# Promoted microbial activity with organic carbon accumulation in macro-aggregates of paddy soils under long term rice cultivation

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**Abstract:**

While soil organic carbon (OC) accumulation and stabilization had been increasingly concerned as ecosystem properties, how this could be linked to soil biological activity enhancement had been poorly assessed. In this study, topsoil samples were collected from a series of rice soils shifted from salt marsh respectively for 0, 50, 100, 300 and 700 years from a coastal area of eastern China. Particle size fractions of soil aggregates were separated using a low energy dispersion protocol. These fractions were analyzed for OC recalcitrance with FTIR spectroscopy and for OC lability with chemical procedures. Soil microbial community of bacterial, fungal and archaeal were portrayed with molecular fingerprinting using specific gene primers. Soil respiration and enzyme activities were measured with lab incubation protocols. While the aggregate size fractions 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. Total OC was enriched highly in coarse sand fraction (40-60 g $kg^{-1}$), moderately in clay fraction (20-25 g $kg^{-1}$), but depleted in silt fraction (~10 g $kg^{-1}$). Recalcitrant OC pool was higher (33-40% of total OC) in both coarse sand and clay fractions than in fine sand and silt fractions (20-29% of total OC). However, the ratio of labile OC to total OC showed a weakly decreasing trend with decreasing size of aggregate fractions. Total soil DNA content in the size fractions followed a similar trend to that of OC. Gene abundance of bacteria and of archaeal were concentrated in both sand and clay fractions, but their diversity generally similar between the fractions. Being highest generally in coarse sand fraction, gene abundance of fungi decreased

sharply but the diversity gently, with decreasing size of the aggregate fractions. Soil respiration quotient (ratio of respired $CO_2$-C to total OC) was highest in silt fraction, followed by the fine sand fraction but lowest in coarse sand and clay fractions in the rice soils cultivated over 100 years. Whereas, microbial metabolic quotient was lower in sand sized fraction than in other fractions. Scaled by total DNA concentration, soil respiration was higher in silt fraction than in other fractions for the rice soils. For the size fractions other than clay fraction, OC scaled DNA concentration and archaeal gene abundance, and normalized enzyme activity were seen increased but OC and DNA scaled soil respiration decreased, more or less with prolonged rice cultivation. Moreover, both microbial gene abundance and normalized enzyme activity were well correlated to total OC and labile OC content only in the coarse sand fractions though chemical stability and respiratory of OC were similar between coarse sand and clay fractions. Thus, biological activity was generally promoted with labile organic carbon accumulation in the coarse sand sized macro-aggregates of the rice soils, positively responding to prolonged rice cultivation management. Yet, the mechanism underspin this trend and the effects on soil functions deserve further studies under field conditions.

**Key words:** rice soil, carbon stabilization, soil bioactivity, soil aggregates, size fractions, rice cultivation, microbial community, chronosequence

## 1 Introduction

Soil organic matter (SOM), as a continuum of organic substances with different degrees of decomposition (Lehmann and Kleber, 2015), provided a key driver for soil aggregation and thus soil ecosystem functions and services (Banwart et al., 2014). Soil aggregates had been considered as fundamental soil particle units where organic matter, minerals and microbes interacted to store C and nutrient 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). It had been increasingly considered as a primary mechiansm for soil carbon sequestration that OC tended physically protected against microbial access and decomposition (Blanco-Canqui and Lal, 2004; Six et al., 2004; Kong et al., 2005; Six and Paustian, 2014). This could be concerned with separate allocation of mineral associated OM fractions (Lehmann et al., 2008; Dungait et al., 2012; Vogel et al., 2014) between micro-aggregates within macro-aggregates. Soil aggregation shaped the micro-habitats for soil microbial communities (Six et al., 2000; Ettema and Wardle, 2002; Balser et al., 2006; Kögel-Knabner et al., 2008), with changes in OC 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 bio-activity as a whole, determined by size, diversity and biochemical activity of soil microbes (Six et al., 2006; Lagomarsino et al., 2012; Bardgett and van der Putten, 2014). Particularly, particulate OC (POC) had been increasingly considered as an indicator of soil quality and health under different stresses

85 or human disturbance (Cambardella and Elliot 1992; Marriott and Wander, 2006). As a
86 labile OC pool, POC had been suggested as a measurement of OC accumulation and
87 stabilization with co-existing microbial activity of soils in different ecosystems (Gajda
88 2010; Six and Paustian 2014). Soil aggregation, affected by land use and management
89 practices, could result in changes in allocation of POC inter- and/or intra-
90 microaggregates in size fractions of soil (Yang et al., 2009; Lagomarsino et al., 2012;
91 Six and Paustian 2014; Smith et al., 2014). Unfortunately, the link between changes in
92 carbon pools and those in microbial biological activity with OC stabilization in soil
93 aggregates had not yet been well understood and quantitatively assessed (Six and
94 Paustian 2014; Smith et al., 2014).
95 Soil aggregation could be characterized by distributions of particle size fractions, which
96 could differ in soil microbial biomass and the activity among them, in response to OC
97 accumulation and stabilization of soil in agro-ecosystems (Salinas-Garcia et al., 1997;
98 Kandeler et al., 1999; Smith et al. 2014). Such difference could mimic the micro-scale
99 interactions driving OC stabilization and nutrient cycling in soils (Kandeler et al., 2006;
100 Lagomarsino et al., 2012; Six and Paustian, 2014). For this, separation should be
101 required with least low energy dispersion of bulk soil into particle size fractions of
102 aggregates (Kandeler et al., 2000), but without any chemical dispersion (Smith et al.
103 2014). Stemmer et al. (1998) developed such a low energy ultrasonic dispersion
104 protocol, which could allow the least disturbed size fraction separation for analyzing
105 microbial community and enzyme activity in soil aggregates (Kandeler et al., 2000).
106 This approach was followed in later studies (Sessitsch et al., 2001; Poll et al., 2003;

