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

Yalong Liu[1,2], Ping Wang[1,2], Lianqing Li[1], Kun Cheng[1], Jufeng Zheng[1], Timothy Filley[3], Xuhui Zhang[1], Jinwei Zheng[1], Genxing Pan[1,4]

[1] Institute of Resource, Ecosystem and Environment of Agriculture, and Department of Soil Science, Nanjing Agricultural University, 1 Weigang, Nanjing 210095, China;

[2] College of Land and Environment, Shenyang Agricultural University, Shenyang 110161, China;

[3] Department of Earth, Atmospheric, and Planetary Sciences, Purdue University, West Lafayette, IN 47907, USA

[4] Center of Terrestrial Ecosystem Carbon Sink and Land remediation, School of Environmental and Resource Sciences, Zhejiang A & F University, Lin'an, Hangzhou 311300, China

Corresponding author: Genxing Pan

Address: Institute of Resource, Ecosystem and Environment of Agriculture, Nanjing Agricultural University, 1 Weigang, Nanjing 210095, China

Tel/Fax: +86 25 8439 6027

Email: pangenxing@aliyun.com

Running title: carbon and microbial activity in aggregates of rice soil

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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

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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

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1 Introduction

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 *e*t 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

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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

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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 chronosuequence, continuous SOC accumulation with increasing rice cultivation intensity, which had been promoted following the desalinization and decalcifiation in

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130 the initial stage after the salt marsh shifted to rice paddy, was characterized. The
131 accumulated SOC was increasingly stabilized with neoformed iron-oxyhydrates
132 accumulated in the rice soils in the long run with prolonged rice cultivation (Cheng
133 et al., 2009; Wissing et al., 2011) and physical protected by micro-aggregates (Wang
134 et al., 2015; Zou et al., 2015). SOC accumulation had been shown driving
135 enhancement of microbial biomass and evolution of microbial community in
136 long-term cultivated paddy soils (Bannert et al., 2011; Jiang et al., 2013; Liu et al.,
137 2015). Nevertheless, the dynamics of SOM and bio-activity in size fractions of soil
138 aggregates had not yet been characterized for understanding carbon sequestration in
139 relation to soil microbial structure and functioning of rice paddy soils.

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

146 **2 Materials and methods**

147 **2.1 Site and soils of the studied chronosequence**

148 The studied soil chronosequence was a series of rice soils shifted from tidal marsh to
149 rice cultivation under different lengths in a coast land located in Cixi Municipality,
150 Zhejiang Province, China (Fig.1). The area is within the typical northern subtropical
151 monsoon climate for Eastern China, with a mean annual temperature of 17.7 °C and

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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).

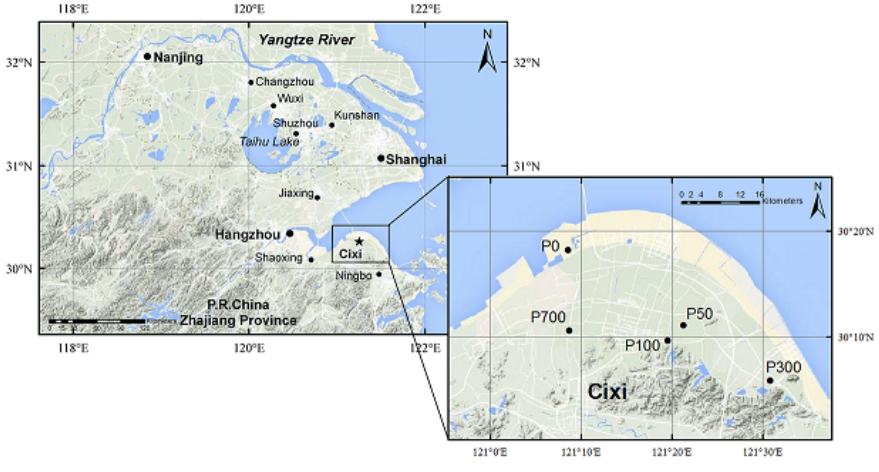

Fig. 1 Sampling sites for the individual soils constituting the rice soil chronosequence from Cixi County, Zhejiang province, China. The suffix number following P (paddy soil) designates the years under rice cultivation after shifting from salt marsh since dyke establishment.

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

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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

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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 recommend-ation 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

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fractions were freeze-dried with a frozen dryer (Thermo, Modulyo D-230, NY, US) and then stored at -70 ^{o}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

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235 bands at 1634 cm^{-1} were due to aromatic C=C vibrations, symmetric stretching of
236 COO−groups, and H-bonded C=O of conjugated ketones. The bands at 1022 cm^{-1}
237 have frequently been assigned to polysaccharide C–O stretching. The proportion of
238 different chemical groups was estimated with a software affiliated with the FTIR
239 manual.

240 **2.5 SEM observation of soil aggregates**

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

248 **2.6 DNA extraction, microbial gene abundance and diversity analysis**

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

255 Quantitative real-time PCR assay was performed on a 7500 real-time PCR
256 system (Applied Biosystems, USA) using SYBR green as a fluorescent dye. Primer

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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:

$$H' = \Sigma Pi\ (\ln Pi) \quad (1)$$

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

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normalized to give a single value of NEA of an individual fraction, which was estimated with the following equation:

$$x_i' = \frac{x_i}{\sum_{i=1}^{n} x_i} (i=1,2,\ldots,5), \quad (2)$$

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 415g kg^{-1}, total N of 6.11 g kg^{-1} and $\delta^{13}C$ abundance of -12‰. For incubation, 300g of an air dried bulk soil sample (passed 2mm 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 ^{o}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 0.15mm sieve for determination of the

