Accumulation of physically protected organic carbon promoted biological activity in macro-aggregates of rice soils under long term rice cultivation

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Running title: carbon and microbial activity in aggregates of rice soil
Abstract:
While carbon stabilization had been increasingly concerned as ecosystem properties, the link between carbon stabilization and soil biological activity had been yet poorly assessed in soil dynamics of carbon and aggregation. In this study, topsoil samples were collected from rice soils derived from salt marsh under different lengths of rice cultivation up to 700 years from a coastal area of China. Particle size fractions (PSF) of soil aggregates were separated using a low energy dispersion protocol. Carbon fractions in the PSFs were analyzed with either FTIR spectroscopy or chemical fractionation. Soil microbial community of bacterial, fungal and archaeal were analyzed with molecular fingerprinting using specific gene primers. Soil respiration and carbon gain from maize straw amendment as well as enzyme activities were respectively measured, using lab incubation protocols. While the PSFs were dominated by fine sand (200-20μm) and silt (20-2μm) fractions, the mass proportion both of sand (2000-200μm) and clay (<2μm) fraction increased with prolonged rice cultivation. Soil organic carbon was enriched mostly in coarse sand fraction (40-60g/kg), followed by the clay fraction (20-25g/kg), but depleted in the silt fraction (~10g/kg). Contents of recalcitrant C pool were higher (33-40% of total SOC) in both coarse sand and clay fractions than in fine sand and silt fractions (20-29% of total SOC). However, the ratio of LOC/SOC showed a weak decreasing trend with decreasing size of the PSFs. Total soil DNA content in the size fractions followed a similar trend to that of SOC. Bacterial and archaeal gene abundance were concentrated in both sand and clay fractions but that of fungi in sand fraction only, but increased with prolonged rice cultivation in both sand and clay fractions. Change
in community diversity with sizes of the PSFs was found of fungi and weakly of bacterial but not of archaeal. Soil respiration quotient (Respired CO$_2$-C to SOC) was highest in silt fraction, followed by the fine sand fraction but lowest in sand and clay fractions in the rice soils cultivated over 100 years. Whereas, scaled by total DNA concentration, respiration was higher in silt fraction than in other fractions for these rice soils. For the size fractions other than clay fraction, soil DNA concentration, archaeal gene abundance, normalized enzyme activity and carbon sequestration was seen increased but SOC- and DNA- content scaled soil respiration decreased, more or less with prolonged rice cultivation. Carbon chemical stability and respiration were in a similar between sand and clay fractions but correlations of total DNA contents and bacterial gene abundance as well as normalized enzyme activity to SOC and labile OC content were only observed in sand fraction only. Our findings suggested that carbon accumulation and stabilization was prevalent in both sand and clay fraction, only the coarse sand fraction was found responsible for bioactivity dynamics in the rice soils.

**Key words:** rice soil, carbon sequestration, carbon stabilization, soil bioactivity, soil aggregates, size fractions, rice cultivation
Soil organic matter (SOM), as a continuum of organic substances with different degree of decomposition (Lehmann and Kleber, 2015), provided a key driver for soil aggregation, mediating soil ecosystem functions and services (Banwart, et al. 2014). Soil aggregates had been considered the fundamental soil particle units that organic matter, minerals and microbes interacted to store C and nutrient as well as moisture, and mediated their cycling in soil-plant systems (Six et al., 2004). Formation and turnover of soil aggregates shaped the micro-habitats for soil microbial communities (Six et al., 2000; Ettema and Wardle, 2002; Balser et al., 2006; Kogel-Knabner et al., 2008). There had been increasing evidences that soil aggregates could be the most responsible to organic carbon sequestration by physical protection against microbial access and decomposition (Blanco-Canqui and Lal, 2004; Six et al., 2004; Kong et al., 2005; Six and Paustian, 2014), with separate allocation of mineral associated OM fractions (Lehmann et al., 2008; Dungait et al., 2012; Vogel et al., 2014). While soil carbon had been physically protected in micro-aggregates, the link between organic carbon stabilization and microbial biological activity in soil aggregates had not yet been quantitatively assessed (Six et al., 2007). Such an assessment could help to understand the relationship between organic carbon sequestration and soil functions.

Soil carbon dynamics, known related to aggregate stability (Six et al., 2000), could drive changes in interactions of minerals, organic matter and soil microbial community (Tisdall and Oades, 1982; Lützow et al., 2006; Marschner et al., 2008; Schmidt et al., 2011). However, carbon dynamics and aggregate stability in soil
could be further affected by biophysical conditions of pH and redox potential as well as carbon substrate quality under varying management practices (Calderon et al., 2001; Aseri and Tarafdar, 2006). Bioactivity, generally known of the size, diversity and biochemical activity of soil microbes (Bardgett and van der Putten 2014), had been shown largely affected by organic carbon availability and redox potential with or within aggregates (Rillig et al., 2001; Six et al., 2006; Strickland and Rousk, 2010). Thus, the changes in organic matter and microbial bioactivity in soil aggregates could offer key information to understand the soil aggregate dynamics in soils with long term agricultural managements.

Soil aggregates could be parameterized by distributions of particle size fractions (PSFs), through separation with least low energy dispersion (Kandeler et al., 2000). Low energy ultrasonic dispersion could allow such least disturbed size fraction separation (Stemmer et al., 1998), and afford measurements of microbial community and enzyme activity in soil aggregates (Stemmer et al., 1998; Kandeler et al., 2000; Marx et al., 2005). This method had been used to characterize distribution of organic matter, microbial communities, and enzyme activity in aggregates and to address the impacts by different agricultural practices (Kandeler et al., 2000; Sessitsch et al., 2001; Poll et al., 2003). Recently, there had been increasing studies on size fractions of soil aggregates, enhancing our understanding of the micro-scale interactions driving SOC stability and nutrient cycling in soils (Kandeler et al., 2006; Lagomarsino et al., 2012; Six and Paustian, 2014). The distribution of soil microbial biomass and activity in particle size fractions could be
important in determining how agro-ecosystems accumulated and stabilized SOC
(Salinas-Garcia et al., 1997; Kandeler et al., 1999). Numerous studies had focused on
the relationship between microbes and SOC in soil particle size fractions under
different tillage conversion or long term soil managements (Kandeler et al., 1999;
Matocha et al., 2004; Zhang et al., 2013). However, interactions of organic matter,
microbial and enzyme activities in aggregate size fractions of long term cultivated
soils and their dynamics with soil development had been not yet fully understood.
Rice paddy soils had been known of high SOC storage and sequestration
potential compared to dry-land croplands (Pan et al., 2004; Pan et al., 2009; Wissing
et al., 2013). In early studies, greater persistence of OC in rice paddies than in dry
croplands had been often attributed to enhanced aggregation and thus the aggregate
stability (Lu et al., 1998; Yang et al., 2005), and to increased humification of SOC
(Olk et al., 2000). SOC stabilization in paddy soils had been increasingly understood
linking to chemical stabilization with OC bound to free oxyhydrates (Zhou et al.,
2009; Cui et al., 2014), to physical protection with enhanced aggregate stability (Li
et al., 2007; Zhou et al., 2008), or their interactions (Song et al., 2012; Song et al.,
2013) as well as chemical recalcitrance (Song et al., 2012). Moreover, there had been
increasing knowledge of co-evolution of soil microbial community and diversity
with SOC accumulation and stabilization in rice paddies (Zhang et al., 2007; Zheng
et al., 2007; Liu et al., 2011). In a recent study by Kalbitz et al. (2013) using a
chronosquence, continuous SOC accumulation with increasing rice cultivation
intensity, which had been promoted following the desalinization and decalcification in
the initial stage after the salt marsh shifted to rice paddy, was characterized. The accumulated SOC was increasingly stabilized with neoformed iron-oxyhydrates accumulated in the rice soils in the long run with prolonged rice cultivation (Cheng et al., 2009; Wissing et al., 2011) and physical protected by micro-aggregates (Wang et al., 2015; Zou et al., 2015). SOC accumulation had been shown driving enhancement of microbial biomass and evolution of microbial community in long-term cultivated paddy soils (Bannert et al., 2011; Jiang et al., 2013; Liu et al., 2015). Nevertheless, the dynamics of SOM and bio-activity in size fractions of soil aggregates had not yet been characterized for understanding carbon sequestration in relation to soil microbial structure and functioning of rice paddy soils.

Taking a rice soil chronosequence as a case, we looked into the changes in organic matter (SOM) stabilization and microbial activity in different size fractions across the sequence and to infer how SOM accumulation and stabilization relate to soil bio-activities and to their dynamics along long term rice cultivation up to 700 years. We aimed to address if organic carbon stabilization could confront soil bioactivity in rice soils.