107 Matocha et al., 2004; Marx et al., 2005; Zhang et al., 2013), addressing the impacts of
108 different management practices or environmental disturbances on OC persistence,
109 microbial communities and enzyme activity in aggregates agricultural soils. However,
110 the interactions between these attributes in aggregate size fractions with carbon
111 stabilization and their trend with continuing management in long term cultivated soils
112 had been not yet well characterized.
113 Soil matrix or microsite properties played an important role in the spatial allocation of
114 organic matter and microbial community and thus the link between OC pools and
115 microbial bio-activity among different fractions of soil aggregates (Smith et al. 2014).
116 Rice paddy soils were developed with dynamic redox regime and neo-formation of
117 iron/manganese oxyhydrates due to hydromorphic pedogenesis under long term
118 hydroagric paddy management (Li 1992). These soils were thus classified as a
119 particular soil group of hydroagric Anthrosols in the new Chinese Soil Taxonomy
120 (Gong et al., 1999). Recently, these soils had been known of high SOC storage and
121 sequestration potential, compared to dry-land croplands (Pan et al., 2004; Pan et al.,
122 2010; Wissing et al., 2013). This had been often attributed to enhanced aggregation and
123 thus the aggregate stability (Lu et al., 1998; Yang et al., 2005) as well as to increased
124 humification of OC (Olk et al., 2000). OC accumulation and stabilization in paddy soils
125 with management practices could be attributed to a number of processes. These were
126 shown with either increased binding to free oxyhydrates (Zhou et al., 2009; Cui et al.,
127 2014) and enhanced chemical recalcitrance (Zhou et al., 2009a, 2011; Song et al., 2012),

or enhanced physical protection with increased aggregate stability (Li et al., 2007; Zhou et al. 2008) or their interactions (Song et al., 2012; Song et al., 2013).

Moreover, OC could be continuously accumulated in rice soils with prolonged rice cultivation in the long run. In a rice soil chronosequence, OC accumulation was promoted following the desalinization and decalcifiation in the initial stage after the salt marsh shifted to rice paddy (Kalbitz et al., 2013). Wherein, the accumulated OC was increasingly stabilized with neoformed 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, total OC accumualtion was well represented by an increase in proportion of water-stable macro-aggregates (>250μm) and the associated POC pool (Zhou et al., 2007). In rice paddies under long term fertilization trials from South China, physically protected OC in the coarse sand size fraction of soil aggregates contributed to bulk soil OC accumualtion and stabilziationin (Zhou et al., 2008).

Importantly, co-evolution of soil microbial community and diversity was observed with OC accumulation and stabilization in rice paddies (Zhang et al., 2007; Zheng et al., 2007; Liu et al., 2011). In line with the trend of OC accumulation in paddy soils, microbial biomass and community diversity was enhanced across a chornosequence under prolonged rice cultivation (Bannert et al., 2011; Jiang et al., 2013). Using a similar chronosequence, the enhanced biological activity could be well portraied with an increase in mean weight diameter of soi aggregates and in POC pool across the soils with prolonged rice cultiavtion (Wang et al., 2015). This indicated a potential role of

physically protected labile OC pool in enhancing biological activity with bulk OC accumualtion in rice soils (Zou et al., 2015). Rcently, changes in mcirobial gene abundance and community compsoition had been reported for the bulk soils (Liu et al., 2016a) and for aggregate size fractions of soils (Liu et al., 2016b), from such a rice soil chronosequence. Thus, physical protection could involve a change in the spatial distribution of OC pools rather than in the chemical recalcitrance, among aggregate size fractions. Accordingly, changed allocation of both OC pools and microbial community could contribute to OC stabilization with increased microbial abundance and microbial carbon use efficiency, $qCO_2$ (Schlesinger & Andrews, 2000), as a result of enhanced aggregation (Lehmann 2011). However, the link of microbial activity to OC accumualtion and stabilization among different aggregate fractions and the evolution with increasing length of rice cultivation had been unknwon. Such information would be of key importance for understanding carbon stabilization in relation to sustainable management of rice paddy soils as carbon biogeochemical cycling had driven ecosystem functions and services provided by soils (Smith et al., 2015).

In this study, two hypotheses are tested. First, microbial bioactivity and carbon stability in soil aggregates could differ among their size fractions, leading to changes in spatial allocation of OC pools among aggregate size fractions in rice paddies. Physical protection of OC could improve microbial microhabitat conditions and thus microbial carbon use efficiency through enhanced aggregation. And it could enable an existence of labile OC pool within micro-aggregates in macro-aggregates or between micro-aggregates (Six and Paustian 2014; Smith et al., 2014). Thus biological activity could

be enhanced with physically protected carbon in macro-aggregates, rather than in micro (clay sized) aggregates with chemically stabilized organic carbon; Second, a strong link of microbial activity to labile OC pool would be promoted with enhancement of physically stabilized OC in macro-aggregates, resulting from 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 OC stabilization (with increasing mineral bound OC, accumulation of recalcitrance OC pool as well as POC pool). This study aimed to help understand that carbon stabilization would not confront but improve biological activity in soils under rice cultivation over centuries.

## 2 Materials and methods

### 2.1 Methodology rational

Using a recommended sonification separation procedure, we looked into the changes in aggregate size fraction composition for aggregate stability, in OC functional group composition for chemical recalcitrance, and in soil respiration for microbial energy use, in order to characterize OC accumulation and stabilization in rice soils. Meanwhile, changes with OC accumulation/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 stabilization and bioactivity among the aggregate fractions were quantitatively assessed using the parameters of carbon- or gene abundance- scaled respiration and enzyme activity. Finally, the evolution of such 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

In this study were investigated a series of soils of a paddy chronosequence, shifted from tidal marsh to rice cultivation for different lengths in a coast land located in Cixi Municipality, Zhejiang Province, China (Fig.1). Lying 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 1,367 mm during 2004-2014 (http://cdc.nmic.cn/home.do). 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. These soils allowed a chronosequence study

207 for rice soil development, such as a pedological characterization by Cheng et al. (2009)
208 and a morphological, mineralogical and microbiological investigation by Kölbl et al.
209 (2014).

