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LONG-TERM CHANGE IN LAND MANAGEMENT FROM SUBTROPICAL WETLAND TO PADDY FIELD SHIFTS SOIL MICROBIAL COMMUNITY STRUCTURE AS DETERMINED BY PLFA AND T-RFLP

ABSTRACT: Conversion of land from wetland to agricultural management practices can lead to significant changes in nutrient rich topsoil, which may have an impact on microbial community structure in soils. However, little is known about how long-term (*ca*. 40 years) rice cultivation, one of major agricultural management practices in many regions, influence soil microbial biomass and community structure. Soil samples were collected from a wetland and paddy field in Anhui province in eastern China to examine soil physical and chemical characteristics and associated soil microbial biomass and community composition. Microbial community composition was assessed using phospholipid fatty acid (PLFA) analysis, terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes. Results indicated that soil moisture content, pH, soil organic carbon, total nitrogen and NH_4^+-N contents were significantly lower in the paddy field in comparison to the wetland. Total microbial biomass showed a slightly significant decrease in the paddy field, however, there were significant shifts in the composition of the microbial communities based on the PLFA and T-RFLP fingerprintings in the both ecosystems. Signature PLFA analysis revealed that the sum of bacterial PLFAs and the relative proportions of Gram negative bacterial specific PLFAs significantly decreased in the paddy field, nonetheless, the relative numbers of actinobacterial, Gram positive and fungal PLFAs as well as the ratio between the bacterial and fungal PLFAs were not affected by the long-term agricultural management. These results revealed that long-term rice cultivations not only drastically decreased soil nutrients but also leaded to shifts in the soil microbial community structure, which would be helpful to provide a better understanding of wetland conservation and management practices.

KEY WORDS: soil microbial community structure, bacteria, PLFA, T-RFLP, paddy field, wetland

1. INTRODUCTION

Wetlands are one of the major terrestrial ecosystems and they play crucial roles in nutrient cycling as a major global carbon pool (Gorham 1991). As populations have been rapidly growing and human activities have intensified since the Industrial Revolution, large amounts of agricultural exploitation and management practices have strongly diminished the area of wetlands, which will further reduce their function as sinks for global carbon. Previous studies have shown that the conversion of natural ecosystems from forest or grasslands to cropping systems as well as increasing intensity of tillage are known to decrease soil organic carbon (SOC) (Murty

et al. 2002, Ogle *et al*. 2005). Comparatively few studies have documented the effect of conversion of wetland to agricultural uses on the soil characteristics and associated microbial community structure.

Soil microorganisms are critical components of terrestrial ecosystems as they play critical roles in organic matter decomposition and nutrient cycling (Zelles 1999). The biomass and composition of soil microbial community have been shown to vary according to the soil environment (Le Mer and Roger 2001, Pennanen 2001, Fierer *et al*. 2003). For example, a significant relationship between soil bacterial communities and soil moisture content was observed in a replicated field trial with winter flooding effects (Bossio and Scow 1998). Pennanen *et al.* (2001) found that the size and activity of soil microbes are directly affected by a shift in soil pH. Some researchers indicated that SOC and soil moisture content were the major determinants of soil community structure (Drenovsky *et al.* 2004, Frostegård and Bååth 1996, Steenwerth *et al*. 2008).

Compared with the wetland, the paddy field is a unique agro-ecosystem, where the field is flooded during most of the period of rice cultivation and is left drained during the off-crop season (Asakawa and Kimura 2008). The conversion of wetland to cropping ecosystems directly and indirectly affects soil environments and thus may alter the biomass and composition of soil microbial community. However, until recently, little is known about how the conversion from subtropical wetland to rice cultivation affect soil properties and associated microbial community structure and biomass, especially at the long-term scale.

The objective of this study was to investigate the changes in soil characteristics and associated microbial community after long-term conversion of a subtropical wetland to paddy field of the southern Anhui province in eastern China. We hypothesized that long-term agricultural cultivation would result in physical and chemical changes occurring in the topsoil, which would further lead to shifts in microbial biomass and community composition. We tested these hypotheses using physical and chemical analyses of the topsoil as well as phospholipid fatty acid analysis (PLFA), terminal restriction fragment length polymorphism (T-RFLP) of 16S rRNA genes.

