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Microbial activity promoted with organic carbon accumulation in macroaggregates of paddy soils under long-term rice cultivation

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Abstract. While soil organic carbon (SOC) accumulation and stabilization has been increasingly the focus of ecosystem properties, how it could be linked to soil biological activity enhancement has been poorly assessed. In this study, topsoil samples were collected from a series of rice soils shifted from salt marshes for 0, 50, 100, 300 and 700 years from a coastal area of eastern China. Soil aggregates were fractioned into different sizes of coarse sand (200–2000 µm), fine sand (20–200 μ m), silt (2–20 μ m) and clay (<2 μ m), using separation with a low-energy dispersion protocol. Soil properties were determined to investigate niche specialization of different soil particle fractions in response to longterm rice cultivation, including recalcitrant and labile organic carbon, microbial diversity of bacterial, archaeal and fungal communities, soil respiration and enzyme activity. The results showed that the mass proportion both of coarse-sand $(2000-200 \,\mu\text{m})$ and clay $(< 2 \,\mu\text{m})$ fractions increased with prolonged rice cultivation, but the aggregate size fractions were dominated by fine-sand $(200-20 \,\mu\text{m})$ and silt $(20-2 \,\mu\text{m})$ fractions across the chronosequence. SOC was highly enriched in coarse-sand fractions (40-60 g kg⁻¹) and moderately in clay fractions $(20-25 \text{ g kg}^{-1})$, but was depleted in silt fractions ($\sim 10 \, \text{g kg}^{-1}$). The recalcitrant carbon pool was higher (33-40% of SOC) in both coarse-sand and clay fractions than in fine-sand and silt fractions (20-29% of SOC). However, the ratio of labile organic carbon (LOC) to SOC showed a weakly decreasing trend with decreasing size of aggregate fractions. Total soil DNA (deoxyribonucleic acid) content in the size fractions followed a similar trend to that of SOC. Despite the largely similar diversity between the fractions, 16S ribosomal gene abundance of bacteria and of archaeal were concentrated in both coarse-sand and clay fractions. Being the highest generally in coarse-sand fractions, 18S rRNA gene abundance of fungi decreased sharply but the diversity gently, with decreasing size of the aggregate fractions. The soil respiration quotient (ratio of respired CO₂-C to SOC) was the highest in the silt fraction, followed by the fine-sand fraction, but the lowest in coarse-sand and clay fractions in the rice soils cultivated over 100 years, whereas the microbial metabolic quotient was lower in coarse-sandsized fractions than in other fractions. Soil respiration was higher in the silt fraction than in other fractions for the rice soils. For the size fractions other than the clay fraction, enzyme activity was increased with prolonged rice cultivation, whereas soil respiration appeared to have a decreasing trend. Only in the coarse-sand fraction was both microbial gene abundance and enzyme activity well correlated to SOC and LOC content, although the chemical stability and respiratory of SOC were similar between coarse-sand and clay fractions. Thus, biological activity was generally promoted with LOC accumulation in the coarse-sand-sized macroaggregates of the rice soils, positively responding to prolonged rice cultivation management. The finding here provides a mechanistic understanding of soil organic carbon turnover and microbial community succession at fine scale of soil aggregates that have evolved along with anthropogenic activity of rice cultivation in the field.

1 Introduction

Soil organic matter (SOM), as a continuum of organic substances that accumulated over time from decomposition of plants and microorganisms (Lehmann and Kleber, 2015), provided a key driver for soil aggregation and thus soil ecosystem functions and services (Banwart et al., 2014). Soil aggregates have been considered as fundamental soil particle units where organic matter, minerals and microbes interacted to store carbon and nutrients, as well as moisture (Tisdall and Oades, 1982; Lützow et al., 2006; Marschner et al., 2008; Schmidt et al., 2011), and mediated their cycling in soil-plant systems (Six et al., 2004). One of the primary mechanisms for soil carbon sequestration could be the increased physical protection of SOC within aggregates that decreased decomposition rates (Blanco-Canqui and Lal, 2004; Six et al., 2004; Kong et al., 2005; Six and Paustian, 2014). This could have something to do with separated allocation of mineralassociated SOC fractions between microaggregates within macroaggregates (Lehmann et al., 2008; Dungait et al., 2012; Vogel et al., 2014). Soil aggregation further shaped the microhabitats for soil microbial communities (Six et al., 2000; Ettema and Wardle, 2002; Balser et al., 2006; Kögel-Knabner et al., 2008), with changes in SOC substrate availability, chemical recalcitrance and redox potential with or within aggregates (Rillig et al., 2001; Six et al., 2006; Strickland and Rousk, 2010). Consequently, changes in composition of soil aggregate fractions could lead to changes in bioactivity reflected by the size, diversity and biochemical activity of the microbial community (Six et al., 2006; Lagomarsino et al., 2012; Bardgett and van der Putten, 2014). Particulate organic carbon (POC) has been increasingly considered as an indicator of soil quality and health under different stresses or human disturbance (Cambardella and Elliot, 1992; Marriott and Wander, 2006). As a labile carbon pool, POC has also been suggested as a measurement of SOC accumulation and stabilization with co-existing microbial activity of soils in different ecosystems (Gajda, 2010; Six and Paustian 2014). Soil aggregation, affected by land use and management practices, could result in changes in allocation of POC inter- and/or intra-microaggregates in size fractions of soil (Yang et al., 2009; Lagomarsino et al., 2012; Six and Paustian, 2014; Smith et al., 2014). Unfortunately, the link between changes in carbon pools and those in microbial biological activity with SOC stabilization in soil aggregates has not yet been well understood and quantitatively assessed (Six and Paustian 2014; Smith et al., 2014).

Soil aggregation could be characterized by measuring distributions of defined particle size fractions, which could differ in soil microbial biomass and activity, in response to SOC accumulation and stabilization of soil (Salinas-Garcia et al., 1997; Kandeler et al., 1999; Smith et al., 2014). Such difference could mimic the microscale interactions driving SOC stabilization and nutrient cycling in soils (Kandeler et al., 2006; Lagomarsino et al., 2012; Six and Paustian, 2014). To examine these interactions, aggregate separation methods should use the least low-energy dispersion of bulk soil into particle size fractions (Kandeler et al., 2000), and avoid use of any chemical dispersion methods (Smith et al., 2014). Stemmer et al. (1998) developed a low-energy ultrasonic dispersion protocol, which allowed the least disturbed separation for analyzing microbial community composition and enzyme activity in the obtained size fractions of soil aggregates (Kandeler et al., 2000). This approach was followed in later studies (Sessitsch et al., 2001; Poll et al., 2003; Matocha et al., 2004; Marx et al., 2005; Zhang et al., 2013), addressing the impacts of different management practices or environmental disturbances on SOC persistence, microbial communities and enzyme activity in aggregate agricultural soils. However, the interactions between these attributes in aggregate size fractions with carbon stabilization and their trend with continuing management in long-term cultivated soils has not yet been well characterized.

Soil matrix or microsite properties have been well known to have an important role in the spatial allocation of organic matter and microbial community and thus the link between SOC pools and microbial bioactivity among different fractions of soil aggregates (Smith et al., 2014). Rice paddy soils were developed with a dynamic redox regime and neoformation of iron/manganese oxyhydrates due to hydromorphic pedogenesis under long-term hydroagric paddy management (Li, 1992). These soils were thus classified as a particular soil group of hydroagric Anthrosols in the new Chinese Soil Taxonomy (Gong et al., 1999). Recently, these soils have been shown to have high SOC storage and sequestration potential compared to dry-land croplands (Pan et al., 2004, 2010; Wissing et al., 2013). This has previously been attributed to enhanced aggregation and aggregate stability (Lu et al., 1998; Yang et al., 2005) as well as to increased humification of SOC (Olk et al., 2000). However, SOC accumulation and stabilization in paddy soils with management practices has been addressed with a number of processes. These processes were understood with either increased binding to free oxyhydrates (Zhou et al., 2009b; Cui et al., 2014) and enhanced chemical recalcitrance (Zhou et al., 2009a, 2011; Song et al., 2012), or enhanced physical protection with increased aggregate stability (L. Li et al., 2007; Zhou et al., 2008) or their interactions (Song et al., 2012, 2013).

Moreover, SOC could be continuously accumulated in rice soils with prolonged rice cultivation in the long run. In a rice soil chronosequence, SOC accumulation was promoted shortly following the desalinization and decalcification in the initial stage after the salt marshes shifted to rice paddies (Kalbitz et al., 2013). Across this chronosequence, the accumulated SOC was increasingly stabilized with neo-formed iron oxyhydrates (Cheng et al., 2009; Wissing et al., 2011), as rice cultivation prolonged. Whereas, in a rice paddy with well-managed fertilization from southeastern China, SOC accumulation was well represented by an increase in proportion of water-stable macroaggregates (>250 μ m) and the associated POC pool (Zhou and Pan, 2007). In rice paddies under long-term fertilization trials from southern China, physically protected SOC in the coarse-sand-sized fractions of soil aggregates contributed to bulk-soil SOC accumulation and stabilization (Zhou et al., 2008).