Fig. 1

211 In this study, individual soils of the chronosequence were identified based on dyke
212 establishment history recorded in Cixi County Annals (with brief information in
213 Chinese available at www.cixi.gov.cn), including an initial tidal marsh soil before rice
214 cultivation (P0), and rice soils of P50, P100, P300 and P700 shifted for rice cultivation
215 respectively 50, 100, 300 and 700 years before present (Fig.1). These soils were apart
216 from each other in a distance no more than 40-km in nearly the same topography. All
217 the soils developed on comparable parent materials of paleo-deposit from Yangtze
218 River, with a particle composition of silt (75%-84%), followed by clay but low in sand
219 content (Chen and Zhang, 2009). Soil texture ranged from silty loam to silty clay-loam.
220 The clay mineral assemblage consisted of illite (40-50%), chlorite (20-30%) and
221 kaolinite (10-20%) with a minor amount of smectite and quartz (Zhang et al., 2010b).
222 As situated in a relatively small area with a traditional summer rice-winter rape rotation,
223 rice production management of the chronosequence could be considered relatively
224 consistent across sites, with similar cultivars and management practices including crop
225 protection, irrigation and fertilization (Cheng et al., 2009). Of course, influence of salt
226 on rice production could occur in the early stage of rice cultivation on the tidal marsh
227 derived soils while the ground water table had been enough low without restricting rice
228 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., 2013; Wissing et al., 2011; Kölbl et al., 2014), interaction of organic matter with minerals (Wissing et al., 2011; 2014) as well as organic carbon pools (Wissing et al., 2011; Wang et al., 2015) had been already 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 OC substrates in soil samples, the sampling was done in early November 2011, when the soil was moist following rice harvest. While sampling in field, an undisturbed soil core was collected using an Eijkelkamp soil core sampler (Agrisearch Equipment, Giesbeek, The Netherlands) while a bulk soil sample using a stainless steel shovel. A topsoil was collected in triplicates respectively from three adjacent individual fields. Finally, all soil samples were shipped to lab within two 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 had been assessed in our previous study by Wang et al. (2015) and Liu et al. (2016a and 2016b).

Table 1

### 2.4 Particle size fractionation of soil aggregates

In this study, the undisturbed soil cores were used for 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), Kandeler, et al (1999, 2000 and 2006). A portion of field moist soil core (50 g equivalent d.w.), removed of discernible straw material if any, 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 J $g^{-1}$ for 5 min. Aggregates in diameter of 2000-200 μm and of 200-20 μm, were respectively separated by wet sieving and by subsequent sedimentation after siphonage, and assigned to coarse and fine sand sized fraction. The remainder was centrifuged to firstly collect the aggregates in diameter of 20-2 μm (assigned to silt sized fraction) and further centrifuged to collect those in diameter of ≤ 2 μm (assigned to clay sized fraction). The samples of the obtained size fractions were freeze-dried with a frozen dryer (Thermo, Modulyo D-230, NY, US) and then stored at -70 ºC. Here, water stable macro-aggregates larger than 2000μm were not taken into consideration as they were insignificant in rice soils under prevailing water submergence and pudding activities under long term hydroagric management (Deng and Xu, 1965). The classes of the size fractions were kept basically consistent with our previous studies (Li et al., 2007a, b; Zheng et al., 2007; Pan et al., 2008 and Chen et al., 2014).

**2.5 Organic carbon pool and FTIR spectroscopy analysis**

Total 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 ($KMnO_4$), 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 coarse sand fraction of P0 soil was not provided due to the very small sample obtained via the sonification and separation procedure.

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 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 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. Following Ellerbrock et al. (1999) and Cocozza et al. (2003), the characteristic vibration peak at 1050 $cm^{-1}$ 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 $cm^{-1}$ to phenols. Subsequently, a general semi-quantification of three major functional OC groups of polysaccharides, aliphatic and aromatic compounds was done 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

295 polysaccharides (Mao et al., 2008;Tivet et al., 2013). All the obtained FTIR spectra are
296 given in Supplement Fig. 1.

297 **2.6 SEM observation of soil aggregates**

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

305 **2.7 DNA extraction, microbial gene abundance and diversity analysis**

306 A portion (0.45 g) of a PSF sample stored at -70 ºC was used for DNA extraction with
307 PowerSoil™ DNA Isolation Kit (MoBio, USA), following the manufacturer guide. The
308 concentration of the DNA extracts was checked with a spectrophotometer (Eppendorf,
309 Germany), and its integrity and size were checked by using 1.0% agarose gel
310 electrophoresis. Extracted DNA was stored at -70 ºC prior to molecular bioassay.
311 Quantitative real-time PCR assay was performed on a 7500 real-time PCR system
312 (Applied Biosystems, USA) using SYBR green as a fluorescent dye. Primer
313 combinations of 338F/518R (Øvreås and Torsvik, 1998), ITS1F/ITS4 (Gardes and
314 Bruns, 1993) and Ar109F/Ar915R (Lueders and Friedrich, 2000) were used for
315 bacterial 16S rRNA, fungal Internal Transcribed Spacer (ITS) region and archaeal 16S
316 rRNA genes respectively in the Real-time PCR assay.

317 PCRs were carried out on all PSF's DNA samples with specific primers to amplify the
318 16S rRNA genes from bacteria (27F and 1492R) and archaea (Ar109F and Ar915R)
319 and the ITS regions from fungi (ITS1F and ITS4). The forward primer from each pair
320 had a fluorescent label (6-FAM) attached to the 5' end. Amplification of the 16S rRNA
321 gene and ITS regions, purification, digestion and amplicon separation for T-RFLP
322 analysis are described in the supplementary materials and methods.
323 From the T-RFLP profiles, the Shannon diversity index (*H'*) of the individual T-RFs
324 was calculated following Blackwood et al. (2007), using an equation:

325 $$H' = \Sigma Pi\ (\ln Pi) \quad (1)$$

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

327 **2.8 Soil enzyme activity**

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

336 $$x_i' = \frac{x_i}{\sum_{i=1}^{n} x_i}\ (i=1,2,\ldots,5), \quad (2)$$

337 where, *i* was the number of each soil sample (P0, P50, P100, P300, P700), *x* was the
338 enzyme activity and *x*′ was 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 $CO_2$ production using an anaerobic laboratory incubation protocol, following Zheng et al. (2007). A size fraction sample (20g d.w. equivalent) was placed into a 125ml glass jar and 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 slurry were incubated in an incubator, as described in Section 2.8, at 25 ± 1 $^oC$ for 37 days. During incubation, a 0.25 ml sample of the headspace gas was collected by a pressure syringe every 5 days since 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 remove all the emitted gas in the jar. $CO_2$ 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 total

361 $CO_2$ evolved was estimated from the cumulative sum of the gas evolved in all
362 monitoring intervals and was used to calculate the anaerobic soil respiration expressed
363 in terms of soil mass.

364 **2.10 Data treatment and statistical analysis**

365 All data was treated with EXCEL 2013 and expressed as mean plus/minus standard
366 deviation of triplicate samples. The significant differences between particle size
367 fractions in a single soil and between soils of a single particle size fraction were
368 respectively statistically analyzed by one-way ANOVA with Tukey's test, using a SPSS
369 software package 20.0. A statistical significance was defined at 95% confidence level.