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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:

$$^{13}C\ (‰) = [(R_{sample}/R_{standard}) - 1] \times 1000 \quad (3)$$

where, R_{sample} and $R_{standard}$ 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:

$$C_4 = \frac{\delta - \delta C_3}{\delta C_4 - \delta C_3} \times C_t \quad (4)$$

$$C_{gain} = C_4 \times P_{mass} \quad (5)$$

where, C_t is the organic carbon content (g kg^{-1}) , δC_3 and **δ** is the relative abundance of ^{13}C before and after incubation, of a particle size fraction; δC_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_{gain} was the amount (gC) of maize derived carbon after incubation while P_{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

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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_2 circulation were inserted into the stopper. The headspace was repeatedly evacuated and flushed with N_2 gas into the jar at a rate of 300ml min^{-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 ^oC 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_2 gas was again flushed into the jar at a rate of 300ml min^{-1} for 30 min to removing all the emitted gas in the jar (Wang et al., 1999). CO_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^oC and a FID temperature of 200^oC, with N_2 as the carrier gas at a flow rate of 40ml min^{-1} and a make-up gas mixture of H_2 and air at a flow rate of 35 ml min^{-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_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

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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.

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Soil	2000-200μm	200-20μm	20-2μm	<2μm	MWD(μm)
P0	2.78±0.59c	46.53±1.30a	41.00±2.46a	9.69±0.57d	86.5± 6.2c
P50	5.10±0.25b	44.31±0.02b	40.79±0.41a	9.8±0.14d	109.5±2.1b
P100	5.34±0.10b	43.17±0.53c	39.72±0.72a	11.78±0.09c	110.8±1.3b
P300	6.87±1.04a	41.53±1.64d	38.67±0.33a	12.92±0.27b	125.8±7.8a
P700	7.63±1.40a	39.91±5.16d	36.97±3.59a	15.49±0.16a	132.2±8.5a

(Fig. 2). 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.

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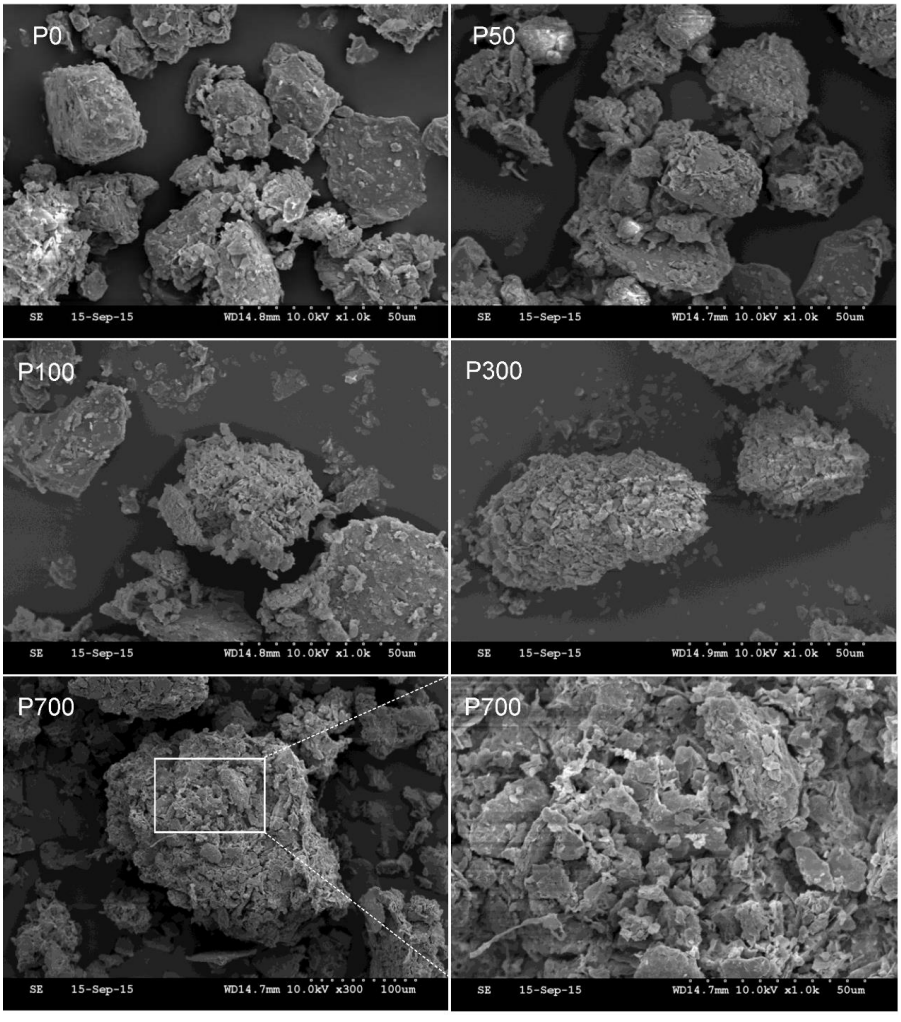

Fig. 2 Scanning electron microscopy images of aggregates after dispersion in water from the studied soil chronosequence, with age varying from 0 to 700 yr of paddy cultivation history. The number of years of rice cultivation were marked in the upper left corner of the images.