Importantly, the co-evolution of the soil microbial community and diversity has been observed with SOC accumulation and stabilization in rice paddies (Zhang et al., 2007; Zheng et al., 2007; Liu et al., 2011). In line with the trend of SOC accumulation in paddy soils, microbial biomass and community diversity was shown to be enhanced across a chronosequence under prolonged rice cultivation (Bannert et al., 2011; Jiang et al., 2013). Using a similar chronosequence, the enhanced biological activity could be well portrayed with an increase in mean weight diameter of soil aggregates and in the POC pool across the soils with prolonged rice cultivation (Wang et al., 2015). This indicated a potential role of the physically protected labile carbon pool in enhancing biological activity with bulk SOC accumulation in rice soils (Zou et al., 2015). Recently, changes in microbial gene abundance and community composition were reported for bulk soils (Liu et al., 2016a) and for aggregate size fractions of soils (Liu et al., 2016b), from such a rice soil chronosequence. It could be speculated that physical protection could involve a change in the spatial distribution of pools rather than in the chemical recalcitrance, of organic carbon located among aggregate size fractions. The changed allocation of both carbon pools and the microbial community could contribute to SOC stabilization with increased microbial abundance and the carbon use efficiency, qCO_2 (Schlesinger and Andrews, 2000), as a result of enhanced aggregation (Lehmann, 2011). Such information would be of key importance for understanding carbon stabilization in relation to sustainable management of rice paddy soils with respect to carbon biogeochemical cycling and ecosystem functions provided by soils (Smith et al., 2015).

In this study, two hypotheses were tested. First, we sought to examine whether microbial bioactivity and carbon stability in soil aggregates could differ in their size fractions, leading to changes in spatial allocation of SOC pools among aggregate size fractions in rice paddies. In this case, physical protection of SOC could improve microbial microhabitat conditions and thus microbial carbon use efficiency through enhanced aggregation. Furthermore, it could enable an existence of labile carbon pool within microaggregates that build up macroaggregates or between microaggregates within macroaggregates (Six and Paustian 2014; Smith et al., 2014). Thus, biological activity could be enhanced with physically protected carbon in macroaggregates, as compared to microaggregates (clay sized) with chemically stabilized organic carbon. Second, we sought to examine whether the strong link between microbial activity and the size of labile carbon pool in macroaggregates could be promoted with enhancement of physically stabilized SOC through continuing hydroagric paddy management under long-term rice cultivation. In a series of soils formed on similar paleo-deposits rich in silt, continuous rice cultivation could result in a directional change in soil aggregation, and thus in microhabitat conditions as well as nutrients. This directional pedogenetic development would in turn affect a more or less directional change in SOC stabilization (with increasing POC pool and accumulation of recalcitrant carbon and mineral bound carbon). This study aimed to understand that carbon stabilization could not confront but could improve biological activity in soils under rice cultivation over centuries.

2 Materials and methods

2.1 Methodology rational

Using a recommended sonication separation procedure, we examined changes in aggregate size fraction composition for aggregate stability, in functional group composition for chemical recalcitrance of SOC, and in soil respiration for microbial energy use, in order to characterize SOC accumulation and stabilization in rice soils. Meanwhile, changes with SOC accumulation and stabilization were explored in microbial activity for soil functioning. For this, we analyzed total microbial gene abundance and estimated overall enzyme activity in aggregate size fractions. Furthermore, the potential link between OC (organic carbon) stabilization and bioactivity among the aggregate fractions were quantitatively assessed using the parameters of carbon-scaled or gene abundance-scaled respiration and enzyme activity. Finally, the evolution of such an interlink was traced by comparing the soils of sequential lengths of rice cultivation up to 700 years in a soil chronosequence.

2.2 Site and soils

The study reported here examined a series of soils along a paddy chronosequence, which were shifted from tidal marsh to rice cultivation for different lengths of time on a coast land area located in Cixi Municipality, Zhejiang Province, China (Fig. 1). Located in the south bank of Hangzhou Bay, the area was within the typical northern subtropical monsoon climate for eastern China, with a mean annual temperature of 17.7 °C and precipitation of 1367 mm during 2004–2014 (http://cdc.nmic.cn/home.do). In this area, coastal tidal marsh has been increasingly reclaimed for rice production, with dyke establishments at different historical stages for the last 2000 years. These soils allowed for chronosequence



Figure 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.

studies for rice soil development, including a pedological characterization by Cheng et al. (2009) and a morphological, mineralogical and microbiological investigation by Kölbl et al. (2014).

Individual soils of the chronosequence were identified based on dyke establishment history recorded in Cixi County Annals (with brief information in Chinese available at www. cixi.gov.cn), including an initial tidal marsh soil before rice cultivation (P0), and rice soils of P50, P100, P300 and P700 shifted for rice cultivation, respectively, 50, 100, 300 and 700 years before present (Fig. 1). These soils were separated from each other at a distance of no more than 40 km in nearly the same topography. All of the soils developed on comparable parent materials of paleo-deposit from the Yangtze River, with a particle composition of silt (75–84%) followed by clay but low in sand content (Chen and Zhang, 2009). Soil texture ranged from silty loam to silty clay loam. The clay mineral assemblage consisted of illite (40-50%), chlorite (20-30%) and kaolinite (10-20%) with a minor amount of smectite and quartz (G. Zhang et al., 2010).

The cropping system in this area followed a traditional summer rice–winter rape rotation. Rice production management on the chronosequence was relatively consistent across the sites, with similar cultivars and management practices including crop protection, irrigation and fertilization (Cheng et al., 2009). The influence of soil salinity on rice production could occur in the early stage of rice cultivation on the reclaimed tidal marsh though the groundwater table was already low enough without restricting rice growth (Kölbl et al., 2014). The directional evolution of soil properties (Cheng et al., 2009; Chen et al., 2011), neo-formation of clay minerals, particularly of iron/manganese oxyhydrates (Wissing et al., 2011, 2013; Kölbl et al., 2014), and interaction of organic matter with minerals (Wissing et al., 2011, 2013) as well as organic carbon pools (Wissing et al., 2011; Wang et al., 2015) have already been characterized.

2.3 Soil sampling

Topsoil (0–15 cm in depth) samples of the five individual soils of the chronosequence were used in the study. To avoid influence of fresh straw material on soil aggregates and carbon substrates in soil samples, the sampling was done in early November 2011, when the soil was moist following rice harvest. While collecting a soil sample from the field, an undisturbed soil core was obtained using an Eijkelkamp soil core sampler (Agrisearch Equipment, Giesbeek, the Netherlands), whereas a bulk-soil sample was obtained using a stainless steel shovel. For each individual soil, a topsoil was collected in triplicates from three adjacent individual fields. Finally, all soil samples were shipped to a lab within 2 days after sampling, and stored at 4 °C before soil analysis in the following 2 weeks. The basic properties of the studied soils are listed in Table 1. Changes of OC stability and microbial activity of bulk soil along the chronosequence has been assessed in our previous study (Wang et al., 2015, and Liu et al., 2016a, b).

2.4 Particle size fractionation of soil aggregates

Soil aggregates were obtained from the undisturbed soil cores by dispersion in water with low-energy sonication, without chemical dispersing agents. Particle size fractions of water-stable aggregates were separated with a modified procedure described by Stemmer et al. (1998) and later on followed by Stemmer et al. (1999), Sessitsch et al. (2001)

Soil	pH (H ₂ O)	SOC (g kg ⁻¹)	Total N $(g kg^{-1})$	$\frac{BD}{(g cm^{-3})}$	$\begin{array}{c} \text{CEC} \\ (\text{cmol}\text{kg}^{-1}) \end{array}$	Fed $(g kg^{-1})$
P0	$8.62\pm0.07a$	$6.32\pm0.58d$	$0.79\pm0.02c$	$1.31\pm0.05a$	$6.32\pm0.34c$	$1.76\pm0.02c$
P50	7.84 ± 0.04 b	$15.96\pm0.66c$	$1.81 \pm 0.06b$	$1.13\pm0.03b$	$12.82\pm0.06\mathrm{b}$	$1.96 \pm 0.01b$
P100	$6.39\pm0.05d$	$17.07\pm0.49\mathrm{b}$	$2.06\pm0.09a$	1.06 ± 0.04 b	$12.54\pm0.12b$	$2.04\pm0.04a$
P300	$6.40\pm0.03d$	$17.97\pm0.81\mathrm{b}$	$2.09\pm0.08a$	$1.07\pm0.07\mathrm{b}$	$13.78\pm0.26a$	$2.08\pm0.05a$
P700	$6.65\pm0.08\mathrm{c}$	$21.07 \pm 1.21 a$	$2.14\pm0.06a$	$1.06\pm0.05b$	$12.97\pm0.27b$	$1.71\pm0.02c$

Table 1. Basic properties of the soils in the chronosequence (mean \pm SD, n = 3).