## 3 Results

### 3.1 Organic carbon characterization in aggregate size fractions

As shown in Table 2, the fine sand (200-20μm) and silt (20-2μm) sized fractions together accounted for up to 80% of a bulk soil across soils. However, the proportion of coarse sand sized (2000-200μm) macro-aggregates and clay sized (< 2μm) fine aggregates increased with prolonged rice cultivation over the chronosequence. As indicated in Fig. 2, soil aggregates from the initial marsh soil (P0), were sharply edged single individual minerals, and mostly uncovered with clear surfaces; However, in the rice soils with increasing rice cultivation lengths, soil aggregates became increasingly round, loosely assembled of fine minerals but covered with more or less amorphous materials. Particularly in P700, soil aggregates were seen in large size, very loosely assembled of unclearly shaped mineral particles with amorphous materials, of which some particulate organic matter including some fungal hyphae on the aggregate surface (magnified P700 image in Fig. 2).

Table 2

Soil properties of total OC, total N and LOC were extensively different among the size fractions and between uncultivated and rice soils (Table 3). Total OC, LOC and total N pools were generally in an order of sand size fraction > clay sized fraction> fine sand fraction >silt sized fraction in a single soil. And these pools of all the particle size fractions except fine sand fraction, were greater in rice soils than in the uncultivated marsh soil. Particularly, OC of rice soils was enriched mostly in coarse sand sized macro- aggregates, moderately in clay sized fraction, fairly in fine sand sized fraction

but depleted in silt sized fraction, respectively in a range of 41-61 g $kg^{-1}$, of 20-24 g $kg^{-1}$, of 8.5-20 g $kg^{-1}$and of 10-11 g $kg^{-1}$. However, C/N ratio was in a significantly decreasing trend with the decreasing size of the aggregate fractions across the chronosequence. The ratio of LOC to total OC, an indicator of C lability in soils, was in a significantly decreasing order of coarse sand fraction>fine sand fraction>silt and clay sized fractions.

The FTIR spectra showed sharp peaks generally at vibration of 1050$cm^{-1}$ (assigned to polysaccharides) but broad shoulders at vibration of 3405$cm^{-1}$ assigned to aromatic carbon across the aggregates fractions (Supplement Fig.1). There was a clear trend of decreasing intensity the polysaccharide peaks but increasing shoulder intensity of aromatic carbon in a single fraction, with increasing rice cultivation. The semi-quantitative 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 small contribution (0.6-3.7%) of aliphatic carbon in a single fraction. Relative proportion of aromatic carbon was lower but of polysaccharide carbon higher in silt fraction than in other fractions, without a significant difference in-between the latter. Consequently, the estimated OC chemical recalcitrance (ratio of aromatic to polysaccharide C) was lowest in silt fraction, followed by fine sand fraction but highest in coarse sand and clay fractions.

Recalcitrance of OC 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 OC for the soil (Table

414 2 and Table 3), had a moderate OC recalcitrance but the coarse sand fraction had similar
415 OC recalcitrance but higher carbon lability and higher C/N ratio. This indicated a
416 greater existence of potentially available carbon pool (POC, for example) in the coarse
417 sand fraction, compared to other fractions.

418 Table 3

419 Table 4

420 Fig. 2

421 **3.2 Microbial biomass carbon, microbial gene abundance and diversity**

422 The measured microbial biomass carbon (MBC) was highest in the coarse sand fraction
423 of macro-aggregates while lowest in the clay sized fraction of fine micro-aggregates
424 over the sequence (Table 3). Generally, MQ, the microbial quotient, was not
425 significantly different between the coarse sand-, fine sand- and silt- sized fractions but
426 significantly higher than the clay sized fractions.

427 The microbial DNA content (equivalent to biomass) and gene abundance of microbial
428 communities in the fractions over the chronosequence are shown in Table 5. Total DNA
429 ranged from 1.57 μg $g^{-1}$ in silt fraction to 4.00 μg $g^{-1}$ in clay fraction of the tidal marsh
430 and from 4.35 μg $g^{-1}$ in fine sand fraction to 35.33 μg $g^{-1}$ in coarse sand size in the rice
431 soils. Fungal ITS gene copies were generally higher in coarse sand fractions, decreasing
432 with the size of aggregate fractions. Whereas, generally in a bimodal pattern among the
433 particle size fractions, total DNA, bacterial and archaeal 16S rRNA gene copy numbers
434 were higher in both coarse sand and clay fractions, compared to other fractions across
435 the chronosequence. Clearly, microbial gene abundance was dominated by bacterial,

436 with archaeal and fungal gene abundance respectively one and two order lower than
437 bacterial across the fractions. Whereas, the ratio of fungal to bacterial gen abundance
438 generally decreased but that of archaeal to bacterial increased with decreasing size of
439 the aggregate fractions.
440 Over the studied chronosequence, DNA contents of a fraction were several folds higher
441 in the rice soils over the initial tidal marsh. Accordingly, gene copy numbers of
442 microbial communities from a fraction were greatly higher in rice soils than in the initial
443 tidal marsh. Bacterial and fungal abundance in coarse sand, fine sand, silt and clay
444 fraction in P50 was increased by 688%, 72%, 498% and 622 %, and 74%, 149%, 7%
445 and 152 %, respectively over P0. A mean increase in the rice soils cultivated for over
446 100 years over P0 in bacterial gene copy numbers was seen significant, by 73% to
447 437 %, 0.4% to 67 %, 225% to 246 % and 147% to 201 %, respectively in coarse sand,
448 fine sand, silt and clay fraction. Comparatively, the change across the soils in fungal
449 gene abundance of aggregates was much smaller, particularly in silt and clay sized
450 fractions. In contrast, archaeal abundance in a single fraction across the soils was found
451 increased over P0 consistently with the prolonged rice cultivation, though smaller in
452 fine sand and silt sized fractions. For the coarse sand fraction only, both of fungal to
453 bacterial ratio and of archaeal to bacterial ratio tended to increase with increasing rice
454 cultivation lengths.
455 Data of microbial Shannon diversity index of the four size fractions of the
456 chronosequence soils are presented in Table S1. In detail, Shannon index of bacterial
457 community was much higher in coarse sand fraction and, to a lesser extent, in clay size
458 fraction than in fine sand and silt fractions across the chronosequence. Fungal

459 community Shannon index was shown highest in coarse sand fraction among the
460 fractions, decreasing generally with the size of aggregate fractions. However, there
461 were no significant changes in archaeal Shannon index among the size fractions across
462 the sequence. Generally, Shannon diversity index of the microbial communities in a
463 single fraction was greatly higher in the rice soils than in the uncultivated tidal marsh.

