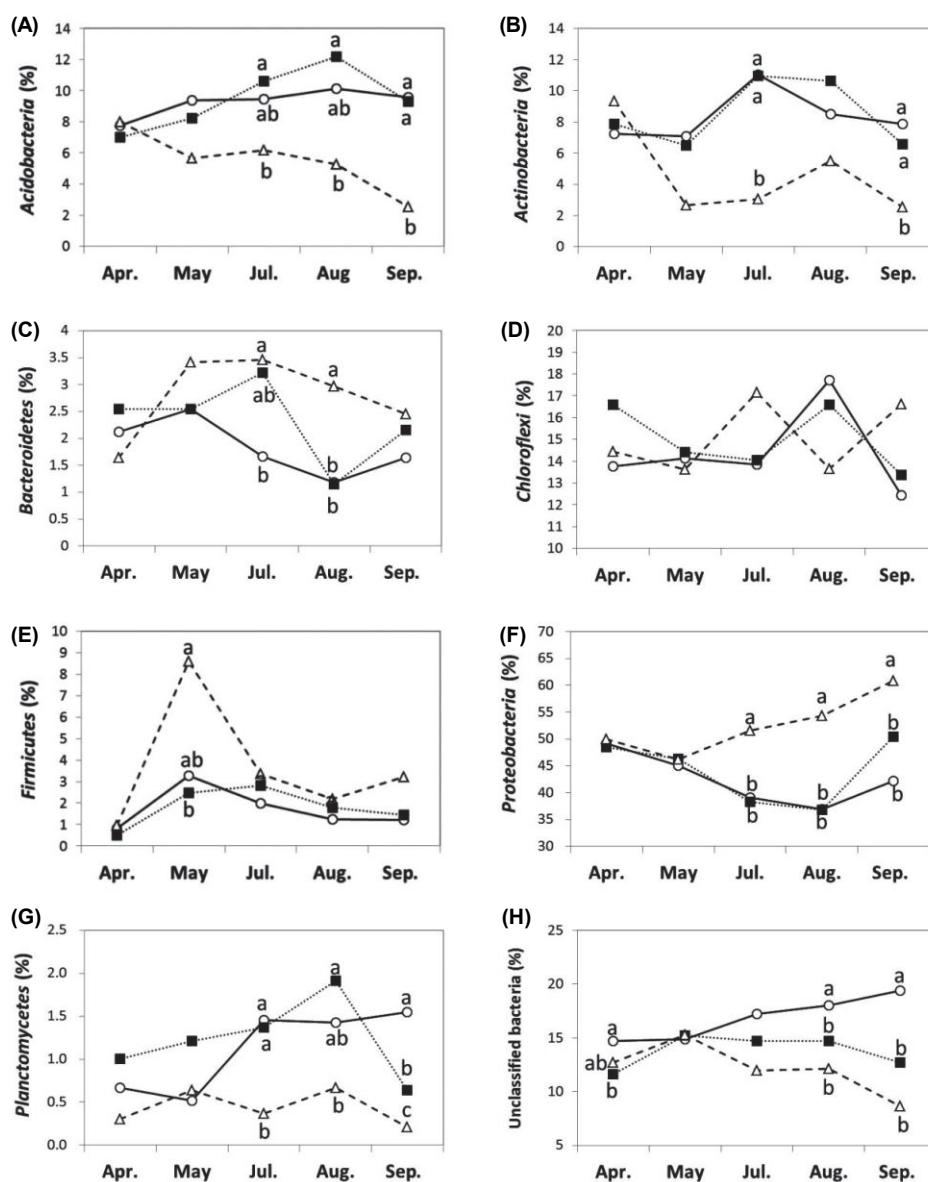

Fig. 3. Seasonal variability in the relative abundances of 16S rRNAs of the major bacterial phyla in rice field soils according to fertilization scheme. Refer to Fig. 1. for the symbols. Values are the means of triplicate measurements. Different lowercase letters indicate significant difference among fertilization treatments for a given season.

addition of rice straw compost reduces or has no effect on the activities of *Acidobacteria*. Previous studies have shown that *Acidobacteria* are a bacterial group adapted to low nutrient conditions, that is, oligotrophs or *K*-strategists (Fierer *et al.*, 2007; Ward *et al.*, 2009). There may be a critical point in soil organic matter content that determines the activities of *Acidobacteria*.

Among the subgroups of *Acidobacteria*, Gp1 and Gp16 were the most prevalent (occupying 2–43% and 18–36% of acidobacterial 16S rRNAs, respectively) (Supplementary data Table S1). Jones *et al.* (2009) analyzed 87 different soils and found that the relative abundances of Gp1 and Gp16 had a negative and positive correlations with soil pH, respectively. The period during which the relative abundance of Gp1 exceeded that of Gp16 was longer in the order of CAPK > APK > NF (Supplementary data Fig. S1). This result agrees with that of a previous study considering that the soil pH was NF > APK = CAPK (Table 1) and is thought to reflect variations in pH according to season and fertilization.