SOC, soil organic carbon; BD, bulk density; CEC, cation exchange capacity; Fed: dithionate-extractable iron oxyhydrates.

and Kandeler, et al. (1999, 2000, 2006). A portion of field moist soil core (50 g equivalent dry weight), cleaned of discernible straw material, was placed into a glass beaker in 100 mL of distilled water. The soil mass was dispersed using a low-energy ultrasonic disaggregator (Zhixin, JVD-650, Shanghai, China) with an output energy of 170 Jg^{-1} for 5 min. A coarse-sand-sized fraction of aggregates with a diameter range of 2000-200 µm was separated by wet sieving and the fine-sand-sized fraction of 200-20 µm was subsequently obtained by sedimentation after siphoning. The remainder was centrifuged to collect the silt-sized fraction of 20-2 µm and the supernatant was centrifuged again to collect the clay-sized fraction of $\leq 2 \,\mu m$. The samples of the obtained size fractions were freeze-dried (Thermo, Modulyo D-230, NY, USA) and then stored at -70 °C. Here, waterstable macroaggregates larger than 2000 µm were not taken into consideration as they were insignificant in rice soils under prevailing water submergence with long-term hydroagric management (Deng and Xu, 1965). The classes of the size fractions were kept basically consistent with our previous studies (L. Li et al., 2007; Z. P. Li et al., 2007; Zheng et al., 2007; Pan et al., 2008; Chen et al., 2014).

2.5 Organic carbon pool and FTIR spectroscopy analysis

Soil organic carbon (SOC) and total nitrogen (TN) of the separated fractions 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 (KMnO₄), following a procedure described by Blair et al. (1995). Microbial biomass carbon (MBC) was measured using the chloroform fumigation-extraction method. The MBC content was estimated as the difference of OC between the unfumigated and fumigated samples using the conversion factor of 0.45, following Joergensen (1996). Herein, MBC of the coarse-sand fractions of *P*0 soil was not provided due to the very small sample obtained via the sonication and separation procedure.

The chemical composition of the organic carbon in the particle size fractions was characterized with FTIR spec-

troscopy 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 pressure 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 $400-4000 \text{ cm}^{-1}$. In all cases, 20 scans per sample were recorded, averaged for each spectrum and corrected against the spectrum with ambient air as background. Following Ellerbrock et al. (1999) and Cocozza et al. (2003), the characteristic vibration peak at $1050 \,\mathrm{cm}^{-1}$ was assigned to polysccharides, those at 1630 cm⁻¹ to aromatic compounds and those at 2927 cm⁻¹ to aliphatic compounds as well as those at $3405 \,\mathrm{cm}^{-1}$ to phenols. Subsequently, a general semi-quantification of three major functional OC groups of polysaccharides, aliphatic and aromatic compounds was performed following Tivet et al. (2013). Nevertheless, it was not able to quantify potential contributions from organic Si or P compounds to the intensity of the band assigned to polysaccharides (Mao et al., 2008; Tivet et al., 2013). All the obtained FTIR spectra are given in Fig. S1 in the Supplement.

2.6 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 a double sticker and coated with gold using Hummer sputter coating equipment (Anatech Ltd., Union City, CA, USA). Images were captured by automatic image capturing software (Hitachi Science Systems LTD., Schaumburg, IL, USA). Magnifications and linear scale are indicated in the micrographs.

2.7 DNA extraction, microbial gene abundance and diversity analysis

A portion (0.45 g) of a PSF (particle size fraction) sample stored at -70 °C was used for DNA (deoxyribonucleic

acid) extraction with PowerSoilTM DNA Isolation Kit (Mo-Bio, USA), following the manufacturer's 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 bioassay.

Quantitative real-time PCR (polymerase chain reaction) assay was performed on a 7500 real-time PCR system (Applied Biosystems, USA) using SYBR Green (an asymmetrical cyanine dye commonly used as a nucleic acid stain in molecular biology) 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 in the real-time PCR assay.

PCRs were carried out on all PSF 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 (terminal restriction fragment length polymorphism) analysis is described in the Supplement.

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

$$H' = \Sigma \mathrm{Pi}(\ln \mathrm{Pi}),\tag{1}$$

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

2.8 Soil enzyme activity

Soil enzyme activities relevant to cycling of C, N and P in soils were measured. In detail, activities of 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 as the normalized enzyme activity (NEA) of an individual fraction, which was estimated with the following equation:

$$x'_{i} = \frac{x_{i}}{\sum_{i=1}^{n} x_{i}} (i = 1, 2, \dots, 5),$$
(2)

where *i* is the number of each soil sample (*P*0, *P*50, *P*100, *P*300, *P*700), *x* the enzyme activity and x' the normalized enzyme activity of each soil sample. Subsequently, an arithmetic mean of enzyme activity of each sample was obtained for the NEA.

2.9 Soil respiration

For assessing microbial use of carbon in aggregates of different size fractions, soil respiration was determined by measuring CO₂ production following an anaerobic laboratory incubation protocol by Zheng et al. (2007). A size fraction sample (20 g equivalent dry weight) was placed into a 125 mL glass jar and submerged with 40 mL of distilled water before being gently mixed. The jar was then sealed with a butyl rubber stopper and two Teflon tubes for gas sampling and N₂ circulation were inserted into the stopper. The headspace was repeatedly evacuated and flushed with N2 gas into the jar at a rate of 300 mL min⁻¹ for 30 min, which created an anaerobic condition. The jars with soil slurry were incubated in an incubator (LRH-250-S, Medicine Machinery Co. Ltd, Guangdong, China) at 25 ± 1 °C for 37 days. During incubation, a 0.25 mL sample of the headspace gas was collected with a syringe every 5 days starting the third day after incubation was initiated. After each gas sampling, N2 gas was again flushed into the jar at a rate of 300 mL min^{-1} for 30 minto remove all the emitted gas in the jar. CO2 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 A. Zhang et al. (2010), the determination was done with an oven temperature of 80 °C and a FID temperature of 200 °C, with N₂ as the carrier gas at a flow rate of 40 mLmin^{-1} and a makeup gas mixture of H₂ 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 total CO₂ 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 were analyzed using EXCEL 2013 and expressed as mean \pm standard deviation of triplicate samples. The significant differences between particle size fractions in a single soil and between soils of a single particle size fraction were statistically analyzed by one-way ANOVA with Tukey's test, using the SPSS software package 20.0. A statistical significance was defined at a 95 % confidence level.

3 Results

3.1 Organic carbon characterization in aggregate size fractions

The fine-sand- $(200-20 \,\mu\text{m})$ and silt-sized $(20-2 \,\mu\text{m})$ fractions together accounted for up to 80 % of bulk soil across all soils (Table 2). However, the proportion of coarse-sand-sized $(2000-200 \,\mu\text{m})$ macroaggregates and clay-sized (<2 μ m) fine aggregates increased with prolonged rice cultivation over the



Figure 2. Scanning electron microscopy images of aggregates separated with sonication dispersion in water from a topsoil sample of the studied chronosequence. *P0*, *P50*, *P100*, *P300* and *P700* represent, respectively, the uncultivated mash soil and the rice soils cultivated for 50, 100, 300 and 700 years.

chronosequence. As indicated in Fig. 2, soil aggregates from the initial marsh soil (*P*0) were sharply edged single individual minerals and mostly uncovered with clear surfaces; however, in rice soils with increasing rice cultivation lengths, soil aggregates became increasingly round, loosely assembled fine minerals but covered with more or less amorphous materials. Particularly in *P*700, soil aggregates were seen in large size, very loosely assembled unclearly shaped mineral particles with amorphous materials, with some particulate organic matter including some fungal hyphae on the aggregate surface (magnified *P*700 image in Fig. 2).

Soil properties of SOC, total N and LOC were significantly different among the size fractions and between the uncultivated and rice soils (Table 3). SOC, LOC and total N pools all generally followed the order coarse-sand-sized fractions > clay-sized fractions > fine-sand-sized fractions > siltsized fractions in a single soil. With the exception of the fine-sand fraction, all these pools were greater in rice soils than in the uncultivated marsh soil. Particularly, SOC of rice soils was enriched mostly in the coarse-sand-sized macroaggregates, moderately in the clay-sized fractions and fairly in the fine-sand-sized fractions, but were depleted in the siltsized fraction, respectively in a range of 41–61, 20–24, 8.5– 20 and 10–11 g kg⁻¹. However, the C / N ratio showed a significantly decreasing trend with the decreasing size of the aggregate fractions across the chronosequence. The ratio of LOC to SOC, an indicator of C lability in soils, followed a

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

Table 2. Particle size distribution (%) of aggregates of the studied chronosequence soils. Lowercase letters indicate a significant (p < 0.05) difference between soils for a single fraction in a column.

decreasing order, i.e., coarse-sand fractions > fine-sand fractions > silt- and clay-sized fractions.