464 **3.3 Enzyme activity and basal respiration**

465 All analyzed enzyme activities (Table S2) were seen increased in the rice soils over the
466 initial tidal marsh. Furthermore, NEA was 0.07 in the coarse sand and 0.10 in the fine
467 sand fraction, and 0.07 and 0.14 in the silt and clay fractions in P0. In contrast, NEA
468 was 0.18-0.30 in coarse sand and 0.12-0.30 in fine sand fraction, but 0.17-0.30 in silt
469 and 0.19-0.24 in clay fraction of the rice soils. Moreover, NEA in a single size fraction
470 showed a significantly increasing trend with prolonged rice cultivation (Table 6).
471 Soil respiration of a single fraction was much higher for the rice soils than for the marsh
472 soil, and in sand sized macro-aggregate fraction than in silt and fine sand fraction over
473 the soils (Table 6). In detail, soil respiration was 662 $mgCO_2\ kg^{-1}$ and 565 $mgCO_2\ kg^{-1}$
474 in coarse and fine sand fraction, and 298 $mgCO_2\ kg^{-1}$ and 496 $mgCO_2\ kg^{-1}$ in silt and
475 clay fraction, respectively in P0. While in rice soils, soil respiration was in a range of
476 1588-2914 mg $CO_2\ kg^{-1}$ in coarse sand, and of 1076-1256 $mgCO_2\ kg^{-1}$ in fine sand
477 fraction, and of 740-1354 $mgCO_2\ kg^{-1}$ in silt and of 1028-1434 $mgCO_2\ kg^{-1}$ in clay
478 fraction, of the rice soils. Basal respiration in a single size fraction generally increased
479 with rice cultivation length (Table 6).
480 Using the data in Table 3, the estimated RQ (the ratio of respired C to total OC) and
481 $qCO_2$ (the ratio of respired OC to MBC) were seen variable across the size fractions

and among the soils (Supplement Table 1). Generally, RQ was lower both in sand- and clay- sized fractions than in fine sand- and silt- sized fractions. Value of $qCO_2$ was lowest in the coarse sand sized fraction but 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.

## 4 Discussions

### 4.1 Carbon accumulation versus stabilization in soil aggregates

In this study, level of OC, soil respiration and microbial gene abundance/diversity differed significantly among different size fractions of water stable aggregates from the chronosequence. Similar to the findings by Li et al. (2007b) and Zheng et al. (2007), OC was seen accumulated highly in sand sized and moderately in clay sized fractions but depleted in silt sized aggregate fractions (Table 3). As shown in Fig. 3, soil organic carbon content (level of OC accumulation) in a fraction was found very significantly positively linearly correlated to OC recalcitrance from the FTIR analysis (Table 4). Whereas, respiration quotient as a rate indicator of carbon turnover for microbial energy use (Kennedy and Papendick, 1995), was in a very significantly negative logarithm function of OC 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 OC turnover (Qian et al., 2013). The correlations hereby could suggest the accumulation of OC 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-degradable or residue OC in soils (Lützow et al., 2006; Mikutta et al., 2006).

Fig. 3

However, calculated using the OC contents (Table 3) and the fraction mass percentage (Table 2) of a single fraction, only the amount of OC allocated in sand and clay sized fractions were closely correlated to the bulk OC contents (Table 1) of the soils (Fig. S1).

511 This was in general agreement with the finding for similar rice paddy soils from an
512 adjacent area (Pan et al., 2008). The increased allocation of OC to clay sized fraction
513 could be attributed to the accelerated formation of clay and hydroxyl Fe/Mn minerals
514 (Wissing et al., 2013) due to long term paddy management (Kölbl et al., 2014).
515 Furthermore, the enrichment index (EI) of OC, calculated with OC content in a fraction
516 divided by that in the bulk soil, was higher than 1 in both sand and clay sized fraction
517 but much lower than 1 in silt fractions. When plotting the EI values against LOC content
518 (Table 3) for all the fractions (Fig. 4), enrichment of OC was seen relevant to labile OC
519 pool in the fractions. Moreover, the EI values were seen significantly but weakly
520 positively correlated both to F/B ratio of gene abundance (Table 5) and to OC
521 recalcitrance (Table 4). These evidenced that accumulation of labile OC, mostly POC,
522 contributed significantly to OC pool in sand sized macro-aggregates (Zhou et al., 2008)
523 though hereby the apparent recalcitrance was in a similar range to that in clay fractions
524 (Table 4). It had been well understood that light fraction or macro-aggregates in soil
525 were rich in new or relatively labile carbon substrates, more or less related to root fungal
526 activities, which were largely physically protected in micro-aggregates within macro-
527 aggregates (Elliott et al., 1986; Jastrow et al., 1998; Six et al., 2000). As shown by Wang
528 et al. (2015), OC accumulation in bulk soil could be well accounted for by the changes
529 in POC of the studied chronosequence.

Fig. 4

531 Synthesizing data from Tables 2 and 3, OC protected in the sand and fine sand fractions
532 constituted 51%-62% while chemically protected or mineral bound OC in the clay sized

fractions 11%-19%, to the total OC pool of soils over the studied sequence. In a study of a river bed sediments from a Californian river basin (Wakeham and Canuel, 2016), light fractions contributed largely to the total OC pool but the heavy (clay) fraction contained smaller amount but old OC. Six et al. (2002a) addressed that organic matter accumulated mainly as unprotected particulate pool in micro-aggregates in size lager than 53μm though intimately associated with silt and clay with high chemical recalcitrance. The higher enrichment of OC related to LOC in macro-aggregates of sand size fraction and smaller enrichment in clay sized fraction in this study supported the general understanding of relatively unprotected labile carbon in macro-aggregates but relatively recalcitrant carbon in micro-aggregates as clay complexes (Six et al., 2002a). Micro-aggregates 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).

Physical protection of labile carbon in macro-aggregates rather than inherent chemical stability of OC (a minor mass fraction of the clay sized micro-aggregates, Table 2) had been increasingly concerned 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 here, OC accumulated and stabilized mainly through physical protection of new or relatively labile carbon in macro-aggregated though old or mineral bound OC preserved in fine aggregates of clay size (Marschner et al., 2008). This study also confirmed our previous understanding that sand-sized fraction of aggregates could play a prevalent role in soil carbon sequestration (Zhou et al 2008).