2 Materials and methods

2.1 Site and soils of the studied chronosequence

The studied soil chronosequence was a series of rice soils shifted from tidal marsh to rice cultivation under different lengths in a coast land located in Cixi Municipality, Zhejiang Province, China (Fig.1). The area is within the typical northern subtropical monsoon climate for Eastern China, with a mean annual temperature of 17.7 °C and
annual precipitation of 1,367 mm during 2004-2014 (http://cdc.nmic.cn/home.do).

Lying in the south bank of Hangzhou Bay, the parent material was estuarine sediments deposited within the Hangzhou Estuary, East China Sea (Fig.1). In the area, coastal tidal marsh had been increasingly reclaimed for rice production, with dyke establishments at different historical stages for the last 2000 years. The studied chronosequence had been already identified and pedologically characterized by Cheng et al. (2009), and soil development had been in depth studied in morphology, mineralogy and microbiology (Kölbl et al., 2014). Changes of SOC stability and microbial activity along the chronosequence had been assessed in our previous research by Wang et al. (2015) and Liu et al. (2015).

In this study, individual rice soils of the chronosequence were identified based on dyke establishment history recorded in Cixi County Annals (with brief

**Fig. 1** Sampling sites for the individual soils constituting the rice soil chronosequence from Cixi County, Zhejiang province, China. The suffix number following P (paddy soil) designates the years under rice cultivation after shifting from salt marsh since dyke establishment.
information in Chinese available at www.cixi.gov.cn), including an initial tidal marsh soil before rice cultivation (P0), rice soils of P50, P100, P300 and P700 shifted for rice cultivation on dyke establishment respectively 50, 100, 300 and 700 years before present (Fig.1). These soils were apart from each other in a distance no more than 40-km in nearly the same topography. All the rice soils developed on comparable parent materials of paleo-deposit from Yangtze River under more or less consistent biogeographical condition. Soil texture ranged from silty loam to silty clay-loam. Particle composition of the soils was dominated by silt (75%-84%), followed by clay but low in sand content (Chen and Zhang, 2009). The clay mineral assemblage consisted of illite (40-50%), chlorite (20-30%) and kaolinite (10-20%) with a minor amount of smectite and quartz (Zhang et al., 2010b).

As situated in a relatively small area with a traditional summer rice-winter rape rotation, rice production management on the soils of the chronosequence could be considered relatively consistent across sites, with similar cultivars and management practices including crop protection, irrigation and fertilization (Cheng et al., 2009). Of course, influence of salt on rice production could occur in the early stage of rice cultivation on the tidal marsh derived soils while the ground water table had been enough low without restricting rice growth (Kölbl et al., 2014).

2.2 Soil sampling
All the five individual soils of the chronosequence were sampled in early November 2011, when the soil was moist following rice harvest. During soil sampling of topsoil (0-15 cm in depth) for each soil, an undisturbed soil core was collected using an
191 Eijkelkamp soil core sampler (Agrisearch Equipment, Giesbeek, The Netherlands) while a bulk soil sample using a stainless steel shovel. The sampling was done in triplicates respectively from three adjacent individual fields. All soil samples were shipped to the lab within two days after sampling, and stored at 4 °C before soil analysis in the following 2 weeks.

A bulk sample was divided into two portions, one for physical-chemical analysis and the other for biochemical and microbial incubation study. For soil property analysis, a portion of soil samples were removed of gravels, roots and visible plant detritus, ground to pass through a 2-mm mesh sieve and further to pass the mesh as required by the protocol.

2.3 Particle size fractionation of soil aggregates

The undisturbed soil cores were used for dispersion in water with low energy sonication procedure, without chemical dispersing agents, following the recommendation by Smith et al. (2014). In this study, particle size fractions of water stable aggregates were separated with a modified procedure described by Stemmer et al. (1998). A portion of field moist soil core (50 g equivalent d.w.) were placed into a glass beaker and dispersed in 100 ml of distilled water using a low-energy ultrasonic disaggregator (Zhixin, JVD-650, Shanghai, China) with output energy of 170 J g⁻¹ for 5 min. A fraction of 2000-200 μm was separated by wet sieving and the fraction of 200-20 μm was subsequently obtained by sedimentation after siphonage. The remainder was centrifuged to collect the fraction of 20-2 μm and the supernatant was centrifuged to collect the fraction of <2 μm. The samples of the obtained size
fractions were freeze-dried with a frozen dryer (Thermo, Modulyo D-230, NY, US) and then stored at -70 °C.

### 2.4 Organic carbon pool and FTIR spectroscopy analysis

Total soil organic carbon (SOC) and total nitrogen (TN) of the separated PSFs were determined with a CNS elemental analyzer (Elementar Vario-max CNS Analyser, Germany Elementar Company). Labile organic carbon (LOC) content was measured by 0.33 M potassium permanganate oxidation (KMnO$_4$), following a procedure described by Blair et al. (1995).

Chemical composition of organic carbon in the particle size fractions were characterized with FTIR spectroscopy using a Bruker FTIR spectrophotometer (Bruker TENSOR 27 Spectrometer, Ettlingen, Germany). Briefly, a portion of frozen-dried aggregate sample was powdered in an agate mill, and 1 mg of the homogenized sample powder was mixed thoroughly with 100 mg KBr. The pellet prepared with a press was placed in a sample holder and FTIR spectra were recorded. FTIR scanning was conducted in ambient conditions at 22±1°C. The resolution was set to 4 cm$^{-1}$ and the operating range was 400 to 4000 cm$^{-1}$. In all cases, 20 scans per sample were recorded, averaged for each spectrum and corrected against the spectrum with ambient air as background. Absorption peaks were assigned to organic functional groups following Ellerbrock et al. (1999) and Cocozza et al. (2003). The absorption intensity band from 3700 to 3000 cm$^{-1}$ represented vibrations of H-bonded hydroxyl O-H in phenols. The bands at 2931 cm$^{-1}$ are preferentially assigned to asymmetric and symmetric aliphatic-C CH$_3$ and CH$_2$ stretching. The
12 bands at 1634 cm\(^{-1}\) were due to aromatic C=C vibrations, symmetric stretching of COO\(^-\) groups, and H-bonded C=O of conjugated ketones. The bands at 1022 cm\(^{-1}\) have frequently been assigned to polysaccharide C–O stretching. The proportion of different chemical groups was estimated with a software affiliated with the FTIR manual.

2.5 SEM observation of soil aggregates

The aggregate assembly of a portion of an undisturbed soil core was examined under a scanning electron microscope (Model Hitachi S-3000N) at an electron acceleration voltage of 20 kV. Prior to scanning, a sample was mounted on a stub using double sticky stickers and coated with gold using Hummer sputter coating equipment (Anatech Ltd., Union City, CA). Pictures were captured by automatic image capturing software (Hitachi Science Systems LTD., Schaumburg, IL). Magnifications and linear scale are indicated in the micrographs.

2.6 DNA extraction, microbial gene abundance and diversity analysis

A portion (0.45 g) of a PSF sample stored at -70 °C was used for DNA extraction with PowerSoil\textsuperscript{TM} DNA Isolation Kit (MoBio, USA), following the manufacturer guide. The concentration of the DNA extracts was checked with a spectrophotometer (Eppendorf, Germany), and its integrity and size were checked by using 1.0% agarose gel electrophoresis. Extracted DNA was stored at -70 °C prior to molecular microbiological assay.

Quantitative real-time PCR assay was performed on a 7500 real-time PCR system (Applied Biosystems, USA) using SYBR green as a fluorescent dye. Primer
combinations of 338F/518R (Øvreås and Torsvik, 1998), ITS1F/ITS4 (Gardes and Bruns, 1993) and Ar109F/Ar915R (Lueders and Friedrich, 2000) were used for bacterial 16S rRNA, fungal Internal Transcribed Spacer (ITS) region and archaeal 16S rRNA genes respectively in the Real-time PCR assay.

PCRs were carried out on all PSF’s DNA samples with specific primers to amplify the 16S rRNA genes from bacteria (27F and 1492R) and archaea (Ar109F and Ar915R) and the ITS regions from fungi (ITS1F and ITS4). The forward primer from each pair had a fluorescent label (6-FAM) attached to the 5’ end. Amplification of the 16S rRNA gene and ITS regions, purification, digestion and amplicon separation for T-RFLP analysis are described in the supplementary materials and methods.