The FTIR spectra showed sharp peaks generally at a vibration of $1050\,\mathrm{cm}^{-1}$ (assigned to polysaccharides) but broad shoulders at a vibration of 3405 cm^{-1} (assigned to aromatic carbon across the aggregate fractions) (Fig. S1). There was a clear trend of decreasing intensity in the polysaccharide peaks but increasing shoulder intensity of aromatic carbon in a single fraction, with increasing rice cultivation. The semiquantitative data of carbon chemical groups obtained with FTIR analysis is presented in Table 4. Herein, carbon groups in aggregates were dominated by polysaccharides (60-70%), followed by aromatic carbon (20-39%) with a small contribution (0.6–3.7%) of aliphatic carbon in a single fraction. The relative proportion of aromatic carbon was lower and polysaccharide carbon higher in the silt fractions as compared to the other fractions. Consequently, the estimated SOC chemical recalcitrance (ratio of aromatic to polysaccharide C) was the lowest in the silt fractions, followed by the fine-sand fractions, and the highest in the coarse-sand and clav fractions.

Recalcitrance of SOC of in a single fraction was generally lower in uncultivated marsh soil than in the shifted rice soils, but tended to increase with increasing length of rice cultivation. The fine-sand fraction, bearing the majority of total SOC for the soil (Tables 2 and 3), had a moderate carbon recalcitrance but the coarse-sand fraction had similar carbon recalcitrance but a higher carbon lability and higher C / N ratio. Compared to other fractions, this indicated a greater existence of potentially available carbon pools (POC, for example) in the coarse-sand fraction,.

3.2 Microbial biomass carbon, microbial gene abundance and diversity

MBC was the highest in the coarse-sand fraction of macroaggregates and the lowest in the clay-sized fraction of fine microaggregates over the sequence (Table 3). Generally, the microbial quotient (MQ) was not significantly different between the coarse-sand-, fine-sand- and silt-sized fractions but was significantly higher than the clay-sized fractions.

The microbial DNA content (equivalent to biomass) and gene abundance of microbial communities in the fractions

over the chronosequence are shown in Table 5. Total DNA ranged from $1.57 \,\mu g \, g^{-1}$ in the silt fraction to $4.00 \,\mu g \, g^{-1}$ in the clay fraction of the tidal marsh and from $4.35 \,\mu g \, g^{-1}$ in the fine-sand fraction to $35.33 \,\mu g \, g^{-1}$ in the coarse-sand fraction in rice soils. Fungal ITS gene copies were generally higher in the coarse-sand fractions, decreasing with the size of aggregate fractions. Although there was a general bimodal pattern among the particle size fractions, total DNA, bacterial and archaeal 16S rRNA gene copy numbers were higher in both coarse-sand and clay fractions, compared to other fractions across the chronosequence. Clearly, microbial gene abundance was dominated by bacterial, with archaeal and fungal gene abundance respectively one and two orders of magnitude lower than bacterial copy numbers across the fractions. The ratio of fungal to bacterial gene abundance generally decreased while that of archaeal to bacterial gene abundance increased with decreasing size of the aggregate fractions.

Over the studied chronosequence, DNA contents of a fraction were several folds higher in the rice soils as compared to that of the initial tidal marsh. Accordingly, gene copy numbers of microbial communities from a fraction were much higher in rice soils than in the initial tidal marsh. Bacterial and fungal abundance in coarse-sand, fine-sand, silt and clay fractions in P50 was increased by 688, 72, 498 and 622 %, and 74, 149, 7 and 152 %, respectively over P0. A mean increase in the rice soils cultivated for over 100 years over P0 in bacterial gene copy numbers was seen statistically significant, with percentages ranging from 73 to 437, 0.4 to 67, 225 to 246 and 147 to 201%, respectively, in the coarse-sand, fine-sand, silt and clay fractions. Comparatively, changes in fungal gene abundance of aggregates were much smaller across the soils, particularly in the silt- and clay-sized fractions. In contrast, archaeal gene abundance in a single fraction across the soils increased over P0 consistently with prolonged rice cultivation, although the increase was smaller in fine-sand- and silt-sized fractions. For the coarse-sand fraction only, both the fungal-to-bacterial ratio and the archaealto-bacterial ratio tended to increase with increasing rice cultivation lengths.

Data of the microbial Shannon diversity index of the four size fractions of the chronosequence soils are presented in

Table 3. SOC, total N and LOC in $g kg^{-1}$ and SMBC in $mg kg^{-1}$ of the size fractions (PSFs) of the soil chronosequence. Different capital and lowercase 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.

PSF	Soil	SOC	Total N	LOC	SMBC
Coarse sand	P0 P50	11.07 ± 1.20 Ad 53.44 ± 1.00 Ab	1.04 ± 0.11 Ad 4.15 ± 0.40 Aa	6.22 ± 0.18 Ac	not determined 794.7 ± 47.0 A c
$(2000-200 \mu m)$	F 30	$33.44 \pm 1.09 \text{AU}$	4.13 ± 0.49 Aa	27.03 ± 1.01 Aa	$1052 \pm 72.7 \text{ AL}$
	P100	$41./4 \pm 1.31AC$	$3.37 \pm 0.38 \text{AD}$	19.09 ± 1.10 AD	1052 ± 73.7 AD
	P 300	$40.64 \pm 1.5 / Ac$	2.72 ± 0.12 Ac	$18.80 \pm 1.45 \text{Ab}$	1385 ± 88.1 Aa
	P700	60.79 ± 1.88 Aa	$4.43 \pm 0.22 \text{Aa}$	28.64 ± 1.90 Aa	1480 ± 166.2 Aa
Fine Sand	P0	$9.90 \pm 0.43 \mathrm{Ac}$	$1.01\pm0.14\mathrm{Ac}$	$4.34\pm0.14Bb$	$188.0\pm8.0\mathrm{Ac}$
(200–20 µm)	P50	8.45 ± 0.27 Cc	0.73 ± 0.11 Dd	3.66 ± 0.57 Cb	$309.2 \pm 16.5 Bb$
	P100	$16.48\pm0.41\text{Cb}$	1.57 ± 0.14 Cb	7.36 ± 0.32 Ca	441.1 ± 13.4 Ba
	P300	15.16 ± 1.45 Cb	1.51 ± 0.13 Bb	7.03 ± 0.30 Ca	$445.9\pm28.2\mathrm{Ba}$
	P700	$19.86 \pm 1.11 \text{Ca}$	$1.81\pm0.12Ca$	$7.99\pm0.65\mathrm{Ba}$	$449.9\pm25.9Ba$
Silt	P0	$5.13\pm0.19\text{Bb}$	$0.52\pm0.14Bd$	$1.53\pm0.13\text{Db}$	$166.7 \pm 4.5 \text{Ad}$
(20–2 µm)	P50	$10.73\pm0.55\mathrm{Ba}$	1.20 ± 0.11 Cb	$4.50\pm0.13\mathrm{Ca}$	$296.2\pm15.0\mathrm{Bc}$
	P100	$10.13\pm0.44 \mathrm{Da}$	$1.15\pm0.09\mathrm{Cc}$	$4.10\pm0.26\mathrm{Da}$	$287.0\pm2.7\mathrm{Cc}$
	P300	11.37 ± 0.58 Da	1.33 ± 0.11 Ba	$4.39\pm0.29 \text{Da}$	$392.1 \pm 15.0 \text{Ba}$
	P700	$10.57\pm0.43 \text{Da}$	$1.11\pm0.08 \mathrm{Dc}$	$3.95\pm0.69\mathrm{Ca}$	$348.3 \pm 10.5 \text{Cb}$
Clay	<i>P</i> 0	$9.29 \pm 0.29 \mathrm{Ac}$	1.17 ± 0.15 Ad	$2.96\pm0.27\mathrm{Cc}$	$155.6 \pm 18.1 \text{Ac}$
(<2 µm)	P50	$19.80 \pm 1.47 \text{Bb}$	$2.27\pm0.14\mathrm{Bc}$	$7.99\pm0.28\mathrm{Bb}$	$284.9 \pm 19.7 \text{Bb}$
	P100	$22.94 \pm 1.43 \mathrm{Ba}$	$2.70\pm0.12\mathrm{Bb}$	$9.19\pm0.35\mathrm{Ba}$	$279.4 \pm 5.0 \mathrm{Cb}$
	P300	23.45 ± 1.46 Ba	2.92 ± 0.12 Aa	$9.36 \pm 0.40 \text{Ba}$	324.8 ± 13.1 Ca
	P700	$24.36 \pm 1.65 Ba$	$2.73\pm0.16Bb$	$9.05\pm0.47Ba$	$325.7\pm8.1\mathrm{Ca}$

Abbreviation: SOC: soil organic carbon, LOC: labile organic carbon, SMBC: soil microbial biomass carbon.