555 **4.2 Bio-activities versus OC stabilization between sand and clay sized fractions**

556 Biological activity of soil microbes including soil respiration and soil enzyme activity
557 had been well known varying across size fractions of soil aggregates (Kandeler et al.,
558 1999; Sessitsch et al., 2001; Poll et al., 2003; Allison and Jastrow, 2006). In this study,
559 total DNA content was found significantly positively but linearly correlated with
560 content either of organic carbon and nitrogen, or of labile organic carbon, across the
561 size fractions of the studied sequence (Fig. S2). However, gene abundance of bacterial,
562 fungal and archaeal communities could be correlated neither to total pool of organic
563 carbon and labile organic carbon nor to carbon recalcitrance and lability (LOC/total
564 OC), across the sequence. Likewise, OC level did not necessarily affect microbial
565 populations along soil reclamation gradients with exotic carbon amendments (Yin et al.,
566 2000; Torsvik and Øvreås, 2002). Indeed, different carbon lability and accessibility
567 could shape microbial communities within and between size fractions of aggregates
568 (Six et al., 2000; Ettema and Wardle, 2002; Balser et al., 2006; Kögel-Knabner et al.,
569 2008).

570 Soil matrix and micro-habitat conditions (aggregation and associated nutrients and C
571 substrate as well as redox potential) played a critical role in changes in soil microbial
572 abundance and structure (Lehmann et al, 2011; Smith et al., 2014). Here, a clearly
573 marked difference in microbial abundance and community could be found between the
574 rice soils and the initial marsh soil before shift to rice cultivation, either for bulk soils
575 (Wang et al., 2015) or for aggregates fractions (Liu et al., 2016b). This could be
576 coincident with the shift in soil physical and chemical conditions between the rice soils

577 and the initial marsh soil, with the latter was alkaline in reaction, poor aggregation due
578 to depleted OC and high salinity (Data in Table 1).
579 Among the soils studied, both the coarse sand and clay sized fractions showed higher
580 enrichment of OC, which was relevant to different association of carbon pools and
581 interaction to minerals. There was a difference in the ratio of LOC to total OC, as a
582 negative indicator of chemical stability, and in OC recalcitrance measured with FTIR,
583 between the coarse sand and clay sized fractions. The trends of carbon stability with
584 microbial respiratory (RQ) were similar between the sand and clay sized fractions (Fig.
585 5). Clearly, this similarity could not be explained by the difference in the trend of LOC
586 to total OC ratio, and of carbon recalcitrance (Table 3).
587 Fig. 5
588 We further compare the bio-activity versus OC accumulation between sand and clay
589 sized fractions of aggregates. Here, a correlation of DNA content (relevant to microbial
590 biomass size) to OC content was very significant for coarse sand fraction but not valid
591 for clay fraction (Fig. 6a). Meanwhile, normalized enzyme activity was in a positively
592 linear function with total OC accumulation for coarse sand fraction but failed again for
593 clay fractions (Fig. 6b). In contrast, DNA content scaled soil basal respiration was in a
594 negatively power function with total DNA content, being higher for the coarse sand
595 than for the clay sized fractions (Fig. 6c), showing a higher increase in carbon use
596 efficiency with the SOM accumulation in sand sized fractions than in clay sized
597 fractions. Moreover, a positively linear correlation of DNA content to the content of
598 LOC (Fig. 6d) was found only for sand sized aggregate fractions but for clay sized

599 fractions.

Fig. 6

601 The failure of bio-activity improvement with OC accumulation in clay sized fractions
602 indicated an insignificant potential to support biological activities in fine aggregates
603 rich in stabilized OC with high recalcitrance. In clay sized fractions of aggregates, DNA
604 content was independent of OC, which could be either inaccessible to microbes or non-
605 degradable due to binding to minerals or as inert OC (Lützow et al., 2006; Kögel-
606 Knabner et al., 2008). On contrary, the DNA of microbes, mainly as bacterial or
607 archaeal in the soils here, could be mostly adsorbed on clay minerals or hidden in
608 minute pores within the fine aggregates (Poll et al., 2003; Chiu et al., 2006). Soil
609 enzyme activities could represent an overall microbial activity for soil functioning
610 (Allison et al., 2010), which was no response to accumulation of OC in the clay
611 fractions though extracellular enzymes could be also adsorbed on to clay particles
612 (Allison and Jastrow, 2006).
613 In contrast, high microbial biomass and enzyme activities were in line with carbon
614 accumulation and stabilization in coarse sand sized macro-aggregates. The high
615 response of total microbial DNA and carbon use efficiency to OC accumualtion in the
616 coarse sand size fraction could suggest an improvement of either carbon substrate
617 supply or of habitat environemnt through increases in mass proportion of macro
618 aggregates with enhanced aggregation in soils (Lehmann et al., 2011). While containing
619 a recalcitrant OC pool similar to clay sized fractions, the macro-aggregates in coarse
620 sand sized fraction preserved also 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 found linked to the increase in particulate OC content, which was enhanced via physical protection with increasing aggregate stability (Wang et al., 2015). Although habitats within macro-aggregates offered protection of the young and labile carbon against microbial decomposition (Gupta and Germida, 2015), enhanced aggregation could lead to increased population and activities of specific microbial groups in between micro-aggregates within macro-aggregates (Six et al., 2002b).

The metabolic quotient $qCO_2$ was proposed as an indicator of energy use by live soil microbial organisms (Schlesinger & Andrews, 2000). The data in Table 3 and Supplement Table 1 clearly demonstrated the lowest $qCO_2$ in the coarse sand sized fraction but the highest $qCO_2$ in the clay sized fraction, among the size fractions of aggregates. Again, $qCO_2$ of the coarse sand sized fraction was in a generally decreasing trend with OC accumulation under prolonged rice paddy management. With soil aggregation improved, macro-aggregates could provide increasingly diverse soil microhabitats with varying types of OC substrates accessible to microbes under sustainable agricultural management (Six and Paustian, 2014). Improvement of spatial allocation within and between micro-aggregates 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

643 (Marx et al., 2005; Allison and Jastrow, 2006; Shi et al., 2006; Yu et al., 2012). Thus,
644 carbon stabilization (indicated of carbon recalcitrance or respiration quotient) was not
645 confronting microbial activity (Janzen, 2006) in macro-aggregates, where highly
646 enriched OC (particularly of labile OC pool) was physically protected, in rice soils
647 under long term paddy management. This could explain a potential co-evolution of
648 improved bio-activity with enhanced carbon sequestration in agricultural soils (Rabbi
649 et al., 2010). Of course, the relation between carbon pools and specific microbial
650 communities and biogeochemical activities seemed still unclear (Smith et al., 2014).