From the T-RFLP profiles, the Shannon diversity index ($H'$) of the individual T-RFs was calculated following Blackwood et al., (2007), using an equation:

$$H' = \sum Pi \ln Pi$$

where, $Pi$ is the proportion of each T-RF in a single sample.

2.7 Soil enzyme activity of soil aggregates

In this study were analyzed soil enzyme activities involved mainly in cycling of C, N and P in soils. In detail, invertase, urease and acid phosphatase were determined using the methods described by Guan et al., (1986) while β-glucosidase, β-cellobiosidase and peroxidase were measured using 96 micro-plates colorimetric methods described by Saiya-Cork et al., (2002). For an integrated assessment of microbial biochemical activity, the six different enzyme activities analyzed were
normalized to give a single value of NEA of an individual fraction, which was estimated with the following equation:

\[ x'_i = \frac{x_i}{\sum_{i=1}^{n} x_i} \quad (i=1,2,\ldots,5), \tag{2} \]

where, \( i \) was the number of each soil sample (P0, P50, P100, P300, P700), \( x \) was the enzyme activity and \( x' \) was the normalized enzyme activity of each soil sample.

Subsequently, an arithmetic mean value of enzyme activity of each sample was obtained for the NEA.

2.8 Carbon gain in soil aggregates

Maize shoot biomass was crashed into pieces of 2-3 cm length and further ground in a stainless steel grinder to pass a 1.0 mm sieve, homogenized before use. The prepared maize material contained organic carbon of 415 g kg\(^{-1}\), total N of 6.11 g kg\(^{-1}\) and \( \delta^{13} \)C abundance of -12‰. For incubation, 300 g of an air dried bulk soil sample (passed 2 mm sieve) was thoroughly mixed with 3.9 g of the prepared maize material (corresponding to 5.4 mg C g\(^{-1}\) soil), in a plastic jar sealed with pierced plastic film.

The incubation was performed in a moisture- and temperature-constant incubator (LRH-250-S, Medicine Machinery Co Ltd. Guangdong, China) at constantly 25 ± 1 °C for 180 days. The soil moisture in the jar was adjusted to 60% of soil water holding capacity, which was sustained over the incubation course by weekly adding distilled water to reach the weight balance. After incubation for 180 days, soil samples were air dried at room temperature and then separated to obtain particle size fractions followed the procedure mentioned in Section 2.3. A portion of the separated size fraction sample was sieved through a 0.15 mm sieve for determination of the
relative abundance of $^{13}$C, with an isotope ratio mass spectrometer (Finnigan MAT253) in Institute of Geochemistry Chinese Academic of Science, Guiyang, China. For this determination, the samples were removed of inorganic carbon, using a dilute HCL solution.

The result of $^{13}$C abundance was expressed in δ per mil scale according to the equation:

$$^{13}\text{C} \, (\text{‰}) = \left[\left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1\right) \times 1000 \right]$$  

where, $R_{\text{sample}}$ and $R_{\text{standard}}$ was the isotope ratio of $^{13}$C/$^{12}$C of a sample and a reference material respectively, and were related to the Pee Dee Belemnite (PDB).

The amount of maize carbon preserved in a particle size fraction of soil aggregate was calculated with the following equations:

$$C_4 = \frac{\delta - \delta_{C_3}}{\delta_{C_4} - \delta_{C_3}} \times C_t$$  

$$C_{\text{gain}} = C_4 \times P_{\text{mass}}$$

where, $C_t$ is the organic carbon content (g kg$^{-1}$), $\delta C_3$ and $\delta$ is the relative abundance of $^{13}$C before and after incubation, of a particle size fraction; $\delta C_4$ is the native relative $^{13}$C abundance of the used maize material; And, $C_4$ is the concentration of maize carbon in a particle size fraction after incubation. $C_{\text{gain}}$ was the amount (gC) of maize derived carbon after incubation while $P_{\text{mass}}$ was the mass distribution (%), of a particle size fractions of an incubated bulk soil.

2.9 Soil respiration of aggregates

For assessing microbial use of carbon in different PFSs, soil respiration as measured by CO$_2$ production of a fraction sample was determined using an anaerobic
laboratory incubation protocol, following Zheng et al., (2007). For this, 20g dry weight equivalent of a PSF sample (Section 2.3) was placed into a 125ml glass jar and the sample was submerged with 40ml distilled water before being gently mixed. The jar was then sealed with a butyl rubber stopper and two Teflon tubes for gas sampling and N\textsubscript{2} circulation were inserted into the stopper. The headspace was repeatedly evacuated and flushed with N\textsubscript{2} gas into the jar at a rate of 300ml min\textsuperscript{-1} for 30min, creating an anaerobic condition. The jars with soil samples were randomly arranged in an incubator (LRH-250-S, Medicine Machinery Co Ltd. Guangdong, China) and incubated constantly at 25 ± 1 °C for 37 days. During incubation, a 0.25 ml sample of the gas was collected by pressure syringe every 5 days starting on the third day after incubation was initiated. After each gas sampling, N\textsubscript{2} gas was again flushed into the jar at a rate of 300ml min\textsuperscript{-1} for 30 min to removing all the emitted gas in the jar (Wang et al., 1999). CO\textsubscript{2} concentration in a gas sample was determined with a gas chromatograph (Agilent 4890D) equipped with a stainless steel column (Porapak Q) (80/100 mesh) and flame-ionization detector (FID). Following the procedures described by Zhang et al., (2010a), the determination was done with an oven temperature of 80°C and a FID temperature of 200°C, with N\textsubscript{2} as the carrier gas at a flow rate of 40ml min\textsuperscript{-1} and a make-up gas mixture of H\textsubscript{2} and air at a flow rate of 35 ml min\textsuperscript{-1}. A blank of 40 ml distilled water was used as the control for the gas concentration in the bottle. The incubation was conducted in triplicates. The total CO\textsubscript{2} evolved was estimated from the cumulative sum of the gas evolved in all monitoring intervals and was used to calculate the anaerobic soil respiration.
expressed in terms of soil mass.

2.10 Data treatment and statistical analysis

All data was treated with EXCEL 2013 and expressed as mean plus/minus standard deviation. The significant differences in carbon fractions and in microbial parameters between particle size fractions in a single soil and between soil samples of a single particle size fraction were respectively statistically analyzed by one-way ANOVA with Tukey’s test using a SPSS software package 20.0. Statistical significance was defined at 95% confidence level.

3 Results

3.1 Organic carbon characterization

In Table 1 is presented the results of size fractions distribution of the soils over the chronosequence. While the fine sand (200-20µm) and silt (20-2µm) sized fractions together accounted for up to 80% of a bulk soil across soils, the proportion of coarse sand (2000-200µm) and clay (<2µm) sized aggregates increased with prolonged rice cultivation of the chronosequence. The mean weight diameter (MWD), an indicator of soil aggregate stability, increased from 86.5 µm of P0 to 132 µm of P700 over the chronosequence. This change in mean diameter was supported by the SEM observation.

Table 1 Particle-size distribution (%) of aggregates of the studied chronosequence soils. Low case letters indicate a significant (p<0.05) difference between soils for a single fraction, in a column.
Direct evidence was found for promoting aggregation with the increasing age of rice cultivation. There were dispersed mineral particles in the initial tidal marsh (P0). Mineral particles and organic matter were bound together into micro-aggregates during the initial paddy cultivation stage (50 years). With increasing rice cultivation length, micro-aggregates at a lower hierarchical order exclude the pore spaces between the particles and aggregates that comprise a higher order.

<table>
<thead>
<tr>
<th>Soil</th>
<th>2000-200μm</th>
<th>200-20μm</th>
<th>20-2μm</th>
<th>&lt;2μm</th>
<th>MWD(μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>2.78±0.59c</td>
<td>46.53±1.30a</td>
<td>41.00±2.46a</td>
<td>9.69±0.57d</td>
<td>86.5±6.2c</td>
</tr>
<tr>
<td>P50</td>
<td>5.10±0.25b</td>
<td>44.31±0.02b</td>
<td>40.79±0.41a</td>
<td>9.8±0.14d</td>
<td>109.5±2.1b</td>
</tr>
<tr>
<td>P100</td>
<td>5.34±0.10b</td>
<td>43.17±0.53c</td>
<td>39.72±0.72a</td>
<td>11.78±0.09c</td>
<td>110.8±1.3b</td>
</tr>
<tr>
<td>P300</td>
<td>6.87±1.04a</td>
<td>41.53±1.64d</td>
<td>38.67±0.33a</td>
<td>12.92±0.27b</td>
<td>125.8±7.8a</td>
</tr>
<tr>
<td>P700</td>
<td>7.63±1.40a</td>
<td>39.91±5.16d</td>
<td>36.97±3.59a</td>
<td>15.49±0.16a</td>
<td>132.2±8.5a</td>
</tr>
</tbody>
</table>
Fig. 2 Scanning electron microscopy images of aggregates after dispersion in water from the studied soil chronosequence, with age varying from 0 to 700 yr of paddy cultivation history. The number of years of rice cultivation were marked in the upper left corner of the images.