2. SITE DESCRIPTION

Soil sampling sites were located in subtropical wetland of shengjinhu (30°20'48" N, 117°12'38"E) and in the neighborhood paddy fields in the south of Anhui province in the eastern China. The region belongs to national wetland reservation, occupying 125 ha. Rice cultivation from the conversion of a part of wetland has begun since the late 1960's, i.e., it is something like 40 years of agricultural land management history. This area belongs to subtropical monsoon climate and climate is warm and moisture. The mean annual precipitation is from 1300 to 1500 mm and the majority is concentrated from April to August. The mean annual temperature ranges from 14.5 to 16.6°C and frost-free period is over 240 days a year. Large amounts of wetland reclamation began in the late 1960's and have remained ever since. The agricultural practice in this region is an intensive double rice cultivation system, that is, rice growing seasons are from April to July and from August to October. The N fertilizers are applied to rice fields with the approximate ratio of base-tiller-ear being 60–30–10% during the rice growing seasons and the total amount of N, P_2O_5 and K_2O fertilizers is about 300, 75 and 75 kg ha^{-1} each year. The plant communities are dominated by. The plant communities are dominated by sedges (such as *Carex angustisquama* Franch. and *Cyperus dulourii*), eel grass (*Vallisneria spiralis* L.) and pondweed (*Potamogeton malaianus* Miq.) in the natural wetland.

3. METHODS

3.1. Soil sampling and soil characteristics analyses

Soil sampling was collected in late October 2007 from the wetland and paddy fields 1 km away after rice had been harvested. For each site, three soil samples were collected from spots at 10 m apart. Twenty bulk soil samples were taken from the depth of 0–10 cm with a soil auger (diameter 5 cm) at each spot. All plant residues were removed by

hand, and each sample was placed in polyethylene bags, homogenized by manual mixing, and stored on ice for transport to the laboratory. A part of subsamples from each soil sample were stored at 4°C for physical and chemical analyses which were performed within 2 weeks of sample collection, the remaining soil were stored at –20°C for subsequent PLFA and molecular analyses.

All bulk soil samples were passed through a 2 mm sieve. Soil moisture content was determined gravimetrically by drying subsamples at 105°C overnight and pH was determined in a 1:2 soil/water slurry. The SOC and total N contents were measured according to the methods given by Chen *et al.* (2002). After soils were extracted with 0.5 M K_2SO_4 (5 ml g^{-1} soil) (Mulvaney 1996) and the suspension was filtered (Advantec No. 5B, Tokyo, Japan), the NH_4^+-N concentrations in the extract were determined using a flow injection analyzer (Auto Analyzer 3, Bran+Luebbe Com. Ltd., Germany).

3.2. PLFA – Phospholipid fatty acid analysis

PLFAs were analyzed with the method described by Zelles and Bai (1993) and Zhang *et al.* (2008). Briefly, 3 g of fresh soil sample were extracted with a chloroform/methanol/citrate buffer mixture (1:2:0.8) and lipids were separated into neutral lipids, glycolipids, and phospholipid in a silicic acid column. Phospholipids were subjected to mild alkaline methanolysis to recover fatty-acid methyl esters (FAMEs) which were further analyzed with gas chromatography mass spectrometry (GC-MS). Helium was used as carrier gas at a flow rate of 0.9 ml min^{-1} and small aliquots of hexane to suspended lipids were used as internal standard. The time-temperature program for the oven was as follows: initial temperature 50°C for 2 min, increase 12°C min^{-1} until 180°C, increase 6°C min^{-1} until 220°C, and then increase 15°C min^{-1} until 280°C, final temperature 280°C for 10 min. Calculations of quantitative concentrations (nmol PLFA g^{-1} d.w. soil) of single PLFAS were carried out based on the soil moisture content. For each sample, individual PLFA values were expressed as a percentage of the total PLFAs (mole %) in the sample.