Table 4. Relative proportion (%) of carbon chemical groups and carbon recalcitrance (ratio of aromatic to polysaccharide carbon) in size fractions by FTIR analysis. Different capital and lowercase letters indicate a significant (p < 0.05) difference respectively between fractions of a single soil, and between soils for a single fraction.

Size fraction	Soil	Total aromatic	Aliphatic	Polysaccharide
Coarse sand	P0	$28.58 \pm 1.41 \text{Bc}$	$0.03 \pm 0.00 \mathrm{Ac}$	71.41±5.76ABa
(2000–200 µm)	P50	$38.55 \pm 5.73 \text{Aab}$	$0.50\pm0.09\mathrm{Aa}$	60.94 ± 2.54 Cb
	P100	$34.43 \pm 3.78 \text{ABab}$	$0.27\pm0.03\mathrm{Ab}$	$65.31 \pm 4.72 \text{Bab}$
	P300	$32.67\pm0.78 \text{ABb}$	$0.28\pm0.04\mathrm{Ab}$	$67.04 \pm 4.66 \text{BCab}$
	P700	$38.47 \pm 1.59 \text{Aa}$	$0.37\pm0.03Ab$	$61.17 \pm 4.30 \text{Cb}$
Fine sand	P0	$26.30\pm1.57\mathrm{Ba}$	$0.05\pm0.01 Ab$	73.64±4.83ABa
(200–20 µm)	P50	$26.98 \pm 1.15 \text{Ba}$	$0.04\pm0.00\mathrm{Bb}$	$72.98 \pm 4.43 \text{ABa}$
	P100	$29.62 \pm 1.07 \mathrm{Ba}$	$0.13\pm0.03\mathrm{Ba}$	$70.24\pm3.47\mathrm{ABa}$
	P300	$29.60 \pm 1.42 \text{Ba}$	$0.07\pm0.02\mathrm{Bb}$	$70.32\pm4.60\mathrm{ABa}$
	P700	$29.33 \pm 1.28 \text{Ba}$	$0.17\pm0.02Ba$	$70.51 \pm 4.09 Ba$
Silt	P0	$23.22\pm1.27\mathrm{Ca}$	$0.01\pm0.00\mathrm{Ba}$	76.76±3.81Aa
(20–2 µm)	P50	$23.98 \pm 1.50 \mathrm{Ca}$	$0.01\pm0.00\mathrm{Ca}$	$76.02\pm4.29\mathrm{Aa}$
	P100	$22.61 \pm 1.32 Ca$	$0.00\pm0.00\mathrm{Db}$	77.37 ± 4.73 Aa
	P300	$23.61 \pm 1.14 Ca$	$0.00\pm0.00\mathrm{Db}$	$76.39 \pm 4.21 \text{Aa}$
	P700	$19.87\pm0.83\mathrm{Cb}$	$0.00\pm0.00\mathrm{Db}$	$80.14\pm3.87\mathrm{Aa}$
Clay	<i>P</i> 0	$33.78 \pm 1.69 \text{Aa}$	$0.00\pm0.00\mathrm{Bb}$	$66.20 \pm 3.2B2a$
$(< 2 \mu m)$	P50	$35.46 \pm 1.36 \text{Aa}$	$0.03\pm0.00\mathrm{Ba}$	$64.52 \pm 4.23 \text{Ba}$
	P100	$36.10\pm1.74\mathrm{Aa}$	$0.04\pm0.01\mathrm{Ca}$	$63.85\pm4.57\mathrm{Ba}$
	P300	$36.02\pm1.72\mathrm{Aa}$	$0.03\pm0.01\mathrm{Ca}$	63.96 ± 4.65 Ca
	P700	$36.86 \pm 1.88 \text{Aa}$	$0.05\pm0.01\mathrm{Ca}$	$63.08\pm3.73\mathrm{Ca}$

Table 5. DNA content ($\mu g g^{-1}$), and the copy numbers of bacterial (BA; copies $\times 10^9 g^{-1}$), fungi (FA; copies $\times 10^7 g^{-1}$) and archaeal (ArA; copies $\times 10^8 g^{-1}$) of the size fractions. Different capital and lowercase letters in a single column indicate a significant (p < 0.05) difference respectively between fractions of a single soil, and between soils for a single fraction.

Fraction	Soil	DNA	BA	FA	ArA
Coarse sand	P0	$3.32 \pm 0.07 \mathrm{Ae}$	$5.86\pm0.75 Ad$	$8.92 \pm 1.50 \text{Ab}$	$0.81\pm0.03\mathrm{Ce}$
(2000–200 µm)	P50	$35.33\pm0.42\mathrm{Aa}$	$46.18\pm9.21\mathrm{Aa}$	$15.50\pm2.60\mathrm{Aa}$	$6.37\pm0.81Bd$
	P100	$24.72\pm2.14\mathrm{Ac}$	$31.45\pm5.79\text{Ab}$	$10.49\pm0.87 Ab$	$13.54\pm0.73\mathrm{Bc}$
	P300	$16.20\pm0.05 Ad$	$10.12 \pm 2.39 \mathrm{Ac}$	$8.12\pm0.32Ab$	$16.01 \pm 1.06 \mathrm{Ab}$
	P700	$31.95\pm0.64Ab$	$14.25\pm1.03\text{Ac}$	$9.40\pm0.71 Ab$	$21.17\pm0.48Ba$
Fine sand	P0	$3.63\pm0.28Ab$	$4.90\pm0.45 Ab$	$3.23\pm0.27\mathrm{Bc}$	$2.83\pm0.18\mathrm{Ac}$
(200–20 µm)	P50	$4.35\pm0.40\text{Db}$	$8.42\pm1.75\mathrm{Ba}$	$8.04\pm0.25\mathrm{Ba}$	$5.27 \pm 1.12 Bd$
	P100	$13.63\pm3.30\mathrm{Ba}$	7.75 ± 1.18 Ca	$8.37\pm0.67\mathrm{Aa}$	8.16 ± 2.27 Cab
	P300	$9.97\pm0.33\mathrm{Ba}$	$4.92\pm1.10\text{Bb}$	$6.23\pm0.23Bb$	3.57 ± 0.24 Cb
	P700	$12.83\pm0.33\mathrm{Ca}$	$8.16 \pm 1.64 Ba$	$2.43\pm0.19\text{Cd}$	$7.68\pm0.66\mathrm{Ca}$
Silt	P0	$1.57\pm0.28\mathrm{Bc}$	$1.78\pm0.15\mathrm{Bc}$	$3.98\pm0.57\mathrm{Ba}$	$0.29\pm0.02 \text{Dd}$
(20–2 µm)	P50	$10.02\pm1.58\mathrm{Ca}$	$10.64\pm2.95\mathrm{Ba}$	$4.25\pm0.30\mathrm{Ca}$	$2.48\pm0.44\mathrm{Cc}$
	P100	$8.25\pm0.12 Cab$	$5.78\pm0.36\mathrm{Cb}$	$2.17\pm0.20\mathrm{Bb}$	$8.65\pm0.09\mathrm{Ca}$
	P300	7.78 ± 0.31 Cb	$5.91\pm0.81\text{Bb}$	$2.47\pm0.45Bb$	$6.60\pm0.27\mathrm{Bb}$
	P700	$9.25\pm0.64 \text{Da}$	$6.16\pm0.29Bb$	$3.68\pm0.19Ba$	$9.44 \pm 1.41 \text{Ca}$
Clay	<i>P</i> 0	4.00 ± 1.89 Ad	$5.27 \pm 0.61 \mathrm{Ac}$	0.52 ± 0.03 Cd	$1.83 \pm 0.10 \mathrm{Bc}$
$(< 2 \mu m)$	P50	$17.62\pm0.26Bb$	$38.05\pm4.92\text{Aa}$	$1.31\pm0.07 \mathrm{Dc}$	$14.08\pm2.13Ab$
	P100	$16.20\pm0.38Bb$	$15.86\pm3.31Bb$	$1.94\pm0.30\text{Bb}$	$44.66\pm13.68\mathrm{Aa}$
	P300	$11.17\pm0.90\mathrm{Bc}$	$13.03\pm2.58Ab$	$1.39\pm0.40\mathrm{Cb}$	$22.16\pm6.17\mathrm{Aa}$
	P700	$25.67\pm0.57\mathrm{Ba}$	$15.63\pm2.24Ab$	$2.48\pm0.31Ca$	36.00 ± 3.82 Aa

Table S1 of the Supplement. In detail, a Shannon index of the bacterial community was much higher in the coarse-sand fraction and, to a lesser extent, in the clay-sized fraction than in the fine-sand and silt fractions across the chronosequence. Fungal community Shannon indices generally decreased with the size of aggregate fractions. In contrast, there were no significant changes in the archaeal Shannon index among the size fractions across the sequence. Generally, the Shannon diversity index of the microbial communities in a single fraction was much higher in the rice soils than in the uncultivated tidal marsh.