As listed in Table 2, SOC was 11.07 g kg\(^{-1}\) and 9.90 g kg\(^{-1}\) in coarse sand and fine sand fraction, and 5.13 g kg\(^{-1}\) and 9.29 g kg\(^{-1}\) in silt and clay fraction, in the initial tidal marsh (P0). While in rice soils (P50- P700), SOC ranged from 40.64 g kg\(^{-1}\) to 60.79 g kg\(^{-1}\) in coarse sand fraction, and from 8.45 g kg\(^{-1}\) to 19.86 g kg\(^{-1}\) in
fine sand fraction, and from 10.13 g kg\(^{-1}\) to 11.37 g kg\(^{-1}\) in silt and from 19.80 g kg\(^{-1}\) to 24.36 g kg\(^{-1}\) in clay fractions, showing consistently higher in rice soils than in the uncultivated marsh soil. Similar was the change in total N in the size fractions (total N was 1.04 g kg\(^{-1}\) and 1.01 g kg\(^{-1}\) in coarse sand and fine sand fraction, and 0.52 g kg\(^{-1}\) and 1.17 g kg\(^{-1}\) in silt and clay fraction, in P0. In rice soils (P50 - P700), total N ranged 2.72 - 4.43 g kg\(^{-1}\) in coarse sand, and 8.45 - 19.86 g kg\(^{-1}\) in fine sand, and from 10.13 to 11.37 g kg\(^{-1}\) in silt and from 19.80 to 24.36 g kg\(^{-1}\) in clay. Generally, LOC, SOC and total N contents followed an order of coarse sand fraction > clay fraction > fine sand and silt fractions in a single soil. And C/N ratio was markedly higher in the coarse sand fractions than in the other fractions across the chronosequence. Moreover, the distribution patterns of SOC, LOC and total N associated in the four size fractions were similar across the sequence. SOC, LOC and total N from coarse sand and clay fractions were significantly higher compared to other PSFs. Across the chronosequence, 700 years rice cultivation led to 449 %, 101 %, 106 % and 162 % increases in SOC content in coarse, fine sand, silt and clay size fraction, respectively over the uncultivated marsh soil. Meantime, total N in these size fractions increased by of 326%, 79%, 113% and 133 %, respectively.

Table 2 Contents (g kg\(^{-1}\)) of SOC, total N and LOC of the size fractions of the studied chrono-sequence. Different capital and low case letters indicate a significant (\(p<0.05\)) difference respectively between fractions of a single soil, and between soils for a single fraction, in a single column.

<table>
<thead>
<tr>
<th>Size fraction</th>
<th>Soil</th>
<th>SOC</th>
<th>Total N</th>
<th>LOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coarse sand</td>
<td>P0</td>
<td>11.07±1.20Ad</td>
<td>1.04±0.11Ad</td>
<td>6.22±0.18Ac</td>
</tr>
</tbody>
</table>
The data of soil carbon chemical groups with FTIR analysis is presented in Table 3. Generally, relative proportion of carbon groups followed an order of polysaccharide > phenol > aromatic > aliphatic group in a single soil. For coarse sand fraction, marked differences in carbon chemical groups were found between the tidal marsh and rice soils. For sand sized aggregate fraction, the proportion of polysaccharide group generally decreased but that of aromatic and phenol groups decreased.
increased in the rice cultivated soils, over the uncultivated tidal marsh. However, in
the other fractions, the proportion of carbon chemical groups showed slight changes
with the increasing time of rice cultivation.

Table 3 Relative proportion (%) of carbon chemical groups in size fractions by FTIR analysis.

<table>
<thead>
<tr>
<th>Size fraction</th>
<th>Soil</th>
<th>Aromatic</th>
<th>Phenol</th>
<th>Aliphatic</th>
<th>Polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>0.94±0.03Bc</td>
<td>27.64±1.40Bc</td>
<td>0.03±0.00Ac</td>
<td>71.41±5.76ABa</td>
<td></td>
</tr>
<tr>
<td>P50</td>
<td>3.49±0.47Aab</td>
<td>35.06±5.63Aab</td>
<td>0.50±0.09Aa</td>
<td>60.94±2.54Cbc</td>
<td></td>
</tr>
<tr>
<td>P100</td>
<td>2.82±0.34Ab</td>
<td>31.61±3.58ABab</td>
<td>0.27±0.03Ab</td>
<td>65.31±4.72Bab</td>
<td></td>
</tr>
<tr>
<td>P300</td>
<td>2.49±0.12Ab</td>
<td>30.18±0.72ABb</td>
<td>0.28±0.04Ab</td>
<td>67.04±4.66BCab</td>
<td></td>
</tr>
<tr>
<td>P700</td>
<td>3.66±0.14Aa</td>
<td>34.81±1.56Aa</td>
<td>0.37±0.03Ab</td>
<td>61.17±4.30Cbc</td>
<td></td>
</tr>
<tr>
<td>P0</td>
<td>0.98±0.05Bb</td>
<td>25.32±1.55Ba</td>
<td>0.05±0.01Ab</td>
<td>73.64±4.83ABAa</td>
<td></td>
</tr>
<tr>
<td>P50</td>
<td>1.08±0.06Cb</td>
<td>25.90±1.14Ba</td>
<td>0.04±0.00Bb</td>
<td>72.98±4.43ABAa</td>
<td></td>
</tr>
<tr>
<td>P100</td>
<td>2.10±0.18Ba</td>
<td>27.52±1.00Ba</td>
<td>0.13±0.03Ba</td>
<td>70.24±3.47ABAa</td>
<td></td>
</tr>
<tr>
<td>P300</td>
<td>2.08±0.05Ba</td>
<td>27.52±1.41Ba</td>
<td>0.07±0.02Bb</td>
<td>70.32±4.60ABAa</td>
<td></td>
</tr>
<tr>
<td>P700</td>
<td>2.30±0.10Ba</td>
<td>27.03±1.25Ba</td>
<td>0.17±0.02Ba</td>
<td>70.51±4.09Ba</td>
<td></td>
</tr>
<tr>
<td>P0</td>
<td>0.60±0.03Cb</td>
<td>22.62±1.27Ca</td>
<td>0.01±0.00Ba</td>
<td>76.76±3.81Aa</td>
<td></td>
</tr>
<tr>
<td>P50</td>
<td>1.01±0.03Ca</td>
<td>22.97±1.50Ca</td>
<td>0.01±0.00Ca</td>
<td>76.02±4.29Aa</td>
<td></td>
</tr>
<tr>
<td>Silt</td>
<td>P100</td>
<td>0.95±0.06Ca</td>
<td>21.66±1.31Ca</td>
<td>0.00±0.00Db</td>
<td>77.37±4.73Aa</td>
</tr>
<tr>
<td></td>
<td>P300</td>
<td>1.02±0.10Ca</td>
<td>22.59±1.11Ca</td>
<td>0.00±0.00Db</td>
<td>76.39±4.21Aa</td>
</tr>
<tr>
<td></td>
<td>P700</td>
<td>0.89±0.02Ca</td>
<td>18.98±0.83Cb</td>
<td>0.00±0.00Db</td>
<td>80.14±3.87Aa</td>
</tr>
<tr>
<td></td>
<td>P0</td>
<td>1.24±0.06Ab</td>
<td>32.54±1.69Aa</td>
<td>0.00±0.00Bb</td>
<td>66.20±3.2B2a</td>
</tr>
<tr>
<td>Clay</td>
<td>P50</td>
<td>2.14±0.15Ba</td>
<td>33.32±1.35Aa</td>
<td>0.03±0.00Ba</td>
<td>64.52±4.23Ba</td>
</tr>
</tbody>
</table>
3.2 DNA content, microbial gene abundance and diversity

The microbial DNA content (equivalent to biomass) and gene abundance of microbial communities in the PSFs over the chronosequence are shown in Table 4. Total DNA in the PSFs ranged from 1.57 μg g⁻¹ in silt fraction to 4.00 μg g⁻¹ in clay fraction of the tidal marsh and from 4.35 μg g⁻¹ in fine sand fraction to 35.33 μg g⁻¹ in coarse sand size in the rice soils. Overall, fungal ITS gene copies were generally higher in coarse sand fractions, decreasing with the size of other fractions. Whereas, bacterial and archaeal 16S rRNA gene copy numbers were higher in both coarse sand and clay fractions compared to other fractions across the chronosequence.