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

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382 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}
383 to 24.36 g kg^{-1} in clay fractions, showing consistently higher in rice soils than in the
384 uncultivated marsh soil. Similar was the change in total N in the size fractions (total
385 N was 1.04 g kg^{-1} and 1.01 g kg^{-1} in coarse sand and fine sand fraction, and 0.52 g
386 kg^{-1} and 1.17 g kg^{-1} in silt and clay fraction, in P0. In rice soils (P50 - P700), total N
387 ranged 2.72 - 4.43 g kg^{-1} in coarse sand, and 8.45 - 19.86 g kg^{-1} in fine sand, and
388 from 10.13 to 11.37 g kg^{-1} in silt and from 19.80 to 24.36 g kg^{-1} in clay. Generally,
389 LOC, SOC and total N contents followed an order of coarse sand fraction > clay
390 fraction > fine sand and silt fractions in a single soil. And C/N ratio was markedly
391 higher in the coarse sand fractions than in the other fractions across the
392 chronosequence. Moreover, the distribution patterns of SOC, LOC and total N
393 associated in the four size fractions were similar across the sequence. SOC, LOC and
394 total N from coarse sand and clay fractions were significantly higher compared to
395 other PSFs. Across the chronosequence, 700 years rice cultivation led to 449 %,
396 101 %, 106 % and 162 % increases in SOC content in coarse, fine sand, silt and clay
397 size fraction, respectively over the uncultivated marsh soil. Meantime, total N in
398 these size fractions increased by of 326%, 79%, 113% and 133 %, respectively.
399
400 **Table 2** Contents (g kg^{-1}) of SOC, total N and LOC of the size fractions of the studied
401 chrono-sequence. Different capital and low case letters indicate a significant (p<0.05) difference
402 respectively between fractions of a single soil, and between soils for a single fraction, in a single
403 column.

Size fraction	Soil	SOC	Total N	LOC
Coarse sand	P0	11.07±1.20Ad	1.04±0.11Ad	6.22±0.18Ac

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	P50	53.44±1.09Ab	4.15±0.49Aa	27.85±1.61Aa
	P100	41.74±1.31Ac	3.37±0.38Ab	19.69±1.16Ab
	P300	40.64±1.57Ac	2.72±0.12Ac	18.80±1.45Ab
	P700	60.79±1.88Aa	4.43±0.22Aa	28.64±1.90Aa
	P0	9.90±0.43Ac	1.01±0.14Ac	4.34±0.14Bb
	P50	8.45±0.27Cc	0.73±0.11Dd	3.66±0.57Cb
Fine sand	P100	16.48±0.41Cb	1.57±0.14Cb	7.36±0.32Ca
	P300	15.16±1.45Cb	1.51±0.13Bb	7.03±0.30Ca
	P700	19.86±1.11Ca	1.81±0.12Ca	7.99±0.65Ba
	P0	5.13±0.19Bb	0.52±0.14Bd	1.53±0.13Db
	P50	10.73±0.55Ba	1.20±0.11Cb	4.5±0.13Ca
Silt	P100	10.13±0.44Da	1.15±0.09Cc	4.1±0.26Da
	P300	11.37±0.58Da	1.33±0.11Ba	4.39±0.29Da
	P700	10.57±0.43Da	1.11±0.08Dc	3.95±0.69Ca
	P0	9.29±0.29Ac	1.17±0.15Ad	2.96±0.27Cc
	P50	19.80±1.47Bb	2.27±0.14Bc	7.99±0.28Bb
Clay	P100	22.94±1.43Ba	2.70±0.12Bb	9.19±0.35Ba
	P300	23.45±1.46Ba	2.92±0.12Aa	9.36±0.40Ba
	P700	24.36±1.65Ba	2.73±0.16Bb	9.05±0.47Ba

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

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410 increased in the rice cultivated soils, over the uncultivated tidal marsh. However, in
411 the other fractions, the proportion of carbon chemical groups showed slight changes
412 with the increasing time of rice cultivation.

413
414 **Table 3** Relative proportion (%) of carbon chemical groups in size fractions by FTIR analysis.
415 Different capital and low case letters indicate a significant ($p < 0.05$) difference respectively
416 between fractions of a single soil, and between soils for a single fraction.

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

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P100	2.27±0.12Ba	33.83±1.72Aa	0.04±0.01Ca	63.85±4.57Ba
P300	2.31±0.08Aa	33.71±1.70Aa	0.03±0.01Ca	63.96±4.65Ca
P700	2.44±0.17Ba	34.42±1.82Aa	0.05±0.01Ca	63.08±3.73Ca

417

418 **3.2 DNA content, microbial gene abundance and diversity**

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

427 Over the studied chronosequence, DNA contents of an aggregate size fraction
428 were several folds higher in the rice soils over the initial tidal marsh. Accordingly,
429 gene copy numbers of microbial communities from a PSF were greatly higher in rice
430 soils than in the initial tidal marsh. Bacterial and fungal abundance in coarse sand,
431 fine sand, silt and clay fraction in P50 was increased by 688%, 72%, 498% and
432 622 %, and 74%, 149%, 7% and 152 %, respectively over P0. An increase in
433 bacterial gene copy numbers over P0 was seen significant across the rice soils
434 cultivated for 100-700 years, by 73% to 437 %, 0.4% to 67 %, 225% to 246 % and
435 147% to 201 %, respectively in coarse sand fraction, fine sand fraction, silt fraction
436 and clay fraction. However, those in fungal gene abundance were more or less

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437 inconsistent across the rice soils, by 9% to 18 %, 25% to 159 %, 45% to 8 % and 167%
438 to 377 % in coarse sand, fine sand, silt and clay fractions, respectively. In contrast,
439 archaeal abundance was found increased over P0 consistently across the fractions
440 with the prolonged rice cultivation. In particular, the archaeal abundance in coarse,
441 fine sand, silt and clay increased by 25, 2, 32 and 19 folds in P700 with 700 years
442 rice cultivation over the tidal marsh (Table 4).