651 **4.3 Trend of bioactivity against OC stabilization with prolonged rice cultivation**

652 Being developed on a similar matrix of paleo deposits rich in silt, the rice soils had been
653 subject to a directional development with long term paddy management (Cheng et al.,
654 2009; Wissing et al., 2013). Desalinization initiated when shortly shifted to rice paddy
655 and decalcification proceeded as paddy rice cultivation prolonged. Finally, there was a
656 long existing semi-hydromorphic pedogenesis over several centuries, characterized by
657 mobilization of iron and manganese to form minerals of metal oxyhydrates (Wissing et
658 al., 2013). The resultant directional changes of clay minerals, particularly those of
659 oxyhydrates, of OC pool and the association of both as well as of archaeal and
660 methanogenic archaeal community abundance had been well characterized in the works
661 by Cheng et al.(2009), Chen et al. (2011), Wissing et al. (2011, 2014 and 2014) and
662 Kölbl et al. (2014) as well as by Wang et al. (2015).

663 Coincidently, directional changes were seen also in soil aggregation, and thus in
664 microhabitat conditions as well as in nutrients (Table 1). SEM observation (Fig. 2)

665 evidenced a clear change in size of the randomly sampled aggregates of the soils studied.
666 This was in an agreement with the change in mean weight diameter (MWD), an
667 indicator of soil aggregate stability, with increasing rice cultivation length over the
668 chronosequence (Wang et al. 2015). There were dispersed distinct, sharply-edged but
669 less organic matter-covered mineral particles in the uncultivated tidal marsh (P0).
670 However, aggregates became larger in size and softer, and more porous with minute
671 mineral particles bound together by organic matter in rice soils cultivated over 100
672 years. This is particular the case for P700, where the sand sized macro-aggregates were
673 highly porous and soft, containing smaller sized micro-aggregates and with some
674 string-like particulate organic matter on the surface. The increased aggregate size and
675 thus the mean weight diameter (MWD) could suggest increasing organic matter in-
676 between micro-aggregates in macro-aggregates in rice soils cultivated over centuries.
677 This change, through the improvement of micro-habitat conditions and nutrient storage,
678 could lead to some directional change in the association of microbial community
679 abundance/activity over the long run of rice paddy management. The higher MBC and
680 lower RQ and $qCO_2$ in coarse sand sized macro-aggregates and the decreasing trend of
681 RQ and $qCO_2$ with increasing length of rice paddy management (Supplement Table 1)
682 could suggest some adaptive change in microbial community and improvement of their
683 carbon use efficiency (Chen et al., 2016). Particularly, methanogenic community as
684 particular microbial community of rice soils (Conrad, 2009), had been shown in a
685 directional changes towards prolonged rice paddy management (Liu et al., 2016b).
686 In a previous study, Wang et al. (2015) found bulk soil OC accumulation and promotion

687 of biological activity concurrent with carbon stabilization through POC accumulation,
688 in line with aggregate stability with long-term rice cultivation. Here we synthesize all
689 the analysis data in terms of aggregate size fraction partitioning over the sequence,
690 presented in Fig. 7. After salt marsh soil (P0) shifted to rice cultivation (P50), total OC,
691 enzyme activity and soil respiration showed a more or less consistent increase in both
692 sand and clay sized fractions. The changes in relative portion by sand sized (coarse and
693 fine sand fractions together) aggregates against silt and clay sized ones exerted different
694 patterns between of carbon pools and of microbial activities, across the soils of the
695 chronosequence.
696 Over the sequence, the prevalence of physically protected portion in sand fractions over
697 unprotected portion in silt and clay fractions (Six et al., 2002a) was in a range of 1.5-
698 3.2 and of 1.1-2.6 for total OC and total N, of 0.9-2.2 for total DNA, of 1.2-3.3 for
699 fungal gene copy numbers and of 0.8-1.5 for NEA, respectively. In contrast, the
700 prevalence of archaeal copy numbers and soil respiration was in a range of 2.6-1.0 and
701 2.0-1.3, decreasing with rice cultivation lengths. Therefore, most of analyzed carbon
702 pools and bioactivities were dominated by the macro- and large micro-aggregates in
703 sand sized fractions, which was in general consistent directional change with prolonged
704 paddy management under long term rice cultivation though clay particles were
705 consistently increased (Kölbl et al., 2014).

Fig. 7

707 Long term OC sequestration in agricultural soils had been questioned (Powlson et al.,
708 2011) and OC enriched in coarse sand fractions of aggregates could indeed be subject

709 to fast decomposition in dry condition, for example, after shifting to maize land (Li et
710 al., 2007a). In this study, however, hydroagric paddy management was kept continuing
711 with ever prolonged rice cultivation, which could have driven the ever increasing trend
712 of OC accumulation up to millennium (Wissing et al., 2011; 2013). Consequently, OC
713 accumulation and stabilization could ever take place in sand sized aggregates with
714 physical protection of labile OC pool intra micro-aggregates, with prolonged rice
715 cultivation (Wang et al., 2015). POC, as a pool of relatively fast turnover (Cambardella
716 and Elliott, 1992), had been also kept increasing in paddies cultivated for centuries
717 (Wang et al., 2015). Allison and Jastrow (2006) suggested that microbial biochemical
718 activity and carbon turnover was stronger in POC-enriched size fractions, but weaker
719 in mineral-dominated fractions where enzymes and their carbon substrates were
720 immobilized on mineral surfaces. Long term hydroagric paddy management (Zhang
721 and Gong, 2003) reduced decomposition of root-, crop- or microbial- residue input
722 under reduced conditions (Roth et al., 2011). Moreover, the changes in relative
723 proportion of carbon pools and microbial activities (NEA and soil respiration) by sand
724 sized aggregates further demonstrated that physically protected and stabilized carbon
725 supported high soil bioactivities in macro-aggregates, which had been increasingly
726 prevailed over the smaller sized fractions of soil aggregates.
727 The changes in OC pools and the accessibility to microbes could lead to changes in the
728 relative abundance and activity of microbes, potentially affecting C cycling and storage,
729 in different size aggregates (Six et al., 2006). Unlike the finding by Allison and Jastrow
730 (2006), this study proposed enhanced microbial activity but improved carbon use