Over the studied chronosequence, DNA contents of an aggregate size fraction were several folds higher in the rice soils over the initial tidal marsh. Accordingly, gene copy numbers of microbial communities from a PSF were greatly higher in rice soils than in the initial tidal marsh. Bacterial and fungal abundance in coarse sand, fine sand, silt and clay fraction in P50 was increased by 688%, 72%, 498% and 622 %, and 74%, 149%, 7% and 152 %, respectively over P0. An increase in bacterial gene copy numbers over P0 was seen significant across the rice soils cultivated for 100-700 years, by 73% to 437 %, 0.4% to 67 %, 225% to 246 % and 147% to 201 %, respectively in coarse sand fraction, fine sand fraction, silt fraction and clay fraction. However, those in fungal gene abundance were more or less
inconsistent across the rice soils, by 9% to 18%, 25% to 159%, 45% to 8% and 167% to 377% in coarse sand, fine sand, silt and clay fractions, respectively. In contrast, archaeal abundance was found increased over P0 consistently across the fractions with the prolonged rice cultivation. In particular, the archaeal abundance in coarse, fine sand, silt and clay increased by 25, 2, 32 and 19 folds in P700 with 700 years rice cultivation over the tidal marsh (Table 4).

Table 4 DNA content (μg g\(^{-1}\)), copy numbers of bacterial (BA, \(\text{copies} \times 10^9\ \text{g}^{-1}\)), fungi (FA, \(\text{copies} \times 10^7\ \text{g}^{-1}\)) and archaeal (ArA, \(\text{copies} \times 10^8\ \text{g}^{-1}\)) abundance of the size fractions of the studied chronosequence. Different capital and low case letters indicate a significant (p < 0.05) difference respectively between fractions of a single soil, and between soils for a single fraction.

<table>
<thead>
<tr>
<th>Size fraction</th>
<th>Soil</th>
<th>DNA</th>
<th>BA</th>
<th>FA</th>
<th>ArA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coarse sand</td>
<td>P0</td>
<td>3.32±0.07Ac 5.86±0.75Ad</td>
<td>8.92±1.50Ab 0.81±0.03Ce</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P50</td>
<td>35.33±0.42Aa 46.18±9.21Aa</td>
<td>15.50±2.60Aa 6.37±0.81Bd</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P100</td>
<td>24.72±2.14Ac 31.45±5.79Ab</td>
<td>10.49±0.87Ab 13.54±0.73Bc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P300</td>
<td>16.20±0.05Ad 10.12±2.39Ac</td>
<td>8.12±0.32Ab 16.01±1.06Ab</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P700</td>
<td>31.95±0.64Ab 14.25±1.03Ac</td>
<td>9.40±0.71Ab 21.17±0.48Ba</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fine sand</td>
<td>P0</td>
<td>3.63±0.28Ab 4.90±0.45Ab</td>
<td>3.23±0.27Bc 2.83±0.18Ac</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P50</td>
<td>4.35±0.40Db 8.42±1.75Ba</td>
<td>8.04±0.25Ba 5.27±1.12Bd</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P100</td>
<td>13.63±3.30Ba 7.75±1.18Ca</td>
<td>8.37±0.67Aa 8.16±2.27Cab</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P300</td>
<td>9.97±0.33Ba 4.92±1.10Bb</td>
<td>6.23±0.23Bb 3.57±0.24Cb</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P700</td>
<td>12.83±0.33Ca 8.16±1.64Ba</td>
<td>2.43±0.19Cd 7.68±0.66Ca</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silt</td>
<td>P0</td>
<td>1.57±0.28Bc 1.78±0.15Bc</td>
<td>3.98±0.57Ba 0.29±0.02Dd</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P50</td>
<td>10.02±1.58Ca 10.64±2.95Ba</td>
<td>4.25±0.30Ca 2.48±0.44Cc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P100</td>
<td>8.25±0.12Cab 5.78±0.36Cb</td>
<td>2.17±0.20Bb 8.65±0.09Ca</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P300</td>
<td>7.78±0.31Cb 5.91±0.81Bb</td>
<td>2.47±0.45Bb 6.60±0.27Bb</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P700</td>
<td>9.25±0.64Da 6.16±0.29Bb</td>
<td>3.68±0.19Ba 9.44±1.41Ca</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Microbial Shannon diversity index of the four PSFs of the chronosequence soils are presented in Table S1. In detail, Shannon’s index of bacterial community was much higher in coarse sand fraction and, to a lesser extent, in clay size fraction than in fine sand and silt fractions across the chronosequence. Fungal community Shannon’s index was shown generally decreased with the size of the fractions, being highest in coarse sand fraction among all the fractions. However, there were no significant changes in archaeal Shannon’s index among the PSFs across the sequence. Generally, Shannon diversity index of the microbial communities in a single PSF were greatly higher in the rice soils than in the uncultivated tidal marsh.

### 3.3 Enzyme activity, C gain from maize amendment and basal respiration

All analyzed enzyme activities (Table S2) were seen increased in the rice soils over the initial tidal marsh. Furthermore, NEA (normalized enzyme activity) was 0.07 in the coarse sand and 0.10 in the fine sand fraction, and 0.07 and 0.14 in the silt and clay fractions in P0. In contrast, NEA was 0.18-0.30 in coarse sand and 0.12-0.30 in fine sand fraction, but 0.17-0.30 in silt fraction and 0.19-0.24 in clay fraction of the rice soils. Moreover, NEA in a single size fraction significantly increased with prolonged rice cultivation (Table 5).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>NEA (mg CO₂ g⁻¹)</th>
<th>C Gain (g kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>P50</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Clay</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>P100</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>P300</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>P700</td>
<td>0.07</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Table 5 Normalized enzyme activity (NEA), soil respiration (mg CO₂ g⁻¹) and carbon gain (g kg⁻¹) from maize in incubation of size fractions of the studied chronosequence the four fractions. Different capital and low case letters indicate a significant (p <0.05) difference respectively.
between fractions of a single soil, and between soils for a single fraction, in a single column.

<table>
<thead>
<tr>
<th>Size fraction</th>
<th>Soil</th>
<th>NEA</th>
<th>Soil respiration</th>
<th>Carbon gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td></td>
<td>0.07±0.01Bc</td>
<td>6.62±0.66Ac</td>
<td>0.17±0.03Cc</td>
</tr>
<tr>
<td>P50</td>
<td></td>
<td>0.28±0.03Aa</td>
<td>23.45±8.05AaAb</td>
<td>0.42±0.02Ab</td>
</tr>
<tr>
<td>Coarse sand</td>
<td>P100</td>
<td>0.18±0.01Ab</td>
<td>22.83±5.06AaAb</td>
<td>0.44±0.04Bb</td>
</tr>
<tr>
<td></td>
<td>P300</td>
<td>0.18±0.01Bb</td>
<td>15.88±3.09Ab</td>
<td>0.54±0.03Ba</td>
</tr>
<tr>
<td></td>
<td>P700</td>
<td>0.30±0.05Aa</td>
<td>29.14±1.90Aa</td>
<td>0.56±0.02Ba</td>
</tr>
<tr>
<td></td>
<td>P0</td>
<td>0.10±0.01Bc</td>
<td>5.65±1.53AbBb</td>
<td>0.77±0.07Ac</td>
</tr>
<tr>
<td></td>
<td>P50</td>
<td>0.12±0.03Cc</td>
<td>10.76±1.39Ba</td>
<td>0.50±0.05Ad</td>
</tr>
<tr>
<td>Fine sand</td>
<td>P100</td>
<td>0.21±0.03Ab</td>
<td>12.52±1.03Ba</td>
<td>0.70±0.03Ac</td>
</tr>
<tr>
<td></td>
<td>P300</td>
<td>0.27±0.03Aa</td>
<td>12.56±0.96Aa</td>
<td>1.27±0.06Aa</td>
</tr>
<tr>
<td></td>
<td>P700</td>
<td>0.30±0.02Aa</td>
<td>12.34±1.43Ba</td>
<td>1.07±0.06Ab</td>
</tr>
<tr>
<td></td>
<td>P0</td>
<td>0.07±0.01Bd</td>
<td>2.98±0.53Cc</td>
<td>0.26±0.01Bd</td>
</tr>
<tr>
<td></td>
<td>P50</td>
<td>0.21±0.02Bb</td>
<td>7.40±2.58Bb</td>
<td>0.36±0.03Bc</td>
</tr>
<tr>
<td>Silt</td>
<td>P100</td>
<td>0.17±0.01Ac</td>
<td>12.46±0.63Ba</td>
<td>0.60±0.02Aa</td>
</tr>
<tr>
<td></td>
<td>P300</td>
<td>0.25±0.02Ab</td>
<td>12.56±0.71Aa</td>
<td>0.52±0.02Bb</td>
</tr>
<tr>
<td></td>
<td>P700</td>
<td>0.30±0.02Aa</td>
<td>13.54±0.95Ba</td>
<td>0.45±0.05Bb</td>
</tr>
<tr>
<td></td>
<td>P0</td>
<td>0.14±0.01Ac</td>
<td>4.96±0.53Bb</td>
<td>0.38±0.12Ba</td>
</tr>
<tr>
<td></td>
<td>P50</td>
<td>0.19±0.02Bb</td>
<td>14.25±4.30Aa</td>
<td>0.25±0.02Ca</td>
</tr>
<tr>
<td>Clay</td>
<td>P100</td>
<td>0.20±0.02Aab</td>
<td>14.01±2.89Aa</td>
<td>0.27±0.01Ca</td>
</tr>
<tr>
<td></td>
<td>P300</td>
<td>0.24±0.02Aa</td>
<td>10.28±2.26Aa</td>
<td>0.27±0.03Ca</td>
</tr>
<tr>
<td></td>
<td>P700</td>
<td>0.23±0.01Ba</td>
<td>14.34±1.96Ba</td>
<td>0.31±0.05Ca</td>
</tr>
</tbody>
</table>