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

Size fraction	Soil	DNA	BA	FA	ArA
Coarse sand	P0	3.32±0.07Ae	5.86±0.75Ad	8.92±1.50Ab	0.81±0.03Ce
	P50	35.33±0.42Aa	46.18±9.21Aa	15.50±2.60Aa	6.37±0.81Bd
	P100	24.72±2.14Ac	31.45±5.79Ab	10.49±0.87Ab	13.54±0.73Bc
	P300	16.20±0.05Ad	10.12±2.39Ac	8.12±0.32Ab	16.01±1.06Ab
	P700	31.95±0.64Ab	14.25±1.03Ac	9.40±0.71Ab	21.17±0.48Ba
Fine sand	P0	3.63±0.28Ab	4.90±0.45Ab	3.23±0.27Bc	2.83±0.18Ac
	P50	4.35±0.40Db	8.42±1.75Ba	8.04±0.25Ba	5.27±1.12Bd
	P100	13.63±3.30Ba	7.75±1.18Ca	8.37±0.67Aa	8.16±2.27Cab
	P300	9.97±0.33Ba	4.92±1.10Bb	6.23±0.23Bb	3.57±0.24Cb
	P700	12.83±0.33Ca	8.16±1.64Ba	2.43±0.19Cd	7.68±0.66Ca
Silt	P0	1.57±0.28Bc	1.78±0.15Bc	3.98±0.57Ba	0.29±0.02Dd
	P50	10.02±1.58Ca	10.64±2.95Ba	4.25±0.30Ca	2.48±0.44Cc
	P100	8.25±0.12Cab	5.78±0.36Cb	2.17±0.20Bb	8.65±0.09Ca
	P300	7.78±0.31Cb	5.91±0.81Bb	2.47±0.45Bb	6.60±0.27Bb
	P700	9.25±0.64Da	6.16±0.29Bb	3.68±0.19Ba	9.44±1.41Ca

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	P0	4.00±1.89Ad	5.27±0.61Ac	0.52±0.03Cd	1.83±0.10Bc
	P50	17.62±0.26Bb	38.05±4.92Aa	1.31±0.07Dc	14.08±2.13Ab
Clay	P100	16.20±0.38Bb	15.86±3.31Bb	1.94±0.30Bb	44.66±13.68Aa
	P300	11.17±0.90Bc	13.03±2.58Ab	1.39±0.40Cb	22.16±6.17Aa
	P700	25.67±0.57Ba	15.63±2.24Ab	2.48±0.31Ca	36.00±3.82Aa

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 5 Normalized enzyme activity (NEA), soil respiration (mg CO_2 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

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469 between fractions of a single soil, and between soils for a single fraction, in a single column.

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

470

471 Soil respiration, an indicator of live soil microbial organisms (Schlesinger &
472 Andrews, 2000), ranged from 2.98 mg CO_2 g^{-1} to 6.62 mgCO_2 g^{-1} across the PSFs in
473 the uncultivated tidal marsh and from 7.40 mg CO_2 g^{-1} to 32.45 mg CO_2 g^{-1} in rice
474 soils (Table 5). In detail, soil respiration was 6.62 mgCO_2 g^{-1} and 5.65 mgCO_2 g^{-1} in

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475 coarse and fine sand fraction, and 2.98 mgCO_2 g^{-1} and 4.96 mgCO_2 g^{-1} in silt and
476 clay fraction, respectively in P0. While in rice soils, soil respiration was in a range of
477 15.9-29.1 mg CO_2 g^{-1} in coarse sand, and of 10.8-12.6 mgCO_2 g^{-1} in fine sand
478 fraction, and of 7.4-13.5 mgCO_2 g^{-1} in silt and of 10.3-14.3 mgCO_2 g^{-1} in clay
479 fraction, of the rice soils. Soil respiration in a single size fraction generally increased
480 with rice cultivation length. Over P0, soil respiration increased by 3.4, 1.2, 3.5 and
481 1.9 folds, respectively of coarse sand, fine sand, silt and clay size fractions in P700.
482 However, ratio of respired C to total OC (RQ, a soil respiration quotient) of the
483 four fractions was in a range of 0.15-0.16 gCO_2-C g^{-1}SOC in P0. For the rice soils,
484 however, the RQ was 0.12-0.15 gCO_2-C g^{-1}SOC in coarse sand, and 0.17-0.35
485 gCO_2-C g^{-1}SOC in fine sand fraction, and 0.19-0.35 gCO_2-C g^{-1}SOC in silt fraction
486 and 0.12-0.20 in clay fraction, respectively. Moreover, RQ values of coarse sand
487 fraction and of clay fraction was not significant different among the rice soils
488 cultivated up to 700 years. But, RQ of the fine sand and silt fractions from the rice
489 soils increased by 6 -119 % and by 18-119 %, compared to P0 (Table S3).
490 Carbon gain from amended maize was 0.17 g kg^{-1} in coarse sand and 0.77 g
491 kg^{-1} in fine sand fraction, but 0.26-0.38 g kg^{-1} in silt and clay fraction of P0. Carbon
492 gain from amended maize was 0.42-0.56 g kg^{-1} in coarse sand and 0.50-1.27 in fine
493 sand fraction, but 0.36-0.60 g kg^{-1} in silt fraction and 0.25-0.31 g kg^{-1} in clay fraction
494 of the rice soils. Except the clay fraction, carbon gain potential by a single fraction
495 was higher in rice soils (P50-P700) than in the uncultivated marsh P0, and in P100,
496 P300 and P700 than in P50 for rice soils (Table 5). Amended maize carbon was

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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