***Actinobacteria*:** The relative abundances of actinobacterial 16S rRNAs increased in July and then decreased in the NF and APK plots, while these abundances decreased rapidly in May and remained relatively low in the CAPK plot (Fig. 3B). The actinobacterial genera identified at the study site, such as *Arthrobacter*, *Gaiella*, *Marmoricola*, *Nocardioides*, and *Terrabacter* (Supplementary data Table S1), are generally known as aerobic or absolutely aerobic heterotrophs (Albuquerque *et al.*, 2011; Goodfellow, 2012; İnce *et al.*, 2014; Won *et al.*, 2014). It is thought that the higher organic matter content in the CAPK plot maintained the soil in more reducing condition, as indicated by a lower redox potential in a previous study (Bossio *et al.*, 1999), and decreased the competitiveness of aerobic heterotrophs.

***Chloroflexi*:** The relative abundances of 16S rRNAs of *Chloroflexi* ranged from 12 to 18% and showed no significant difference among treatments (Fig. 3D).

Among the 16S rRNAs of *Chloroflexi*, those affiliated with *Dehalogenimonas* were more abundant in the CAPK plot than those in the NF and APK plots (Supplementary data Fig. S2). The higher abundance of *Dehalogenimonas* in the CAPK plot may be related to the putative presence of organochlorine chemicals and likely more reducing conditions in the CAPK plot (Bossio *et al.*, 1999), as the two type strains of *Dehalogenimonas* are strictly anaerobic bacteria that use hydrogen as an electron donor and chlorinated alkanes as electron acceptors (Moe *et al.*, 2009; Bowman *et al.*, 2013).

***Firmicutes*:** The relative abundances of 16S rRNAs affiliated with *Firmicutes* increased after flooding in all treatments (Fig. 3E). In particular, the increase was much larger in the CAPK plot in May. The activities of the two most abundant genera of *Firmicutes*, *Clostridium* III, and '*Clostridium* sensu stricto' rapidly increased in the CAPK plot (Supplementary data Fig. S3). '*Clostridium* sensu stricto' (also known as *Clostridium* I) is the largest of the clostridial group and exhibits various phenotypes; *Clostridium* III consists of cellulolytic species (Collins *et al.*, 1994). It is thought that the prevalence of anaerobic conditions after flooding and increase in organic matter after compost application may have increased the activities of these clostridial groups, which were also observed in a previous study (Rui *et al.*, 2009).

***Bacteroidetes* and *Proteobacteria*:** The relative abundances of 16S rRNAs affiliated with *Bacteroidetes* and *Proteobacteria* were higher in the CAPK plot than those in the NF and APK plots after flooding and rice planting (Fig. 3C and F). For *Proteobacteria*, this was observed for the alpha-, beta-, gamma-, and epsilon-subgroups but not for the delta-subgroup (Supplementary data Fig. S4). Previous studies showed that the relative abundances of *Bacteroidetes* and *Proteobacteria* increased in soil where the level of available organic matter and rate of mineralization were high because these bacterial groups are generally considered to be adapted to high-nutrient conditions (Padmanabhan *et al.*, 2003; Fierer *et al.*, 2007).

Within the alpha-subgroup, *Bradyrhizobium* and *Methylocystis* spp. were the most abundant and their abundances were higher in the CAPK plot than that in the other plots (Supplementary data Fig. S5). Bao *et al.* (2014b) showed that methane oxidation and nitrogen fixation were simultaneously activated in the roots of rice plants in a paddy field with no N input, likely by the rice gene for microbial symbiosis, *CCaMK* gene (Ca^{2+}/calmodulin-dependent protein kinase). They also showed that the main bacterial groups mediating these processes were type II methanotrophic bacteria of the family *Methylocystaceae* and to a lesser extent *Methylocella* and *Bradyrhizobium* (Bao *et al.*, 2014a). It was suggested that anaerobic conditions decrease N available to the plants by denitrification and immobilization by microorganisms, which may be enhanced by the application of rice straw compost with a high C/N ratio (Devêvre and Horwáth, 2000; Mooshammer *et al.*, 2014).

The relative abundances of betaproteobacterial 16S rRNAs showed no differences among the fertilization treatments in April and May, but were higher in the CAPK plot than those in the other plots from July to September (Supplementary data Fig. S4B). The dominant genera of *Betaproteobacteria*, *Sideroxydans*, and *Thiobacillus* showed higher relative abundances in the CAPK plot than that in the NF plot after flooding (Supplementary data Fig. S6). These two genera are known to be chemoautotrophs that use iron or sulfur compounds as energy sources (Kelly *et al.*, 2005; Weiss *et al.*, 2007). The application of rice straw probably increased the rates of the reductions of ferric iron and sulfate, resulting in the rapid accumulation of ferrous iron and reduced forms of sulfur in pore water (Gao *et al.*, 2004; He *et al.*, 2010; Ye *et al.*, 2015). This, in combination with O_2 produced by plant roots and CO_2 resulting from the microbial metabolism of rice straw, would increase the oxidations of iron and sulfur compounds by chemoautotrophs in the oxic-anoxic interface (Emerson *et al.*, 2013). The increased abundance of the *cbbL* gene, which is involved in autotrophic carbon dioxide assimilation in the plots receiving rice straw in paddy soil, was previously observed (Yuan *et al.*, 2012).