Soil respiration, an indicator of live soil microbial organisms (Schlesinger & Andrews, 2000), ranged from 2.98 mg CO$_2$ g$^{-1}$ to 6.62 mgCO$_2$ g$^{-1}$ across the PSFs in the uncultivated tidal marsh and from 7.40 mg CO$_2$ g$^{-1}$ to 32.45 mg CO$_2$ g$^{-1}$ in rice soils (Table 5). In detail, soil respiration was 6.62 mgCO$_2$ g$^{-1}$ and 5.65 mgCO$_2$ g$^{-1}$ in
coarse and fine sand fraction, and 2.98 mg CO$_2$ g$^{-1}$ and 4.96 mg CO$_2$ g$^{-1}$ in silt and clay fraction, respectively in P0. While in rice soils, soil respiration was in a range of 15.9-29.1 mg CO$_2$ g$^{-1}$ in coarse sand, and of 10.8-12.6 mg CO$_2$ g$^{-1}$ in fine sand fraction, and of 7.4-13.5 mg CO$_2$ g$^{-1}$ in silt and of 10.3-14.3 mg CO$_2$ g$^{-1}$ in clay fraction, of the rice soils. Soil respiration in a single size fraction generally increased with rice cultivation length. Over P0, soil respiration increased by 3.4, 1.2, 3.5 and 1.9 folds, respectively of coarse sand, fine sand, silt and clay size fractions in P700. However, ratio of respired C to total OC (RQ, a soil respiration quotient) of the four fractions was in a range of 0.15-0.16 g CO$_2$-C g$^{-1}$SOC in P0. For the rice soils, however, the RQ was 0.12-0.15 g CO$_2$-C g$^{-1}$SOC in coarse sand, and 0.17-0.35 g CO$_2$-C g$^{-1}$SOC in fine sand fraction, and 0.19-0.35 g CO$_2$-C g$^{-1}$SOC in silt fraction and 0.12-0.20 in clay fraction, respectively. Moreover, RQ values of coarse sand fraction and of clay fraction was not significant different among the rice soils cultivated up to 700 years. But, RQ of the fine sand and silt fractions from the rice soils increased by 6-119 % and by 18-119 %, compared to P0 (Table S3).

Carbon gain from amended maize was 0.17 g kg$^{-1}$ in coarse sand and 0.77 g kg$^{-1}$ in fine sand fraction, but 0.26-0.38 g kg$^{-1}$ in silt and clay fraction of P0. Carbon gain from amended maize was 0.42-0.56 g kg$^{-1}$ in coarse sand and 0.50-1.27 in fine sand fraction, but 0.36-0.60 g kg$^{-1}$ in silt fraction and 0.25-0.31 g kg$^{-1}$ in clay fraction of the rice soils. Except the clay fraction, carbon gain potential by a single fraction was higher in rice soils (P50-P700) than in the uncultivated marsh P0, and in P100, P300 and P700 than in P50 for rice soils (Table 5). Amended maize carbon was...
predominantly sequestered in the fine sand fraction, varying from 33%-49%, and showed no significant change among the soils tested. Proportion of carbon gain in the coarse sand was 10% in P0 and increased to about 20% in the rice soils. In contrast, the proportion in the clay fraction was 24% in P0 and decreased to about 10% in the rice soils (Table S4).

4 Discussions

4.1 Carbon stabilization in soil aggregates

Soil carbon sequestration had been well characterized via stabilization of organic carbon with either physical protection, or chemical binding to clay minerals and/or metal oxyhydrates, or biologically stabilization with increased fungal to bacterial ratio (Six et al., 2002a; Lützow et al., 2006; Plaza et al., 2013). The role of physical protection (Zhou et al., 2008), chemical binding to iron oxyhydrates (Zhou et al., 2009) and microbial stability with increased fungal to bacterial ratio (Liu et al., 2011) had been well addressed for organic carbon sequestration in China’s rice paddy soils. Data from this study could allow a detailed characterization of organic carbon stabilization in different size fractions of soil aggregates. Similar to the findings by Zhang et al., (2007) and Zheng et al., (2007), the present study indicated significant changes in both carbon pools and microbial properties mainly in coarse sand and clay sized fractions of the PFSs, between the soils over the chronosequence.

For the separated PFSs, change in soil organic carbon content was found very significantly positively exponentially correlated (Fig.3a) but respiration quotient significantly negatively linearly correlated (Fig.3b) to carbon recalcitrance, and the
ratio of aromatic and phenol carbon to aliphatic and polysaccharide carbon (Fig. 4), of particle size fractions of soil aggregates of the soils over the chronosequence. This evidenced an overall trend of soil organic stabilization while OM accumulated in the soil aggregates. This was in accordance with our previous finding of soil organic carbon accumulation and stabilization in bulk samples of the studied chronosequence (Wang et al., 2015).

Fig. 3 Correlation of organic carbon (a) and respiration quotient (b) with carbon recalcitrance [the ratio of relative recalcitrant C (Aromatic and Phenol) to relative labile C (Aliphatic and Polysaccharide)] of the particle size fractions of the studied chronosequence.

However, carbon stabilization indicators were seen varying with the different size fractions. The sand sized fractions here were characterized by high OC with
high LOC/SOC ratios, and the clay sized fraction by high OC with high carbon recalcitrance. This seemed in agreement with that SOM accumulated mainly as unprotected POM in micro-aggregates in size larger than 53µm and intimately associated with silt and clay with high chemical recalcitrance (Six et al., 2002). Wakeham and Canuel (2006) reported that the light fractions were higher in total OC but the heavy (clay) fraction contained smaller amount but old OC, of river bed sediments from a Californian river basin. It is worthy to note that respiration quotient, an indicator of biological stability, was no difference between the coarse sand, fine sand and clay sized fractions though respiration was higher in silt sized fraction than in other PSFs (Table 3). Interestingly, the ratio of LOC/SOC, as a negative indicator of chemical stability, was relatively high in coarse sand fraction but low in clay fraction among the PSFs, supporting the general understanding of relatively unprotected labile carbon in macro aggregates but relatively recalcitrant carbon in microaggregates in clay complexes (Six et al., 2007). In contrast, the carbon recalcitrance measured with FTIR was even lower in the coarse sand fractions than in the clay sized fractions. There existed similar carbon stability and microbial decomposition potential between the sand and clay sized fractions (Fig. 4). Obviously, the similar carbon stabilization between the sand sized and clay sized fractions could not be explained by the difference in the trend of LOC/SOC, and of carbon recalcitrance (Table 3). Mikutta et al., (2006) proposed that stabilization of soil organic matter by association with minerals prevailed over chemical recalcitrance. In our previous study, high content of labile carbon (also as particulate
organic carbon) was shown physically protected in line with the enhancement of soil aggregation.