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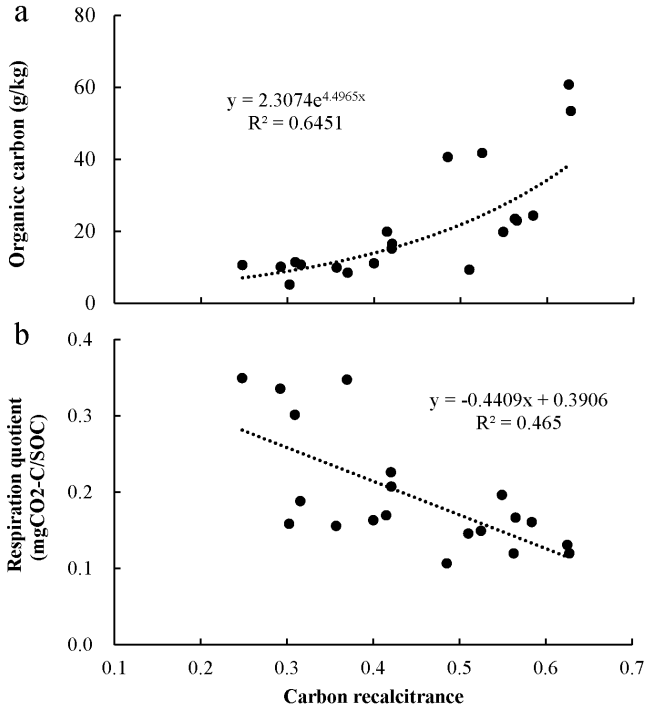
519 ratio of aromatic and phenol carbon to aliphatic and polysaccharide carbon (Fig.4),
520 of particle size fractions of soil aggregates of the soils over the chronosequence. This
521 evidenced an overall trend of soil organic stabilization while OM accumulated in the
522 soil aggregates. This was in accordance with our previous finding of soil organic
523 carbon accumulation and stabilization in bulk samples of the studied chronosequence
524 (Wang et al., 2015).



525
526 **Fig. 3** Correlation of organic carbon (a) and respiration quotient (b) with carbon recalcitrance
527 [the ratio of relative recalcitrant C (Aromatic and Phenol) to relative labile C (Aliphatic and
528 Polysaccharide)] of the particle size fractions of the studied chronosequence.

529 However, carbon stabilization indicators were seen varying with the different
530 size fractions. The sand sized fractions here were characterized by high OC with

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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 lager 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

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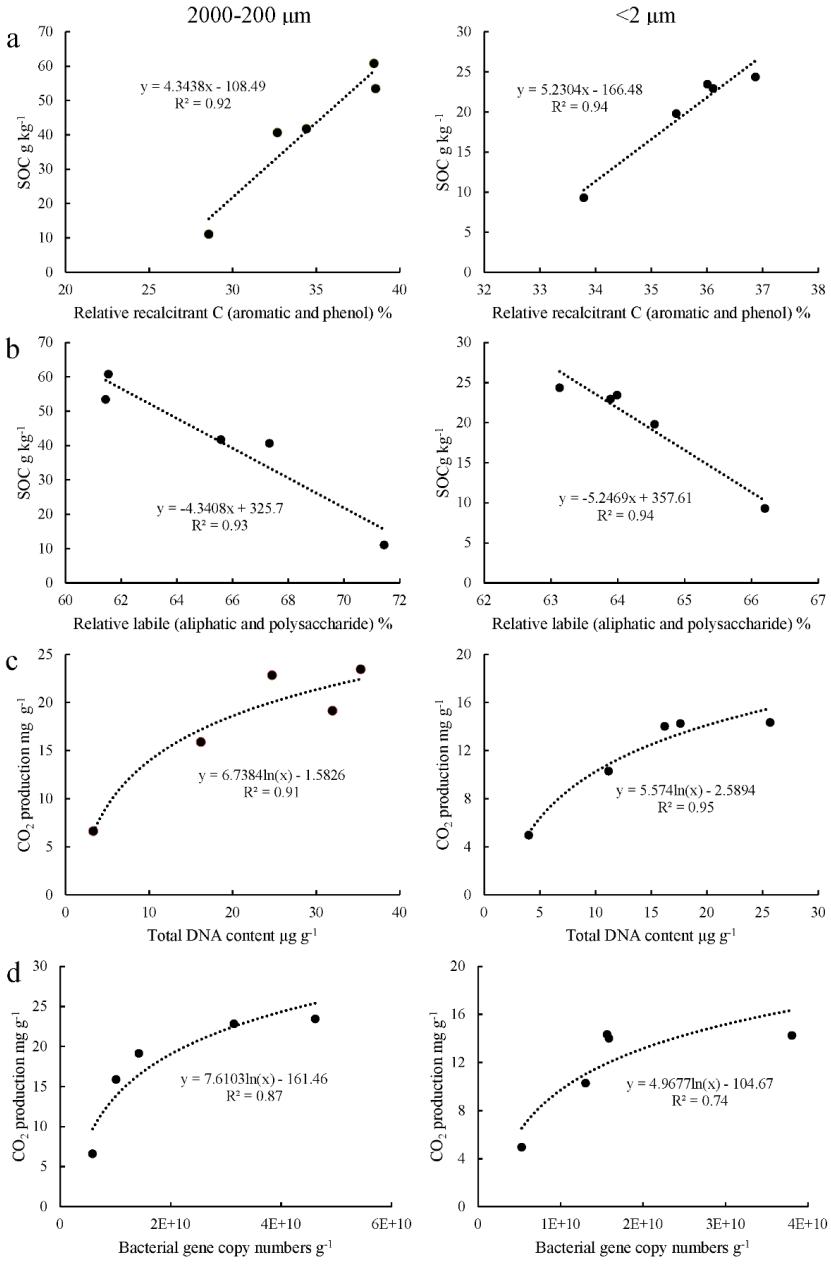
553 organic carbon) was shown physically protected in line with the enhancement of soil

554 aggregation



555

556 **Fig. 4** Inter-correlation between carbon pools and microbial biomass to address the differences of

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557 soil carbon stability and microbial functioning between coarse sand (left) and clay (right) sized
558 aggregates fractions (Soil organic carbon accumulation as a function of relative recalcitrant C
559 (aromatic and phenol) (a) and negatively of relative labile C (aliphatic and polysaccharide) (b);
560 CO_2 production as a plateau function of soil microbial biomass (c) and bacterial abundance (d)).
561 Data was the mean value of triplicates.