The relative abundance of the 16S rRNAs of *Sulfuricurvum*, a member of *Epsilonproteobacteria*, was much higher in the CAPK plot than those in the other plots (Supplementary data Fig. S7). The only known species of the genus, *S. kujiense*, is known to be a chemoautotroph that uses reduced forms of sulfur as energy sources (Kodama and Watanabe, 2004), and its activity was expected to incrase in the CAPK plot due to the same reason for the increase of *Sideroxydans* and *Thiobacillus* as described above.

The relative abundances of deltaproteobacterial 16S rRNAs were lower in the CAPK plot than those in the other plots except for during August (Supplementary data Fig. S4D). Among the dominant genera of *Deltaproteobacteria*, *Anaeromyxobacter* can use oxygen, chlorinated phenol, nitrate, and iron as electron acceptors (Sanford *et al.*, 2002; He and Sanford, 2003) and *Kofleria* includes absolutely aerobic bacteria (Lang, 2014). The application of rice straw probably depletes these electron acceptors more rapidly than in the NF and APK plots and maintains the activities of the bacteria relatively low (Supplementary data Fig. S8).

Planctomycetes: The relative abundance of the 16S rRNAs of *Planctomycetes* was lowest in the CAPK plot (Fig. 3G). The dominant genus affiliated with this phylum, *Pirellula*, was found to grow in low-nutrient medium (Schlesner, 1994). Thus, it is expected that the activities of the genus decreased under the high-nutrient conditions of the CAPK plot (Supplementary data Fig. S9).

Unclassified 16S rRNAs: The OTUs that were not classified at the phylum level with the present classification method accounted for 9–19% of the 16S rRNAs in each plot and their relative abundances were in the order of NF > APK > CAPK after flooding (Fig. 3H), suggesting that the fertilization regimes also affected the abundances of these unclassified bacteria. Among the dominant unclassified OTUs, the relative activity of OTU_16 was lower in the CAPK plot, while that of OTU_7 was higher in the CAPK plot (Supplementary data Fig. S10). Because these OTUs occupied ~2.0% of the 16S rRNAs in the rice field, their functional roles are thought to be important in this ecosystem.

Summary and implications on agriculture and environment

In the present study, we investigated the variations in bacterial communities, based on their 16S rRNAs, in rice field soil according to variations in fertilization and season. The application of rice straw compost maintained the bacterial species richness to be significantly higher than that in case of non-fertilization (NF) and application of inorganic fertilizers (APK) in April and May, although the difference was not observed in July. It was previously shown that the increased microbial diversity in soil confers disease suppression and resistance against stress to the plant (Brussaard *et al.*, 2007; Wall *et al.*, 2015). Whether this has occurred in the present study requires further analysis.

The bacterial community structure in rice field soil was also shown to be significantly affected by the application of rice straw compost. In the plot receiving rice straw compost, the abundances of the bacteria generally known as copiotrophs, such as the members of *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, increased, while those known as oligotrophs such as the members of *Acidobacteria* and *Planctomycetes* decreased. This suggests a faster rate of transformation of organic matter in this plot (Fierer *et al.*, 2007). The effect of this on rice growth remains unclear.

It was also shown that the abundances of organotrophs with respiratory metabolism decreased and those of chemoautotrophs using reduced iron and sulfur as energy sources increased in the plot receiving rice straw compost. This suggests that aerobic mineralization of organic matter decreased and carbon dioxide fixation increased in this plot, which may have led to the reduced emission of carbon dioxide into the atmosphere, contributing to the mitigation of climate change. The effect of rice straw application to soil on carbon sequestration in rice paddy soil also requires further analysis (Lu, 2015).

In contrast, the enhanced emission of methane and the nitrogen deficiency in the plot receiving rice straw compost, which can be inferred from the increased abundances of *Methylocystis* and *Bradyrhizobium*, are negative effects induced by rice straw application, which was also suggested in previous studies (Bossio *et al.*, 1999; Devêvre and Horwáth, 2000; Mooshammer *et al.*, 2014). Additional options for supplying nitrogen, such as the combined application of rice straw and livestock manure (Qian *et al.*, 2014), and for mitigating the emission of methane, such as reduced irrigation (Ahn *et al.*, 2014), should be investigated with the observation of the resulting responses of soil microorganisms.

Considering the drastic effects of fertilization on the abundances of bacterial community found in this study, additional investigations on the interactions among fertilization, soil microorganisms, and plants are required to reduce the use of synthetic chemicals and increase the use of renewable resources in agricultural lands.

Acknowledgements

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