**Fig. 4** Inter-correlation between carbon pools and microbial biomass to address the differences of
soil carbon stability and microbial functioning between coarse sand (left) and clay (right) sized aggregates fractions (Soil organic carbon accumulation as a function of relative recalcitrant C (aromatic and phenol) (a) and negatively of relative labile C (aliphatic and polysaccharide) (b); CO$_2$ production as a plateau function of soil microbial biomass (c) and bacterial abundance (d)). Data was the mean value of triplicates.

indicated by the mean weight diameter of soil aggregates (Wang et al., 2015). All these information above could suggest that organic carbon had been stabilized rather via physical protection in coarse sand fraction of macro-aggregates than via chemical recalcitrance due to mineralogical binding in clay.

Accumulation of SOC under long term rational management practices was well addressed in accompanied with formation of macro-aggregates, which in turn physically protect the SOC from microbial decomposition via forming a physical barrier between the substrates and microbes (Zhou et al., 2009; Tripathi et al., 2014). Physical protection of labile carbon in macro-aggregates rather than inherent chemical stability of OC (a minor mass fraction of the clay sized micro-aggregates, Table 1) had been addressed in many studies for soil carbon sequestration (Six et al., 2004; Kong et al., 2005; Six and Paustian, 2014). Synthesizing data from Table 1 and Table 2, organic carbon physically protected in the sand and fine sand fractions constituted 51%-62% while chemically protected carbon in the clay sized fractions 11%-19%, to the total carbon storage of soils over the studied sequence. Therefore, this study again convinces that, rather than chemical stabilization, physical protection of labile carbon within micro-aggregates in macro-aggregates, against microbial access and decomposition, could be concerned as the major contributor of
soil carbon sequestration in rice soils. This also suggest SOM accumulation was in a continuum between the aggregates from coarse fractions to fine fractions though largely in sand and fine-sand fractions. Such a SOM accumulation continuum could be corresponding to the recent argument by Lehmann and Kleber (2015) that soil organic matter could be considered in a continuum with different accessibilities to microbial decomposition.

4.2 Bioactivity in size fractions of soil aggregates

Biological activity of soil microbes including soil respiration and soil enzyme activity had been well known varying across size fractions of soil aggregates (Kandeler et al., 1999; Sessitsch et al., 2001; Poll et al., 2003; Allison and Jastrow, 2006). Poll et al. (2003) found that fungal biomass, relative fungi gene abundance and xylanase activity tended to increase with decreasing size of aggregate particle fractions. Allison and Jastrow (2006) suggested that microbial biochemical activity and carbon turnover was stronger in POM-enriched size fractions, but weaker in mineral-dominated fractions where enzymes and their carbon substrates were immobilized on mineral surfaces. Soil enzyme activities in different particle size fractions could depend not only on the location of soil microorganisms and their substrates but also on the mechanisms of enzymes to adsorb and bind onto mineral and organic particles. In this study, total DNA content, gene abundance and diversities of microbial community varied greatly between the size fractions (Tables 4 and S1). Total DNA content was found significantly positively but linearly correlated with content either of organic carbon and nitrogen, or of labile organic
carbon, across the size fractions of the studied sequence (Fig. S1). However, gene abundance of bacterial, fungal and archaeal communities could be correlated neither to total pool of organic carbon and labile organic carbon nor to carbon recalcitrance and lability (LOC/SOC), across the sequence. This finding evidenced that carbon and nitrogen level could control the total soil microbial biomass but not the composition of microbial communities of bacteria, fungi and archaeal. This was in general agreement with the finding by Yin et al., (2000) and by Torsvik and Øvreås, (2002) of significant differences in microbial populations along a soil reclamation gradient with different exotic carbon amendments.

Total soil DNA content and fungal gene abundance were highest in coarse sand fractions, while bacterial and archaeal gene abundance higher in sand and clay sized fractions than in other fractions. Here, fungal community appeared to exert selection of size fractions, being predominantly concentrated in coarse sand sized soil aggregates where labile carbon pool and C/N ratio were relatively high (Kandeler et al., 2000; Chiu et al., 2006). Fungal gene abundance positively correlated to C/N in the PSFs ($R^2=0.64$, $p<0.001$) (Fig. S1). Fungal had been considered having a direct and prompt impact on micro-aggregate formation and stabilization of newly input OM (Six and Paustian, 2014). Microaggregates and other primary particles could be bound into macro-aggregates with close association of fungal hyphae and organic matter/materials (Oades, 1984; Tisdall, 1994; Miller and Jastrow, 2000).

As regard to diversity, only Shannon index of fungal diversity was seen
significantly different among the size fractions, being highest in the coarse sand fractions. However, as seen with Tables 2 and 3, the diversity of bacterial, fungal and archaeal were all lowest in the silt fractions among the size fractions, due to the very low soil carbon substrates availability and soil nutrients (Nelson et al., 1994).

Soil respiration had been generally accepted as a size of active microbes in soils using accessible carbon substrates (Schlesinger and Andrews, 2000). In this study, enzyme activity when normalized as NEA, was well correlated to organic carbon contents in soil aggregate fractions (Fig. S3). Soil respiration was higher in sand fraction where SOC and diversities of bacterial community were higher, than clay fraction. The higher bacterial biomass in the larger size fractions was related to the extent of decomposable soil organic matter as LOC contents were higher in coarse fractions than in other fractions (Table 3). However, bacterial and archaeal abundance per unit of SOC were highest, but respiration lowest, in clay fraction. This could explain microbial activity was low due mainly to inert carbon chemical protected in clay sized aggregates (Nelson et al., 1994; Six and Paustian 2002). Microorganisms physically confined in small pores could become less active and protected against grazing by the soil fauna. Moreover, SOM was chemically protected from mineralization by surface adsorption onto clay minerals (Six et al., 2002b; Davidson and Janssens, 2006). Interestingly, soil respiration quotient was seen well negatively correlated to total DNA content and labile carbon content for the rice soils shifted from salt marsh (Fig. S4). This again confirmed that enhanced microbial community with SOC accumulation, exhausted less carbon, indicating
higher carbon use efficiency, particularly with labile carbon in coarse fractions of aggregates (Jastrow et al., 1996). In this study with water stable aggregates from rice soils, microbial activity and carbon use efficiency was generally higher in macro-aggregates than in micro-aggregates. This could lead to an understanding that physically protected carbon as of labile carbon promoted microbial activity in macro-aggregates of the rice soils.

Carbon gain from straw amendment, as one of important soil functions for carbon sequestration, was observed in all fractions of soil aggregates in lab incubation (Table 4). Total carbon gain from amended maize straw was more or less in linear response to relative fungal gene abundance (characterized with fungal to bacterial gene copy number ratio) (data not shown). Here, higher carbon gain in fine sand fraction could be attributed to high fungal dominance and C/N ratio (also high LOC pool). Comparatively, clay fraction with mostly chemically stabilized carbon had a smaller potential to gain exotic carbon. This seemed controversial to the argument by Piccolo et al. (2004) that hydrophobic carbon, high in clay fractions here, could be a sink of amended carbon in soils. Again, the fact that sand sized fraction rather than clay sized fraction, of soil aggregates, preserved more carbon from amended maize verified that carbon sequestration could be predominately contributed by physical protection in macro-aggregates where C/N ratio and fungal dominance and LOC pool are already high (Kandeler et al., 2000). Fontaine et al., (2011) argued that fungi mediated long term carbon sequestration, potentially through their priming effect.
We further compare the bio-activity versus carbon between sand and clay sized aggregate fractions. When plotting DNA content of microbial biomass against OC content, a correlation was very significant for coarse sand fraction but failed for clay fraction (Fig. 5a). Accumulation of SOC in these coarse fractions have been well characterized as physically protected (Six et al., 2000; Six et al., 2004), particularly the POM in large macro-aggregates (Six et al., 2004). The result here could indicate that soil microbial communities in large aggregates could be in an access to SOM physically protected in large aggregates. This is again supported by that finding that normalized enzyme activity from coarse sand fraction was found in a positively linear function with SOC accumulation (Fig. 5b). However, DNA content scaled CO₂ production was in a negatively power function (Fig. 5c) with total soil DNA content, showing an increased carbon use efficiency with the SOM accumulation in large sized fractions. In our previous research, improved microbial activity was found linked to the increase in particulate organic carbon content which was enhanced via physical protection with promoted soil aggregation (Wang et al., 2015). Promoted macro-aggregation, as indicated by increased MWD here, with SOC accumulation could lead to a more heterogeneous soil micro-habitat, a better spatial allocation of various pools of OM and different size groups of microbes and extra-cellulose enzymes within macro-aggregates.