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

566 Accumulation of SOC under long term rational management practices was well
567 addressed in accompanied with formation of macro-aggregates, which in turn
568 physically protect the SOC from microbial decomposition via forming a physical
569 barrier between the substrates and microbes (Zhou et al., 2009; Tripathi et al., 2014).
570 Physical protection of labile carbon in macro-aggregates rather than inherent
571 chemical stability of OC (a minor mass fraction of the clay sized micro-aggregates,
572 Table 1) had been addressed in many studies for soil carbon sequestration (Six et al.,
573 2004; Kong et al., 2005; Six and Paustian, 2014). Synthesizing data from Table 1 and
574 Table 2, organic carbon physically protected in the sand and fine sand fractions
575 constituted 51%-62% while chemically protected carbon in the clay sized fractions
576 11%-19%, to the total carbon storage of soils over the studied sequence. Therefore,
577 this study again convinces that, rather than chemical stabilization, physical
578 protection of labile carbon within micro-aggregates in macro-aggregates, against
579 microbial access and decomposition, could be concerned as the major contributor of

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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

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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

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

628 Soil respiration had been generally accepted as a size of active microbes in soils
629 using accessible carbon substrates (Schlesinger and Andrews, 2000). In this study,
630 enzyme activity when normalized as NEA, was well correlated to organic carbon
631 contents in soil aggregate fractions (Fig. S3). Soil respiration was higher in sand
632 fraction where SOC and diversities of bacterial community were higher, than clay
633 fraction. The higher bacterial biomass in the larger size fractions was related to the
634 extent of decomposable soil organic matter as LOC contents were higher in coarse
635 fractions than in other fractions (Table 3). However, bacterial and archaeal
636 abundance per unit of SOC were highest, but respiration lowest, in clay fraction.
637 This could explain microbial activity was low due mainly to inert carbon chemical
638 protected in clay sized aggregates (Nelson et al., 1994; Six and Paustian 2002).
639 Microorganisms physically confined in small pores could become less active and
640 protected against grazing by the soil fauna. Moreover, SOM was chemically
641 protected from mineralization by surface adsorption onto clay minerals (Six et al.,
642 2002b; Davidson and Janssens, 2006). Interestingly, soil respiration quotient was
643 seen well negatively correlated to total DNA content and labile carbon content for
644 the rice soils shifted from salt marsh (Fig. S4). This again confirmed that enhanced
645 microbial community with SOC accumulation, exhausted less carbon, indicating

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646 higher carbon use efficiency, particularly with labile carbon in coarse fractions of
647 aggregates (Jastrow et al., 1996). In this study with water stable aggregates from rice
648 soils, microbial activity and carbon use efficiency was generally higher in
649 macro-aggregates than in micro-aggregates. This could lead to an understanding that
650 physically protected carbon as of labile carbon promoted microbial activity in
651 macro-aggregates of the rice soils.

652 Carbon gain from straw amendment, as one of important soil functions for
653 carbon sequestration, was observed in all fractions of soil aggregates in lab
654 incubation (Table 4). Total carbon gain from amended maize straw was more or less
655 in linear response to relative fungal gene abundance (characterized with fungal to
656 bacterial gene copy number ratio) (data not shown). Here, higher carbon gain in fine
657 sand fraction could be attributed to high fungal dominance and C/N ratio (also high
658 LOC pool). Comparatively, clay fraction with mostly chemically stabilized carbon
659 had a smaller potential to gain exotic carbon. This seemed controversial to the
660 argument by Piccolo et al. (2004) that hydrophobic carbon, high in clay fractions
661 here, could be a sink of amended carbon in soils. Again, the fact that sand sized
662 fraction rather than clay sized fraction, of soil aggregates, preserved more carbon
663 from amended maize verified that carbon sequestration could be predominately
664 contributed by physical protection in macro-aggregates where C/N ratio and fungal
665 dominance and LOC pool are already high (Kandeler et al., 2000). Fontaine et al.,
666 (2011) argued that fungi mediated long term carbon sequestration, potentially
667 through their priming effect.

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668 We further compare the bio-activity versus carbon between sand and clay sized
669 aggregate fractions. When plotting DNA content of microbial biomass against OC
670 content, a correlation was very significant for coarse sand fraction but failed for clay
671 fraction (Fig. 5a). Accumulation of SOC in these coarse fractions have been well
672 characterized as physically protected (Six et al., 2000; Six et al., 2004), particularly
673 the POM in large macro-aggregates (Six et al., 2004). The result here could indicate
674 that soil microbial communities in large aggregates could be in an access to SOM
675 physically protected in large aggregates. This is again supported by that finding that
676 normalized enzyme activity from coarse sand fraction was found in a positively
677 linear function with SOC accumulation (Fig. 5b). However, DNA content scaled CO_2
678 production was in a negatively power function (Fig. 5c) with total soil DNA content,
679 showing an increased carbon use efficiency with the SOM accumulation in large
680 sized fractions. In our previous research, improved microbial activity was found
681 linked to the increase in particulate organic carbon content which was enhanced via
682 physical protection with promoted soil aggregation (Wang et al., 2015). Promoted
683 macro-aggregation, as indicated by increased MWD here, with SOC accumulation
684 could lead to a more heterogeneous soil micro-habitat, a better spatial allocation of
685 various pools of OM and different size groups of microbes and extra-cellulose
686 enzymes within macro-aggregates.