Biomass indicator for total microbial biomass and several phylogenetic groups were analyzed based on quantitative data of signature fatty acids. The sum of all PLFAs was used as an indicator (Le Mer and Roger 2001) of total microbial biomass (Zelles 1999). Bacteria were identified by saturated fatty acids: i15:0, a15:0, 15:0, i16:0, i17:0, a17:0, 17:0, cy17:0, cy19:0 and the monounsaturated fatty acids: 16:1ω7c, 16:1ω9c and 18:1ω7c. From this section, i15:0, a15:0, i17:0 and a17:0 are specific for Gram positive bacteria, and cy17:0 and cy19:0 for Gram negative bacteria (Zelles 1999). The fatty acid 18:2ω6,9c was used as an indicator of fungal biomass (Frostegård and Bååth 1996). The fatty acid 10-methylbranched 18:0 was used as biomass measure of actinomycetes (Zelles 1999). Fungi-to-bacteria ratio as well as Gram-negative-to-Gram-positive ratio was used to an indicator for shifts in microbial community structure (Fierer *et al.* 2003).

3.3. T-RFLP – Terminal restriction fragment length polymorphism analyses

DNA was extracted from each soil sample using Fastprep bead beater cell disrupter kit as described before (Zhou *et al.* 2008a). Bacterial 16S rRNA genes were amplified via polymerase chain reaction (PCR) using bacterial domain primers 8F and 926R (Liu *et al.* 1997). Both primers were obtained from Takara Biology Com. (DaLian, China) and primer 8F was labeled at the 5' end with phosphoramidite fluorochrome 5-carboxy-fluorescein (FAM). PCR conditions had been given by Zhou *et al.* (2008b) and PCR amplification was performed at Bio-rad MyCycler. The thermo-cycling parameters were as follows: initial denaturation at 94°C for 7 min followed by 33 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 60 s and final extension at 72°C for 10 min. Duplicate PCR reactions were run for each sample and pooled. PCR products were purified with DNA gel purification kit (Qiagen Inc.) and DNA concentrations were determined spectrophotometrically prior to digestion (Biospec-mini, Shimadzu, Japan). Initially, two restriction enzymes (HhaI and MspI) were used for samples to determine the best enzyme for further experiments. MspI produced more peaks and gave higher consistency, thus all further samples were therefore

Table 1. The selected soil physical and chemical characteristics of studied wetland and paddy field of the southern Anhui province in eastern China.
Values represent mean and standard deviation (n = 3). Within a column, data points with different letters are significantly different at P <0.05.

Soil type	Soil moisture content (%)	pH	Soil organic carbon (g kg^{-1})	Total N content (g kg^{-1})	NH_4^+-N (mg kg^{-1})
Wetland	58.71±1.98a	6.02±0.67a	16.58±2.78a	2.77±0.02a	79.12±2.45a
Paddy field	36.13±2.13b	4.25±0.96b	8.32±1.36b	1.58±0.01b	23.53±4.81b

digested with MspI only, which had been generally used in T-RFLP analysis (Hartmann and Winder 2006). Thirty nanograms of each sample were digested with MspI (Promega Com.) according to the manufacturer's instructions. After digestion samples were ethanol precipitated and resuspended in nuclease free water, the lengths of fluorescently labeled fragments were determined with 3730 ABI electrophoretic capillary sequencer (Applied Biosystems) in conjunction with the Genemapper software (Foster City, CA). Terminal reaction fragments (T-RFs) were quantified by peak area integration using a minimum peak height threshold of 50 relative fluorescent units. We excluded T-RFs below size 35 and calculated the proportion of each T-RF in each sample. T-RFs having proportion below 0.1% were excluded for subsequent analyses.

3.4. Statistical analyses

Soil characteristics, PLFA concentrations, the ratios of the difference microbial groups and fungal:bacterial ratio between wetland and cropland soils were analyzed with t-test (SPSS software 11.0, SPSS Inc.). The PLFA and T-RFLP profiles were analyzed using principal component analysis (PCA). The results were accepted as significant at P <0.05.