3.3 Enzyme activity and basal respiration

All analyzed enzyme activities (Table S2) were increased in the rice soils over the levels measured for the initial tidal marsh soil. Furthermore, NEA was 0.07 in the coarse-sand fraction and 0.10 in the fine-sand fraction as well as 0.07 and 0.14 in the silt and clay fractions in *P*0. In contrast, NEA was 0.18–0.30 in the coarse-sand fraction and 0.12–0.30 in the fine-sand fraction, but 0.17–0.30 in silt and 0.19–0.24 in clay fraction of the rice soils. Moreover, NEA in a single size fraction showed a significantly increasing trend with prolonged rice cultivation (Table 6).

Soil respiration of a single fraction was much higher for the rice soils than for the marsh soil, and in the coarse-sandsized macroaggregate fraction than in the silt and fine-sand fractions over the chronosequence (Table 6). In detail, soil respiration was 662 and 565 mg $\text{CO}_2 \text{ kg}^{-1}$ in the coarse and fine-sand fractions, and 298 and 496 mg $\text{CO}_2 \text{ kg}^{-1}$ in the silt and clay fractions, respectively, in *P*0, whereas in rice soils, soil respiration ranged between 1588 and 2914 mg $\text{CO}_2 \text{ kg}^{-1}$ in the coarse sand, 1076 and 1256 mg $\text{CO}_2 \text{ kg}^{-1}$ in the fine-sand, 740 and 1354 mg $\text{CO}_2 \text{ kg}^{-1}$ in the silt and 1028 and 1434 mg $\text{CO}_2 \text{ kg}^{-1}$ in the clay fractions of the rice soils. Basal respiration in a single size fraction generally increased with rice cultivation length (Table 6).

Using the data in Table 3, the estimated RQ (the ratio of respired carbon to total SOC) and qCO_2 (the ratio of respired carbon to MBC) were seen as variable across the size fractions and among the soils (Table S3). Generally, RQ was lower both in coarse-sand- and clay-sized fractions than in fine-sand- and silt-sized fractions. The value of qCO_2 was the lowest in the coarse-sand-sized fraction but the highest in the clay-sized fraction. While there was no overall trend of RQ and qCO_2 in a single fraction between the marsh soil and rice soils, both RQ and qCO_2 in a single fraction followed more or less a decreasing trend with increasing length of rice paddy management.

Table 6. Normalized enzyme activity (NEA) and soil respiration (mg CO₂ kg⁻¹) of aggregate size fractions of the chronosequence soils. Different capital and lowercase letters in a single column indicate a significant (p < 0.05) difference respectively between fractions of a single soil, and between soils for a single fraction.

Size fraction	Soil	NEA	Basal respiration
Coarse sand (2000–200 µm)	P0 P50 P100 P300 P700	$\begin{array}{c} 0.07 \pm 0.01 \mathrm{Bc} \\ 0.28 \pm 0.03 \mathrm{Aa} \\ 0.18 \pm 0.01 \mathrm{Ab} \\ 0.18 \pm 0.01 \mathrm{Bb} \\ 0.30 \pm 0.05 \mathrm{Aa} \end{array}$	$662 \pm 66Ac$ $2345 \pm 805Aab$ $2283 \pm 506Aab$ $1588 \pm 309Ab$ $2914 \pm 190Aa$
Fine sand (200–20 µm)	P0 P50 P100 P300 P700	$\begin{array}{c} 0.10 \pm 0.01 \text{Bc} \\ 0.12 \pm 0.03 \text{Cc} \\ 0.21 \pm 0.03 \text{Ab} \\ 0.27 \pm 0.03 \text{Aa} \\ 0.30 \pm 0.02 \text{Aa} \end{array}$	$565 \pm 153 ABb$ $1076 \pm 139Ba$ $1252 \pm 103Ba$ $1256 \pm 096Aa$ $1234 \pm 143Ba$
Silt (20–2 μm)	P0 P50 P100 P300 P700	$\begin{array}{c} 0.07 \pm 0.01 \text{Bd} \\ 0.21 \pm 0.02 \text{Bb} \\ 0.17 \pm 0.01 \text{Ac} \\ 0.25 \pm 0.02 \text{Ab} \\ 0.30 \pm 0.02 \text{Aa} \end{array}$	$\begin{array}{c} 298 \pm 053 \mathrm{Cc} \\ 740 \pm 258 \mathrm{Bb} \\ 1246 \pm 063 \mathrm{Ba} \\ 1256 \pm 071 \mathrm{Aa} \\ 1354 \pm 095 \mathrm{Ba} \end{array}$
Clay (<2 µm)	P0 P50 P100 P300 P700	$\begin{array}{c} 0.14 \pm 0.01 Ac \\ 0.19 \pm 0.02 Bb \\ 0.20 \pm 0.02 Aab \\ 0.24 \pm 0.02 Aa \\ 0.23 \pm 0.01 Ba \end{array}$	$\begin{array}{c} 496 \pm 053 Bb \\ 1425 \pm 430 Aa \\ 1401 \pm 289 Aa \\ 1028 \pm 226 Aa \\ 1434 \pm 196 Ba \end{array}$

4 Discussions

4.1 Carbon accumulation vs. stabilization in soil aggregates

In this study, the level of SOC, soil respiration and microbial gene abundance/diversity differed significantly among different size fractions of water-stable aggregates from the chronosequence. Similar to the findings of L. Li et al. (2007) and Zheng et al. (2007), SOC accumulated predominantly in the coarse-sand-sized and moderately in the clay-sized fractions, but was depleted in the silt-sized aggregate fractions (Table 3). As shown in Fig. 3a, SOC content in a fraction was positively linearly correlated to organic carbon recalcitrance as determined by the FTIR analysis (Fig. S1; Table 4), whereas respiration quotient as a rate indicator of carbon turnover for microbial energy use (Kennedy and Papendick, 1995) indicated a significant negative logarithm function of SOC level (Fig. 3b). The divergence of the uncultivated marsh soil to the rice soils could be attributed to the land use impact as a determinant factor for SOC turnover (Qian et al., 2013). The correlations hereby could suggest the accumulation of SOC in soil aggregates related to chemical stabilization against biological use for their energy supply, which had been traditionally considered as an inherent carbon sequestration with selective persistence of non-



Figure 3. Correlation of carbon recalcitrance (the ratio of aromatic to polysaccharide and aliphatic carbon) (**a**) and respiration quotient (**b**) to organic carbon level with of the particle size fractions of topsoil of the chronosequence soils.

degradable or residue organic carbon in soils (Lützow et al., 2006; Mikutta et al., 2006).

Calculations using the SOC contents (Table 3) and the fraction mass percentage (Table 2) of a single fraction showed that the amounts of SOC allocated only in the coarse-sand- and clay-sized fractions were closely correlated to the bulk SOC contents (Table 1) of the soils (Fig. S2). This was in general agreement with the findings for similar rice paddy soils from an adjacent area (Pan et al., 2008). The increased allocation of SOC to clay-sized fraction could be attributed to the accelerated formation of clay and hydroxyl Fe/Mn minerals (Wissing et al., 2013) due to long-term paddy management (Kölbl et al., 2014).

Furthermore, the enrichment index (EI) of SOC, calculated with SOC content in a fraction divided by that in the bulk soil, was higher than 1 in both the coarse-sand- and claysized fractions but clearly much lower than 1 in silt fractions. When plotting the EI values against LOC content (Table 3) for all the fractions (Fig. 4), enrichment of SOC was seen as relevant to LOC pools in the fractions. Moreover, the EI values were weakly positively correlated significantly for both the F / B ratio of gene abundance (Table 5) and the SOC recalcitrance (Table 4). These data indicated that accumulation of labile carbon, mostly POC, contributed significantly to the SOC pools in coarse-sand-sized macroaggregates (Zhou et al., 2008), although the apparent recalcitrance was in a similar range to that in clay fractions (Table 4). Light fraction or macroaggregates in soil were considered to be relatively rich



Figure 4. Correlation of organic carbon enrichment index (SOC content in a fraction divided by SOC content of the bulk soil) to content of labile carbon of size fractions of soil aggregates of the chronosequence soils. The open circles are those fractions from the uncultivated marsh soil (*P*0). Closed circles above or below the black long dashed line represent OC enrichment or depletion in a fraction.

in new or labile carbon substrates, more or less related to root fungal activities, and were largely physically protected in microaggregates within macroaggregates (Elliott et al., 1986; Jastrow et al., 1998; Six et al., 2000). As shown by Wang et al. (2015), SOC accumulation in bulk soil could be well accounted for by the changes in POC of the studied chronosequence.