687 Soil enzymes catalyzing the C transformation, nutrient release and redox
688 processes in soils could be considered as soil ecosystem functioning capacity (van
689 der Heijden, et al., 2008). As seen in Fig. 3, microbial enzyme activity and carbon

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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 companied with carbon stabilization underspin 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

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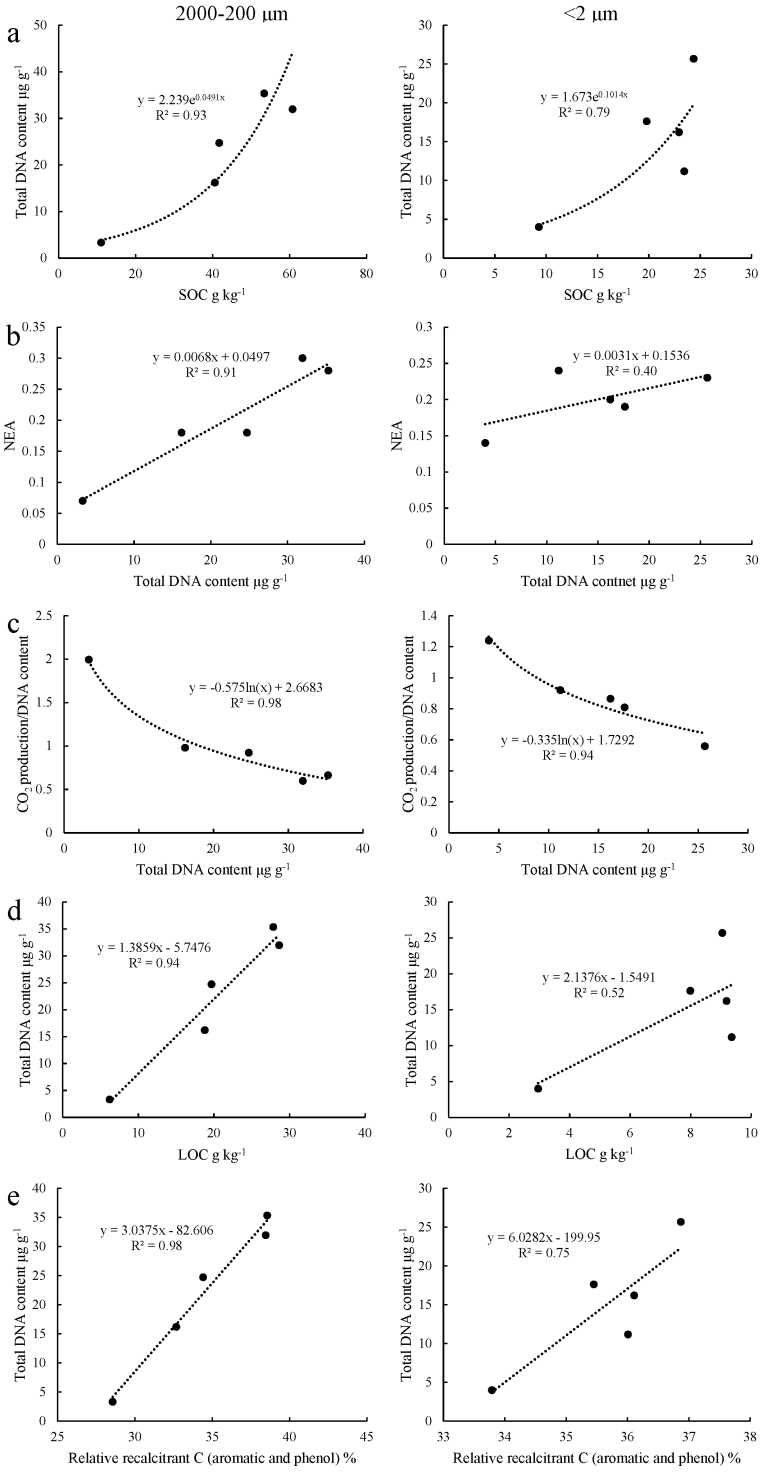

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701 **Fig. 5** Inter-correlation between particulate organic carbon and soil microbial activity to compare
702 the biological activity versus carbon between coarse sand (left) and clay (right) sized aggregate
703 fractions (Soil microbial biomass was as an exponential function of total soil organic carbon (a)
704 and a linear function of labile organic carbon (d). Normalized enzyme activity (b) and DNA
705 content scaled CO2 production (c) as a linear and negative power function of soil microbial
706 biomass. Soil microbial biomass was as a linear function of relative recalcitrant C (aromatic and
707 phenol) (e)). Data was the mean value of triplicates.

708 protection, mentioned above (Wang et al., 2015). With soil aggregation improved,
709 macro-aggregates could provide more diverse soil microhabitats with varying types
710 of OC accessible to microbes under sustainable agricultural management (Six and
711 Paustian, 2014).

712 Many studies had reported that enzyme activity and microbial biomass showed
713 positive relationship with carbon concentrations in bulk soils (Marx et al., 2005;
714 Allison and Jastrow, 2006; Shi et al., 2006; Yu et al., 2012). However, the correlation
715 coefficients between these parameters in clay fraction were much lower than in
716 coarse sand fraction. This suggested that the carbon in this fraction of aggregates
717 was not readily available to microbes, confirming the generally considered C
718 stabilization in clay fraction. With high humification degree and interaction in
719 organo-mineral complexes of clay particles, organic matters were not readily
720 available for soil microbes (Lützow et al., 2006; Kogel-Knabner et al., 2008).
721 Extracellular enzymes could be absorbed by clay minerals (Allison and Jastrow,
722 2006), but the potential activity of soil enzymes in this fraction therefore could not
723 be related to the turnover of OM. Clearly, some mechanism must have prevented
724 enzymes from mineralizing C efficiently in these mineral-associated fractions, which

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725 contained C pools with very slow turnover rates (Six and Jastrow, 2002). Although
726 habits within macro-aggregates offered protection of the young and labile carbon
727 against microbial decomposition (Gupta and Germida, 2015), enhanced aggregation
728 could lead to increased population and activities of specific microbial groups (Six et
729 al., 2002b).