4. RESULTS

There are significant differences in the soils physical and chemical properties between the two sites (Table 1). Soil moisture content, pH and total N content were significantly less in the paddy field compared to the wetland. Wetland soils have twice greater SOC than the paddy field soils. NH_4^+-N con-

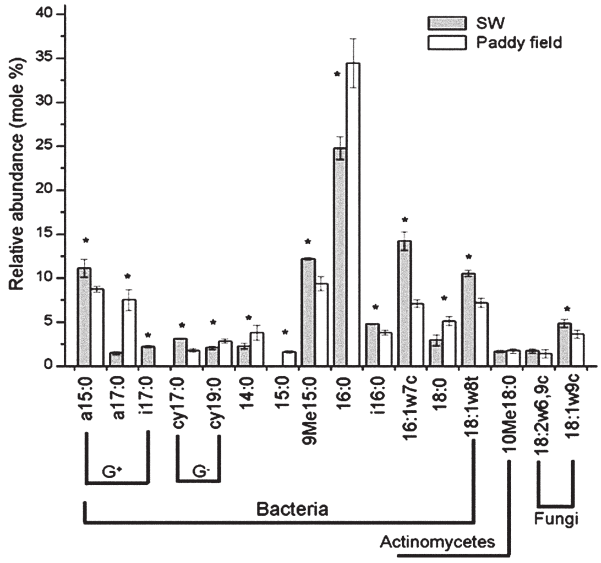

Fig. 1. List of identified PLFAs and percentage of PLFAs (mole %) in subtropical wetland (SW) and paddy field soils. Each value represents mean and standard deviation (n = 3). Asterisk indicates a significant difference at P <0.05 between wetland and paddy field soils.

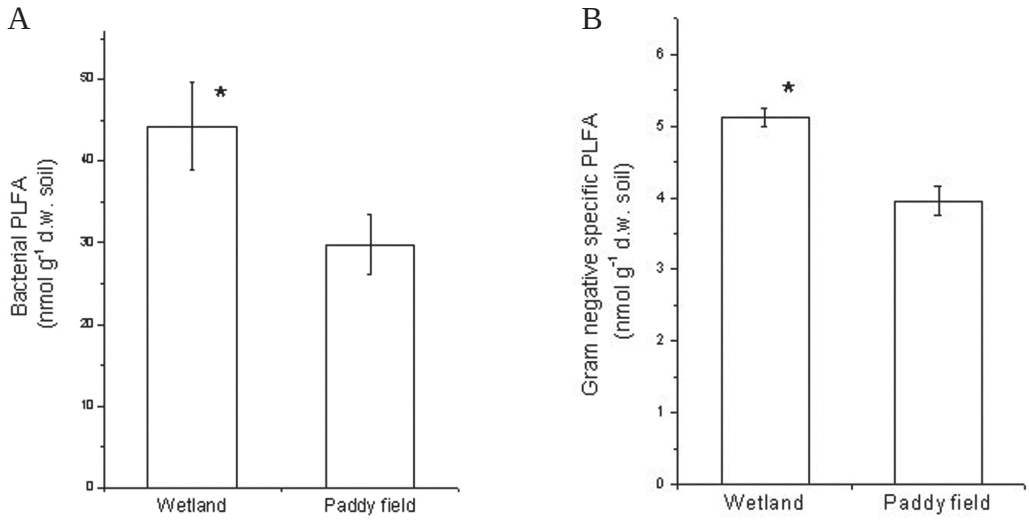

Fig. 2. The sum of bacterial phospholipid fatty acids (PLFAs) (A) and Gram negative specific bacterial PLFAs (B) expressed in nmol g^{-1} dry weight from wetland and paddy field soils. Values represent mean and standard deviation (n = 3). Asterisk indicates a significant difference at P <0.05 between wetland and paddy field soils.

tent was 3.36 times greater in the wetland than the paddy field soils.

PLFA analysis identified a total of 16 different fatty acids in the samples (Fig. 1). PLFA profiles were dominated by the fatty acids 16:0, which accounted for approximately 34 and 24% of the total PLFA in the wetland and the paddy field samples, respectively. In addition, the paddy field fatty acids 16:1ω7c, 9-Me 15:0 and a15:0 together accounted for *ca*. 37% of the total PLFA. The proportions of 8 individual fatty acids were significantly increased among all PLFAs in the paddy field in comparison to the wetland, but other proportions of 6 fatty acids were significantly decreased. The relative numbers of two PLFAs, i.e. 18:2ω6,9c (fungi specific PLFA) and 10-Me 18:0 (Actinobacterial specific PLFA), were similar between the two ecosystems. The total PLFA biomass, which is an indicator of the total microbial biomass, was slightly significantly lower in paddy soils compared to wetland soils (P = 0.08).