Based on the data in Tables 2 and 3, organic carbon protected in the coarse-sand and fine-sand fractions constituted 51-62 % of the total SOC while chemically protected or mineral bound carbon in the clay-sized fractions constituted 11-19% of the total SOC of soils over the studied sequence. In a study of river bed sediments from a Californian river basin (Wakeham and Canuel, 2016), light fractions contributed largely to the total SOC pool but the heavy (clay) fractions contained smaller amounts of mostly old carbon. Six et al. (2002a) reported that organic matter accumulated mainly as unprotected particulate pools in microaggregates having a size larger than 53 µm and organic matter intimately associated with silt and clay had high chemical recalcitrance. The higher enrichment of organic carbon related to LOC in macroaggregates of the coarse-sand-sized fraction and smaller enrichment in clay-sized fraction in this study supported the general understanding of relatively fresh labile carbon in macroaggregates and relatively recalcitrant carbon in microaggregates as clay complexes (Six et al., 2002a). Microaggregates and other primary particles could be bound into macroaggregates with a close association of fungal hyphae and organic matter/materials (Oades, 1984; Tisdall, 1994; Miller and Jastrow, 2000).

Physical protection of labile carbon in macroaggregates rather than inherent chemical stability of SOC (a minor mass fraction of the clay-sized microaggregates; Table 2) has been increasingly considered as a mechanism for soil carbon sequestration (Six et al., 2004; Kong et al., 2005; Six and Paustian, 2014). For the rice soils under long-term rice cultivation that were studied here, SOC accumulated and stabilized mainly through physical protection of new or relatively labile carbon in macroaggregates, whereas old or mineral bound SOC was preserved in clay-sized fine aggregates (Marschner et al., 2008). This study also confirmed our previous understanding that coarse-sand-sized fraction of aggregates could play a prevalent role in soil carbon sequestration (Zhou et al., 2008).

4.2 Bioactivities vs. carbon stabilization between sandand clay-sized fractions

Biological activity of soil microbes including soil respiration and soil enzyme activity were known to vary across size fractions of soil aggregates (Kandeler et al., 1999; Sessitsch et al., 2001; Poll et al., 2003; Allison and Jastrow, 2006). In this study, total DNA content was linearly correlated with content either of SOC and total nitrogen, or of LOC, across the size fractions of the studied sequence (Fig. S3). However, gene abundance of bacterial, fungal and archaeal communities was correlated neither to the size of SOC and LOC nor to measures of carbon recalcitrance and lability (LOC / SOC) across the sequence. Likewise, SOC levels did not necessarily affect microbial populations along soil reclamation gradients with exotic carbon amendments (Yin et al., 2000; Torsvik and Øvreås, 2002). Indeed, different carbon lability and accessibility could shape microbial communities within and between size fractions of aggregates (Six et al., 2000; Ettema and Wardle, 2002; Balser et al., 2006; Kögel-Knabner et al., 2008).

Soil matrix and microhabitat conditions (aggregation and associated nutrients and C substrate as well as redox potential) played a critical role in changes in soil microbial abundance and community composition (Lehmann et al., 2011; Smith et al., 2014). Here, a clearly marked difference in microbial abundance and community could be found between the rice soils and the initial marsh soil before the shift to rice cultivation, either for bulk soils (Wang et al., 2015) or for aggregate fractions (Liu et al., 2016b). This could be coincident with the shift in soil physical and chemical conditions between the rice soil and the initial marsh soil, where the latter has alkaline in reaction, poor aggregation due to depleted SOC and high salinity (data in Table 1).

Among the soils studied, both the coarse-sand- and claysized fractions showed higher enrichment of SOC, which was relevant to a different association of carbon pools and interaction to minerals. There was a difference in the ratio of LOC to total SOC, as a negative indicator of chemical stability, and in carbon recalcitrance measured with FTIR, between the coarse-sand- and clay-sized fractions. The trends of carbon stability with microbial respiratory (RQ) were similar between the coarse-sand- and clay-sized fractions (Fig. 5). Clearly, this similarity could not be explained by the differ-

ence in the trend of the LOC-to-SOC ratio and of carbon recalcitrance (Table 3).

We further compared the bioactivity vs. SOC accumulation between coarse-sand- and clay-sized fractions of aggregates. Here, a correlation of DNA content, as an indicator of microbial biomass, to SOC content was highly significant for the coarse-sand fraction but not for the clay fraction (Fig. 6a). Meanwhile, normalized enzyme activity followed a positive linear function with total SOC content for coarsesand fractions but again not for clay fractions (Fig. 6b). In contrast, soil basal respiration scaled with DNA content reflected a negative power function with total DNA content, being higher for the coarse-sand fraction than for the clay-sized fraction (Fig. 6c). This could suggest a higher increase with SOC accumulation in carbon use efficiency in the coarsesand-sized fractions, compared to clay-sized fractions. Furthermore, a positively linear correlation of DNA content to the content of LOC (Fig. 6d) was found for the coarse-sandsized aggregate fractions but not for clay-sized fractions.

The lack of improvement in bioactivity with SOC accumulation in the clay-sized fractions indicated an insignificant potential to support biological activities in fine aggregates rich in stabilized SOC with high recalcitrance. In the claysized fractions of aggregates, DNA content was independent of SOC, which could be either inaccessible to microbes or non-degradable due to binding to minerals or as inert carbon (Lützow et al., 2006; Kögel-Knabner et al., 2008). In contrast, the DNA of microbes, here mainly as bacterial or archaeal in the soils, could be mostly adsorbed on clay minerals or hidden in small micropores within the fine aggregates (Poll et al., 2003; Chiu et al., 2006). Soil enzyme activities could represent an overall microbial activity for soil functioning (Allison et al., 2010), which was not a response to accumulation of SOC in the clay fractions, although extracellular enzymes also could be adsorbed onto clay particles (Allison and Jastrow, 2006).

High microbial biomass and enzyme activities were in line with carbon accumulation and stabilization in the coarsesand-sized macroaggregates. The large response of total microbial DNA and carbon use efficiency to SOC accumulation in the coarse-sand-sized fraction could suggest an improvement of either carbon substrate supply or of habitat environment through increases in mass proportion of macroaggregates with enhanced aggregation in soils (Lehmann et al., 2011). While containing a recalcitrant carbon pool similar to that in the clay-sized fractions, the macroaggregates in the coarse-sand-sized fractions also preserved a significant amount of labile carbon (Table 3), which could become easily decomposable and potentially used by microbes (Cleveland et al., 2007). For the bulk soil of this chronosequence, improved microbial activity was linked to the increase in POC content, which was enhanced via physical protection with increasing aggregate stability (Wang et al., 2015). Although habitats within macroaggregates offered protection for 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 in-between microaggregates within macroaggregates (Six et al., 2002b).

The metabolic quotient qCO_2 was proposed as an indicator of energy use by live soil microbial organisms (Schlesinger and Andrews, 2000). The data in Tables 3 and S3 clearly demonstrated the lowest qCO_2 in the coarsesand-sized fractions but the highest qCO_2 in the clay-sized fractions, among the size fractions of aggregates. Again, qCO_2 of the coarse-sand-sized fractions was in a generally decreasing trend with SOC accumulation under prolonged rice paddy management. With soil aggregation improved, macroaggregates could provide increasingly diverse soil microhabitats with varying types of carbon substrates accessible to microbes under sustainable agricultural management (Six and Paustian, 2014). Improvement of spatial allocation within and between microaggregates of carbon resource, microbial communities and extracellular enzymes could favor growth of microbiota and their functional performance in well-aggregated soils (Caldwell, 2005; Burns et al., 2013).

Many studies on bulk soils showed correlation of enzyme activity with microbial biomass in agricultural soils including rice paddies under proper management practices (Marx et al., 2005; Allison and Jastrow, 2006; Shi et al., 2006; Yu et al., 2012). Thus, carbon stabilization (indicative of carbon recalcitrance or respiration quotient) could not restrict microbial activity (Janzen, 2006) in macroaggregates, where highly enriched SOC (particularly of LOC pools) was physically protected, in rice soils under long-term paddy management. This could explain a potential co-evolution of improved bioactivity with enhanced carbon sequestration in agricultural soils (Rabbi et al., 2010). As noted by Smith et al. (2014), the relationship between carbon pools and specific microbial communities and biogeochemical activities is still unclear.