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

737 **4.2 Dynamics of C stability and bioactivity with prolonged rice cultivation**

738 In our previous study of bulk soils from the chronosequence, soil organic
739 carbon accumulation was found concurrent carbon stabilization and promotion of
740 biological activity through physically protected labile carbon accumulation with
741 long-term rice cultivation (Wang et al., 2015). And this was found in line with the
742 enhancement of soil aggregation characterized by the change in mean weight
743 diameter of soil aggregates over the sequence (Wang et al., 2015). Here we
744 synthesize all the analysis data in terms of aggregate partitioning over the soils,
745 presented in Fig. 6. After salt marsh soil (P0) shifted to rice cultivation (P50), total
746 SOC, enzyme activity and soil respiration showed a more or less consistent increase

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747 in both sand and clay sized fractions with prolonged rice cultivation. Meanwhile,

748 with prolonged rice cultivation, carbon gain from amended

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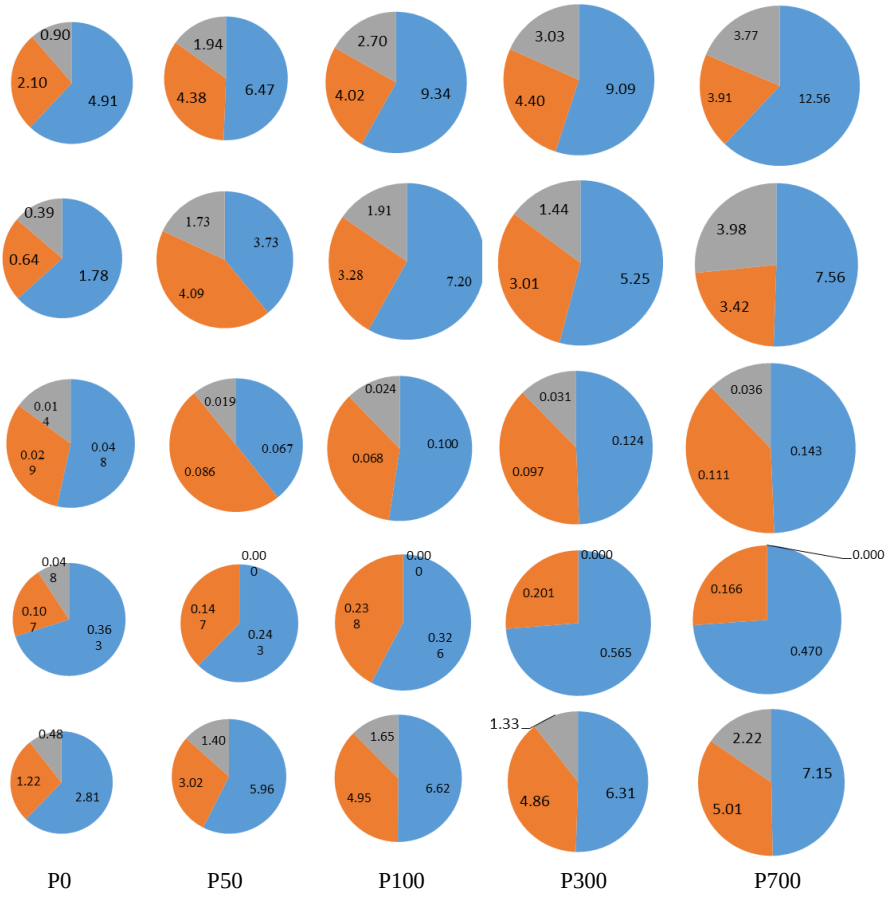

Fig. 6 Change in partitioning of soil organic carbon (a, g/kg), total DNA (b, μg/g) , normalized enzyme activity (c, relative enzyme activity index), carbon gained from maize straw amendment (d, mg/kg) and soil respiration (e,mgCO_2/g) among coarse and fine sand fraction (blue base), silt fraction (brown 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

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762 sized fractions exerted different patterns between of carbon pools and microbial
763 activities, along the chronosequence. Over the sequence, the prevalence of physically
764 protected portion in sand and fine sand fractions over unprotected (referred to Six *et*
765 *al.*, 2002) portion was in range of 1.5-3.2 and of 1.1-2.6 for SOC and total N, of
766 0.9-2.2 for total DNA, of 1.2-3.3 for fungal gene copy numbers, of 0.8-1.5 for NEA
767 and 1.6-2.8 for C gain, respectively. In contrast, the prevalence of archaeal copy
768 numbers and soil respiration was in a range of 2.6-1.0 and 2.0-1.3, decreasing with
769 rice cultivation lengths. Therefore, most of analyzed carbon pools and bioactivities
770 were dominated by the macro-aggregates in sand and fine sand size fractions, which
771 was generally in a consistent directional change with prolonged rice cultivation.

772 The rice soils over the chronosequence were derived from salt marsh, which was
773 indigenously rich in silt mineral particles, with an average silt mass content of 75% -
774 84% (Cheng et al., 2009). During the rice soil development under rice cultivation, silt
775 sized mineral particles were increasingly aggregated with increased OM while clay
776 minerals accumulated due to neoformation of oxyhydrates of iron/manganese as well
777 as minute clay sized minerals from irrigation (Chen and Zhang, 2009; Cheng et al.,
778 2009; Kalbitz et al., 2013; Wissing et al., 2013).With rice cultivation, organic carbon
779 was increasingly accumulated and stabilized in sand sized aggregates with physical
780 protection of labile OC pool intra micro-aggregates, with prolonged rice
781 cultivation(Wang et al., 2015). The changes in relative proportion of carbon pools and
782 microbial activities (NEA, C gain and soil respiration) by sand and fine sand sized
783 aggregates further demonstrated that physically protected and stabilized carbon
784 supported high soil bioactivities, which had been increasingly prevailed over the
785 smaller sized fractions of soil aggregates.

786 The proportion of coarse sand fraction increased, whereas fine sand and silt

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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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