There was a significant relative decrease in bacterial specific PLFAs after long-term conversion of wetland to paddy field (Fig. 2A). A significant decrease was also observed in total Gram negative bacterial-specific PLFAs as a fraction of total bacterial PLFA pool in the paddy field in comparison to the wetland (Fig. 2B). Actinobacterial, Gram positive and fungal PLFAs as well as the ratio between the bacterial and fungal PLFAs were not affected by long-term agricultural management.

The PLFA and T-RFLP profiles were analyzed using PCA to assess the shift of microbial community structure between the two ecosystems (Fig. 3). The PCA plots of the first two principal components (PCs) accounted for 43.7 and 26.2% of the total sample variance based on the analysis of PLFA data (Fig. 2A), and the profiles showed a clear difference between the two ecosystem samples. Similarly, PCA analysis of T-RFLP data also revealed a clear separation of wetland from paddy field samples. The two ecosystems had distinctive PC2 values as wetland had lower values than paddy field (Fig. 2B).

5. DISCUSSION

Microbial communities have been investigated and compared between the geographically different soils, soils with or without a treatment, or different habitats of an ecosystem (e.g. McCaig *et al.* 1999, Ulrich and Becker 2006, Asakawa and Kimura 2008). Comparatively less information is available concerning the change of microbial community after long-term conversion of the wetland to the paddy field. In this study, we hypothesized that long-term rice cultivations would decrease soil physical and chemical properties due to the erosion of surface en-

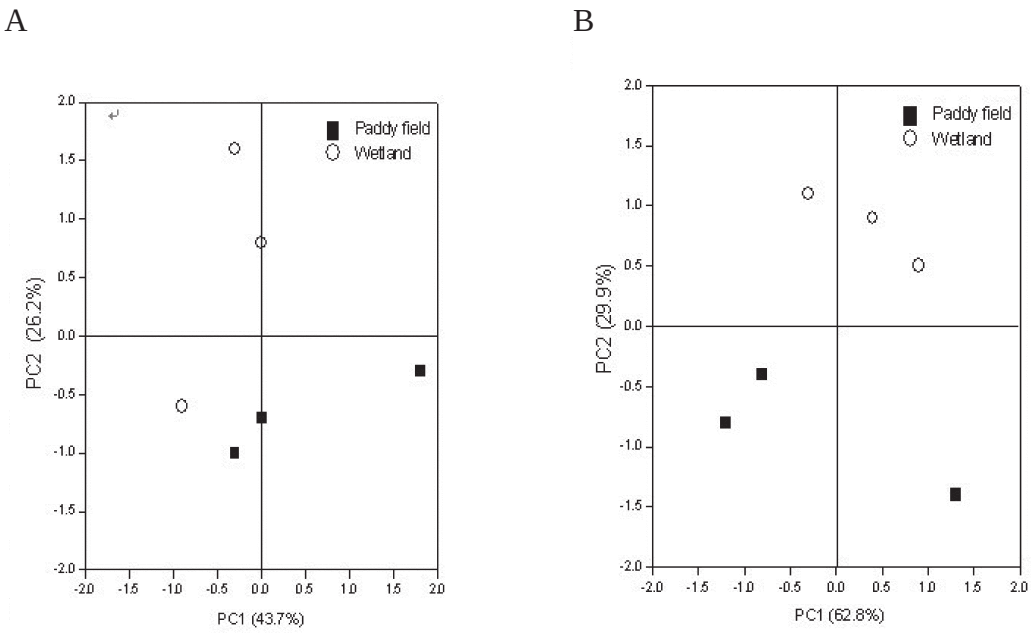

Fig. 3. Ordination plot of principal components analysis (PCA) of PLFA profiles (A) and T-RFLP profiles (B) in soils from subtropical wetland and paddy field in the neighborhood. Numbers in parenthesis are percentage variance by each principal component (PC).