4.3 Trend of bioactivity with carbon stabilization after prolonged rice cultivation

Developed on a similar matrix of paleo-deposits rich in silt, the rice soils have been subject to a directional development with long-term paddy management (Cheng et al., 2009; Wissing et al., 2013). Desalinization initiated after drainage, conversion and decalcification proceeded as paddy rice cultivation was prolonged. Finally, there was a long-existing semi-hydromorphic pedogenesis over several centuries, characterized by mobilization of iron and manganese to form minerals of metal oxyhydrates (Wissing et al., 2013). The resultant directional changes of clay minerals, particularly those of oxyhydrates, the size and nature of SOC pools and the difference in archaeal and methanogenic archaeal community abundance have been well characterized by Cheng et al. (2009), Chen et al. (2011), Wissing et al. (2011, 2013) and Kölbl et al. (2014) as well as by Wang et al. (2015).



Figure 5. 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-sized (right) aggregate fractions. Soil organic carbon accumulation as a function of relative recalcitrant C (aromatic and phenol) (**a**) and negatively for relative labile C (aliphatic and polysaccharide) (**b**); CO_2 production as a plateau function of soil microbial biomass (**c**) and bacterial abundance (**d**). Data were the mean value of triplicates.



Figure 6. Inter-correlation between particulate organic carbon and soil microbial activity to compare the biological activity vs. carbon between coarse-sand (left) and clay-sized (right) 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 CO_2 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 were the mean value of triplicates.



Figure 7. Change in partitioning of soil organic carbon (\mathbf{a} , $g k g^{-1}$), total DNA (\mathbf{b} , $\mu g g^{-1}$), normalized enzyme activity (\mathbf{c} ; relative enzyme activity index) and soil respiration (\mathbf{d} ; $mg CO_2 g^{-1}$) 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 (*P*50–*P*700) shifted from a salt marsh (*P*0) under long-term rice cultivation. The size of a circle in a row is relevant to that of an analyzed parameter among the soils.

The above-mentioned directional changes were also seen in soil aggregation, and thus in microhabitat conditions as well as in nutrients (Table 1). SEM observation (Fig. 2) evidenced a clear change in size of the randomly sampled aggregates of the soils studied. This was in agreement with the change in mean weight diameter (MWD), an indicator of soil aggregate stability, with increasing rice cultivation length over the chronosequence (Wang et al., 2015). There were dispersedly distinct, sharply edged and less organic-matter-covered mineral particles in the uncultivated tidal marsh (P0). However, aggregates became larger in size and softer, and more porous with minute mineral particles bound together by organic matter in rice soils cultivated over 100 years. This is particular the case for P700, where the coarse-sand-sized macroaggregates were highly porous and soft, containing smaller-sized microaggregates with some string-like particulate organic matter on the sur-

face. The increased aggregate size and thus the MWD could suggest increasing organic matter in-between microaggregates in macroaggregates in rice soils cultivated over centuries. This change, through the improvement of microhabitat conditions and nutrient storage, could lead to some directional change in the association of microbial community abundance/activity over the long run of rice paddy management. The higher MBC and lower RQ and qCO_2 in coarsesand-sized macroaggregates and the decreasing trend of RQ and $q CO_2$ with increasing length of rice paddy management (Table S3) could suggest some adaptive change in the microbial community and improvements in their carbon use efficiency (Chen et al., 2016). In particular, the methanogenic community as a particular microbial community in rice soils (Conrad, 2009), has been shown to undergo a directional change towards prolonged rice paddy management (Liu et al., 2016b).

In a previous study (Wang et al., 2015), bulk-soil carbon accumulation and promotion of biological activity was concomitant with carbon stabilization through POC accumulation, in line with aggregate stability with long-term rice cultivation. Here we synthesize all the analysis data with respect to aggregate size fraction partitioning over the sequence (Fig. 7). After salt marsh soil (P0) was converted to rice cultivation (P50), SOC, enzyme activity and soil respiration showed a more or less consistent increase in both coarse-sand- and clay-sized fractions. The changes in relative portion by sand-sized (coarse- and fine-sand fractions together) aggregates against silt- and clay-sized aggregates exerted different patterns between of carbon pools and of microbial activities, across the soils of the chronosequence.

Over the sequence, the prevalence of physically protected organic carbon in coarse and fine-sand fractions as compared to the percentage of physically unprotected organic carbon in the silt and clay fractions (Six et al., 2002a) were in a range of 1.5–3.2 and 1.1–2.6 for SOC and total N, respectively, 0.9–2.2 for total DNA, 1.2–3.3 for fungal gene copy numbers and 0.8–1.5 for NEA. 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 analyzed carbon pools and bioactivities were dominated by the macro- and large microaggregates in size fractions of coarse and fine-sand, which was in general a consistent directional change with prolonged paddy management under long-term rice cultivation, although abundance of clay particles was consistently increased (Kölbl et al., 2014).

Long-term SOC sequestration in agricultural soils has been questioned (Powlson et al., 2011) and SOC enriched in coarse-sand fractions of aggregates could indeed be subject to fast decomposition in dry condition, for example, after shifting to maize cropping (Z. P. Li et al., 2007). In this study, however, hydroagric paddy management was kept continuous with ever prolonged rice cultivation, which could have driven the ever increasing trend of SOC accumulation up to the millennium (Wissing et al., 2011, 2013). Consequently, SOC accumulation and stabilization could take place in coarse-sand-sized aggregates with physical protection of labile carbon pool intra-microaggregates, with prolonged rice cultivation (Wang et al., 2015). POC, as a pool of relatively fast turnover (Cambardella and Elliott, 1992), also has been shown to keep increasing in paddies cultivated for centuries (Wang et al., 2015). Allison and Jastrow (2006) suggested that microbial biochemical activity and carbon turnover was stronger in POC-enriched size fractions but weaker in mineral-dominated fractions, where enzymes and their carbon substrates were immobilized on mineral surfaces. Long-term hydroagric paddy management (Zhang and Gong, 2003) reduced decomposition of root-, crop- or microbial-residue input under low-oxygen conditions (Roth et al., 2011). Moreover, the changes in relative proportion of carbon pools and microbial activities (NEA and soil respiration) by aggregates in the size of coarse and finesand further demonstrated that physically protected and stabilized carbon supported high soil bioactivities in macroaggregates, which has been increasingly prevalent over the smaller-sized fractions of soil aggregates.

The changes in organic carbon pools and the accessibility to microbes could lead to changes in the relative abundance and activity of microbes, potentially affecting C cycling and storage, in different size aggregates (Six et al., 2006). Unlike the findings of Allison and Jastrow (2006), this study proposes enhanced microbial activity but improved carbon use efficiency with reduced respiration quotient for microbial energy in coarse-sand-sized macroaggregates, compared to clay fraction over centuries of rice cultivation. This could be supported by the recent finding that $q CO_2$ was reduced and that the microbial biomass carbon increased in biochar-amended agricultural soils in a case study by Zheng et al. (2016) and in a meta-analysis by Zhou et al. (2016). This study indicated a strong interlink between microbiological activity and labile carbon in large-sized aggregates of paddy soils, though the later has been generally considered as physically protected carbon. As strengthened with prolonged rice paddy management, such a link could help enhance ecosystem functioning and services provided by rice soils (Six and Paustian, 2014; Smith et al., 2015).

Unfortunately, the methodology used here did not allow us to characterize the spatial allocation of carbon substrate, specific microbial communities and extracellular enzyme activities among the aggregate fractions. More importantly, labile OC pools, particularly those intra-aggregates or intermicroaggregates within macroaggregates, could not be further explored. Such data are considered to be critical to unravel the microscale process mediating bioactivities at the aggregate level (Six and Paustian, 2014).

5 Conclusions

Study of soils collected from a rice soil chronosequence derived from salt marsh revealed that soil organic carbon could be accumulated and stabilized both in coarse-sand-sized and clay-sized fractions of soil aggregates. However, microbial abundance and enzyme activity were high and the metabolic quotient was low in the aggregates (with sizes larger than 20 µm) compared to those of silt- and clay-sized fractions, possibly through the enhanced spatial allocation of labile carbon pool for improved microhabitat condition in the largersized aggregates. Thus, carbon stabilization with reduced turnover was not limiting soil bioactivities in macroaggregates, with the exception of silt- and clay-sized microaggregates. This study further supported our previous finding for bulk soils, whereby long-term rice cultivation led to accumulation and stabilization of SOC, and promoted soil biological activities through physical protection of labile carbon in line with enhanced soil aggregation. Thus, labile organic carbons accumulated in macroaggregates could help enhance microbial carbon use efficiency and improve their biogeochemical activity related to ecosystem functioning. More studies are needed on interaction of soil organic matter, minerals and microbial communities to unravel the microscale process mediating bioactivities at the aggregate level.

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