Soil enzymes catalyzing the C transformation, nutrient release and redox processes in soils could be considered as soil ecosystem functioning capacity (van der Heijden, et al., 2008). As seen in Fig. 3, microbial enzyme activity and carbon
substrate use efficiency, especially in coarse sand fraction, could have been improved with the increased microbial biomass under OC accumulation. These suggested that high microbial biomass and high enzyme activities from coarse sand fraction were accompanied with carbon stabilization under OC accumulation. For large sized aggregate fractions, soil DNA content was positively linearly correlated to the content of LOC (Fig. 5D) and to aromatic and phenol (Fig. 5E). LOC in soils could be easily decomposed and potentially used by microbes (Cleveland et al., 2007). In the bulk soils, improved microbial biochemical activity and carbon use efficiency were linked to particulate OC, which was increased with enhanced soil aggregation for physical
a) 2000-200 μm

\[ y = 2.2365x^{0.0663}, \quad R^2 = 0.95 \]

b) <2 μm

\[ y = 0.0053x + 0.0497, \quad R^2 = 0.91 \]

c) Total DNA content μg g\(^{-1}\)

\[ y = -0.3579x + 2.6085, \quad R^2 = 0.98 \]

d) CO\(_2\) production DNA content

\[ y = 1.8596x + 5.7476, \quad R^2 = 0.94 \]

e) LOC kg\(^{-1}\)

\[ y = 3.0375x - 42.606, \quad R^2 = 0.98 \]
Fig. 5 Inter-correlation between particulate organic carbon and soil microbial activity to compare the biological activity versus carbon between coarse sand (left) and clay (right) sized aggregate fractions (Soil microbial biomass was as an exponential function of total soil organic carbon (a) and a linear function of labile organic carbon (d). Normalized enzyme activity (b) and DNA content scaled CO2 production (c) as a linear and negative power function of soil microbial biomass. Soil microbial biomass was as a linear function of relative recalcitrant C (aromatic and phenol) (e)). Data was the mean value of triplicates.

Many studies had reported that enzyme activity and microbial biomass showed positive relationship with carbon concentrations in bulk soils (Marx et al., 2005; Allison and Jastrow, 2006; Shi et al., 2006; Yu et al., 2012). However, the correlation coefficients between these parameters in clay fraction were much lower than in coarse sand fraction. This suggested that the carbon in this fraction of aggregates was not readily available to microbes, confirming the generally considered C stabilization in clay fraction. With high humification degree and interaction in organo-mineral complexes of clay particles, organic matters were not readily available for soil microbes (Lützow et al., 2006; Kogel-Knabner et al., 2008). Extracellular enzymes could be absorbed by clay minerals (Allison and Jastrow, 2006), but the potential activity of soil enzymes in this fraction therefore could not be related to the turnover of OM. Clearly, some mechanism must have prevented enzymes from mineralizing C efficiently in these mineral-associated fractions, which
contained C pools with very slow turnover rates (Six and Jastrow, 2002). Although habits within macro-aggregates offered protection of the young and labile carbon against microbial decomposition (Gupta and Germida, 2015), enhanced aggregation could lead to increased population and activities of specific microbial groups (Six et al., 2002b).

All the above data revealed that bioactivity was not primarily controlled by carbon level but by C lability or accessible carbon, which was predominately physically protected in coarse sand and fine sand fractions. Carbon stabilization characterized by carbon recalcitrance or respiration quotient could not confront microbial activity in soil aggregates, especially in macro-aggregates, where physically protected labile carbon could promote soil bioactivity with inherently accessible to carbon inter micro-aggregates.

4.2 Dynamics of C stability and bioactivity with prolonged rice cultivation

In our previous study of bulk soils from the chronosequence, soil organic carbon accumulation was found concurrent carbon stabilization and promotion of biological activity through physically protected labile carbon accumulation with long-term rice cultivation (Wang et al., 2015). And this was found in line with the enhancement of soil aggregation characterized by the change in mean weight diameter of soil aggregates over the sequence (Wang et al., 2015). Here we synthesize all the analysis data in terms of aggregate partitioning over the soils, presented in Fig. 6. After salt marsh soil (P0) shifted to rice cultivation (P50), total SOC, enzyme activity and soil respiration showed a more or less consistent increase.
in both sand and clay sized fractions with prolonged rice cultivation. Meanwhile, with prolonged rice cultivation, carbon gain from amended
Fig. 6 Change in partitioning of soil organic carbon (a, g/kg), total DNA (b, µg/g), normalized enzyme activity (c, relative enzyme activity index), carbon gained from maize straw amendment (d, mg/kg) and soil respiration (e, mg CO₂/g) among coarse and fine sand fraction (blue base), silt fraction (brown base) and clay fraction (gray base) of soil aggregates, over the chronosequence of rice soils (P50-P700) shifted from a salt marsh (P0) under long term rice cultivation. The size of a circle is relevant to that of an analyzed parameter.

straw exerted a consistent increase in sand fraction though insignificantly observed in clay fractions. This is corresponding to the general trend of the change in these pools along with rice cultivation (Wang et al., 2015). The changes in relative portion by larger sized (coarse and fine sand fractions together) aggregates against silt and clay
sized fractions exerted different patterns between of carbon pools and microbial activities, along the chronosequence. Over the sequence, the prevalence of physically protected portion in sand and fine sand fractions over unprotected (referred to Six et al., 2002) portion was in range of 1.5-3.2 and of 1.1-2.6 for SOC and total N, of 0.9-2.2 for total DNA, of 1.2-3.3 for fungal gene copy numbers, of 0.8-1.5 for NEA and 1.6-2.8 for C gain, respectively. In contrast, the prevalence of archaeal copy numbers and soil respiration was in a range of 2.6-1.0 and 2.0-1.3, decreasing with rice cultivation lengths. Therefore, most of analyzed carbon pools and bioactivities were dominated by the macro-aggregates in sand and fine sand size fractions, which was generally in a consistent directional change with prolonged rice cultivation.

The rice soils over the chronosequence were derived from salt marsh, which was indigenously rich in silt mineral particles, with an average silt mass content of 75% - 84% (Cheng et al., 2009). During the rice soil development under rice cultivation, silt sized mineral particles were increasingly aggregated with increased OM while clay minerals accumulated due to neoformation of oxyhydrates of iron/manganese as well as minute clay sized minerals from irrigation (Chen and Zhang, 2009; Cheng et al., 2009; Kalbitz et al., 2013; Wissing et al., 2013). With rice cultivation, organic carbon was increasingly accumulated and stabilized in sand sized aggregates with physical protection of labile OC pool intra micro-aggregates, with prolonged rice cultivation (Wang et al., 2015). The changes in relative proportion of carbon pools and microbial activities (NEA, C gain and soil respiration) by sand and fine sand sized aggregates further demonstrated that physically protected and stabilized carbon supported high soil bioactivities, which had been increasingly prevailed over the smaller sized fractions of soil aggregates.

The proportion of coarse sand fraction increased, whereas fine sand and silt
fractions decreased, with increasing of SOC accumulation (Table 1). The recent carbon was predominantly stored in macro-aggregates in rice soils (Li et al., 2007; Pan et al., 2008), especially relative labile POM (Zhou & Pan, 2007; Wang et al., 2015). Moreover, changes in the relative abundance and activity of microbes could significantly affect C cycling and storage in different size aggregates (Six et al., 2006). Bacterial and archaeal extracellular excretes and fungal hyphae are primarily responsible for the formation of soil macro-aggregates, which protect plant-derived OM. This study confirmed a much higher response of bioactivity and functioning (enzyme activities and carbon sequestration capacity) from coarse sand fraction than clay fraction over centuries of rice cultivation.

5 Conclusions

This study, taking an example of rice soil chronosequence derived from salt marsh, revealed that soil organic carbon could be accumulated and stabilized both in large and small sized fractions of soil aggregates. However, microbial abundance and activities were high in sand sized fractions rather than silt and clay sized fractions of soil aggregates. With long term rice cultivation, soil carbon particularly labile carbon had been accumulated in majority in macro-aggregates, which supported high microbial abundance and activities. Thus, carbon stabilization was not confronting soil bioactivities in a way that carbon and microbial communities increasingly physically protected in macro-aggregates other than in silt and clay sized aggregates. This study further supported our previous finding for bulk soils that long term rice cultivation led to accumulation of SOC and promoted soil biological activities through physical protection of labile carbon in line with enhanced soil aggregation. And labile organic carbons accumulated in macro-aggregates helped enhancing microbial C use efficiency and improving potentially ecosystem functioning. More studies deserves on
interaction of soil organic matter, minerals and soil microbial communities to unravel the micro-scale process mediating bio-activities at aggregate level.

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