riched soil and the harvest of crop production, which would further lead to changes in microbial biomass and community structure in soils. As we expected, soil characteristics were substantially changed in the paddy field in comparison to the wetland (Table 1). As the paddy field is a unique agro-ecosystem and subjected to dry-wet rotation, it experiences more frequently soil drying compared with wetland. Along with the erosion of soil nutrient and the harvest of crop production, these may lead to a drastic decrease by *ca.* 50% in SOC of paddy soil. Previous studies have shown that agricultural land uses or management practices can significantly decrease SOC stock. For example, Guo and Gifford (2002) reported that soils lost 42 and 59% of their SOC stock upon conversion from forest to crop and from grassland to crop, respectively. Murty *et al.* (2002) also evaluated the decrease of SOC stock in the major US cropland soils at *ca*. 16 and 22–25%, respectively.

Despite these significant changes in soil physical and chemical parameters, the total microbial biomass was only slightly different between the two ecosystems. This result is somewhat surprising, especially given the large changes in soil moisture, organic carbon, and soil nitrogen that occurred (Table 1). However, biomass is not the most sensitive indicator of changes in microbial communities. Changes in specific microbial populations within a community have often been shown to occur even when total microbial community size remains stable (Pennanen 2001, Baniulyte *et al.* 2009). Therefore, molecular methods were used in this study to examine the composition of the soil microbial communities in more detail.

PCA analysis of PLFA data indicated that there was a significant shift in microbial community composition between the samples. The analysis of signature PLFAs revealed a shift within bacterial community between two ecosystems with a significant relative decrease in Gram negative bacteria, but not in Gram positive bacteria. Previous work has shown that Gram negative bacteria dominate in the high nutrient environment (Marilley and Aragno 1999). The decrease in Gram negative bacteria in paddy field may reflect the decrease in plow layer nutrient content, consistent with changes in soil characteristics.

The relative numbers of fungal and actinomycete specific PLFAs remained steady between wetland and paddy field samples. As the majority of soil fungi and actinomycetes are aerobic (Wellington and Toth 1994) and they typically represent a substantial portion of the microbial biomass in terrestrial

soils (Ruzicka *et al.* 2000), the steady populations might reflect a consistent anaerobic environment in wetland and paddy field soils (Le Mer and Roger 2001). The amount of actinomycetes specific PLFAs is a fraction of that of bacterial PLFAs, which was in contrast with recent studies, which reported that the bacterial community in plowed soil of a paddy field had a higher diversity index than other habitats. Microbial communities in rice soils were dominated by actinomycetes and Gram-positive bacteria by PLFA (Kimura and Asakawa 2006) and by Chloroflexi and Actinomycetes groups using the analysis of denaturing gradient gel electrophoresis (DGGE) (Asakawa and Kimura 2008). A slight difference in bacterial community structure based on the analysis of PLFA and clone library data was also observed recently (Baniulyte *et al.* 2009). This might indicate slight difference in the sensitivity of the methods, since PLFA analyzes major microbial communities by cell membrane fatty acids type, not 16S rRNA sequence.

The resolution of PLFA analysis is limited to broad categories of microorganisms, so the composition of the bacterial portion of the microbial communities was examined in more detail using DNA-based techniques. T-RFLP revealed a much clearer separation between wetland and paddy field samples compared to the PCA plot obtained by PLFA data, consistent with a drastic decrease in soil properties of paddy field in comparison to wetland. Many studies have demonstrated that soil physical and chemical characteristics exert a great influence on microbial biomass and community structure (Bossio and Scow 1998, Pennanen 2001), even some factors such as soil moisture and SOC had become the most important determinants of shifts in soil microbial communities (Drenovsky *et al.* 2004, Steenwerth *et al.* 2008).

In conclusion, long term change in land management from subtropical wetland to paddy field significantly reduced pH, SOC, total N, NH_4^+-N contents and the total microbial biomass. Additionally, there were significant shifts in the composition of the microbial communities based on the PLFA and T-RFLP fingerprintings in the both ecosystems. These results revealed that long-term rice cultivations not only drastically decreased soil nutrients but also leaded to shifts in the soil microbial community structure, which would be helpful to provide a better understanding of wetland conservation and management practices.

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