731 efficiency with reduced respiration quotient for microbial energy in coarse sand sized
732 macro-aggregates, compared to clay fraction over centuries of rice cultivation. This
733 could be supported by the recent finding that $qCO_2$ was seen reduced but microbial
734 biomass carbon increased in biochar amended agricultural soils, in a case study by
735 Zheng et al., (2016) and in a meta-analysis by Zhou et al (2016). This study indicated a
736 strong inter-link between microbiological activity and labile OC in large sized
737 aggregates of paddy soils, though the later had been generally considered as physically
738 protected OC. As strengthened with prolonged rice paddy management, such a link
739 could help enhance ecosystem functioning and services provided by rice soils (Six and
740 Paustian 2014; Smith et al., 2015).
741 Of course, the methodology used here could not allow to characterize the spatial
742 allocation of carbon substrate, specific microbial communities and extracellular
743 enzyme activities among the aggregate fractions. Specially, labile OC pools,
744 particularly those intra- aggregates or inter micro-aggregates within macro-aggregates,
745 could not be further explored. Such data had been considered critical to unravel the
746 micro-scale process mediating bio-activities at aggregate level (Six and Paustian 2014).
747 Therefore, the effects on soil functions deserve further studies under field conditions.

748 **5 Conclusions**

749 This study, using a rice soil chronosequence derived from salt marsh, revealed that soil
750 organic carbon could be accumulated and stabilized both in coarse sand- and clay- sized
751 fractions of soil aggregates. However, microbial abundance and enzyme activity were
752 high but metabolic quotient low in sand sized fractions rather than in silt and clay sized

753 fractions of soil aggregates, possibly through the enhanced spatial allocation of labile
754 OC pool for improved microhabitat condition in larger sized aggregates. Thus, carbon
755 stabilization with reduced turnover was not confronting soil bioactivities in a way that
756 carbon and microbial communities biophysically co-evolved in macro-aggregates other
757 than in silt and clay sized micro-aggregates. This study further supported our previous
758 finding for bulk soils that long term rice cultivation led to accumulation and
759 stabilization of SOC and promoted soil biological activities through physical protection
760 of labile carbon in line with enhanced soil aggregation. Thus, labile organic carbons
761 accumulated in macro-aggregates could help enhancing microbial C use efficiency and
762 improving their biogeochemical activity related to ecosystem functioning. More studies
763 are deserved on interaction of soil organic matter, minerals and soil microbial
764 communities to unravel the micro-scale process mediating bio-activities at aggregate
765 level.

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772

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## Figure captions

**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.

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

**Fig. 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.

**Fig. 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 circle are those fractions from the uncultivated marsh soil (P0). Above or below the black long dashed line representing OC enrichment or depletion in a fraction.

**Fig. 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 (right) sized aggregates fractions (Soil organic carbon accumulation as a function of relative recalcitrant C (aromatic and phenol) (a) and negatively of relative labile C (aliphatic and polysaccharide) (b); $CO_2$ production as a plateau

function of soil microbial biomass (c) and bacterial abundance (d)). Data was the mean value of triplicates.

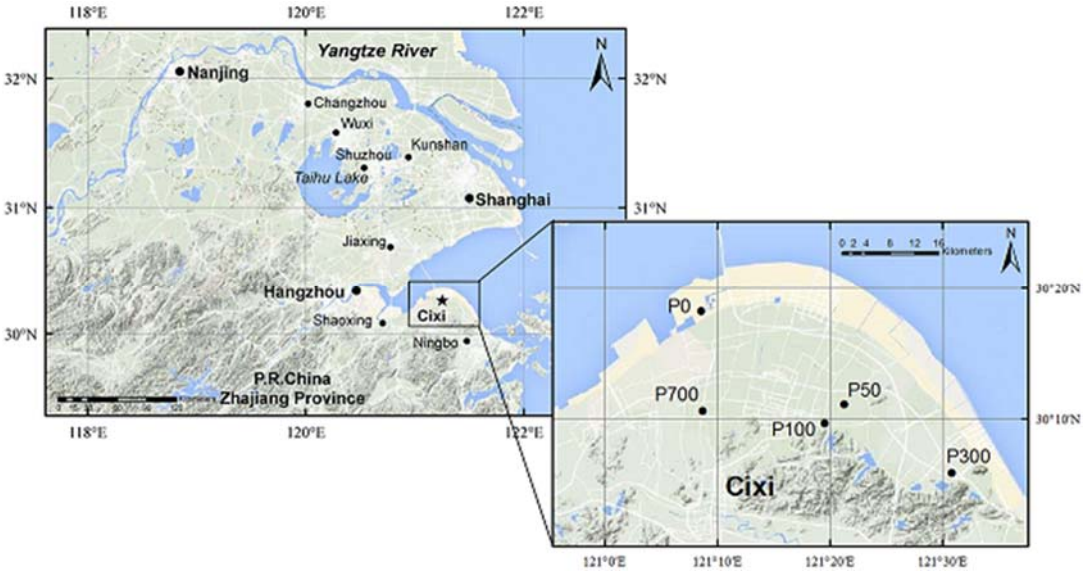
**Fig. 6** Inter-correlation between particulate organic carbon and soil microbial activity to compare the biological activity versus carbon between coarse sand (left) and clay (right) sized aggregate fractions (Soil microbial biomass was as an exponential function of total soil organic carbon (a) and a linear function of labile organic carbon (d). Normalized enzyme activity (b) and DNA content scaled $CO_2$ 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 was the mean value of triplicates.

**Fig. 7** Change in partitioning of soil organic carbon (a, g/kg), total DNA (b, μg/g) , normalized enzyme activity (c, relative enzyme activity index) and soil respiration (d, mg$CO_2$/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 in a row is relevant to that of an analyzed parameter among the soils.

**Supplement material**

**Supplement Figure 1.** FTIR spectrum of aggregate size fractions of the paddy soil chronosequence (a: 2000-200μm; b: 200-20μm; c: 20-2μm; d: <2μm). The code of P0 and P50-P700 denotes respectively the uncultivated marsh soil, and soils shifted under rice cultivation for 50-700 years.

**Supplement Table 1.** Mean soil respiration quotient (portion of respired $CO_2$-C to SOC) and soil metabolic quotient (ratio of respired $CO_2$-C to MBC) of the soil aggregate size fractions estimated using the data in Table 3 in the text. N.d., not determined due to the very small amount of the fraction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Shanghai
Jiaxing
Hangzhou
Shaoxing
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P.R.China
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121°20'E
121°30'E

**Fig. 1**

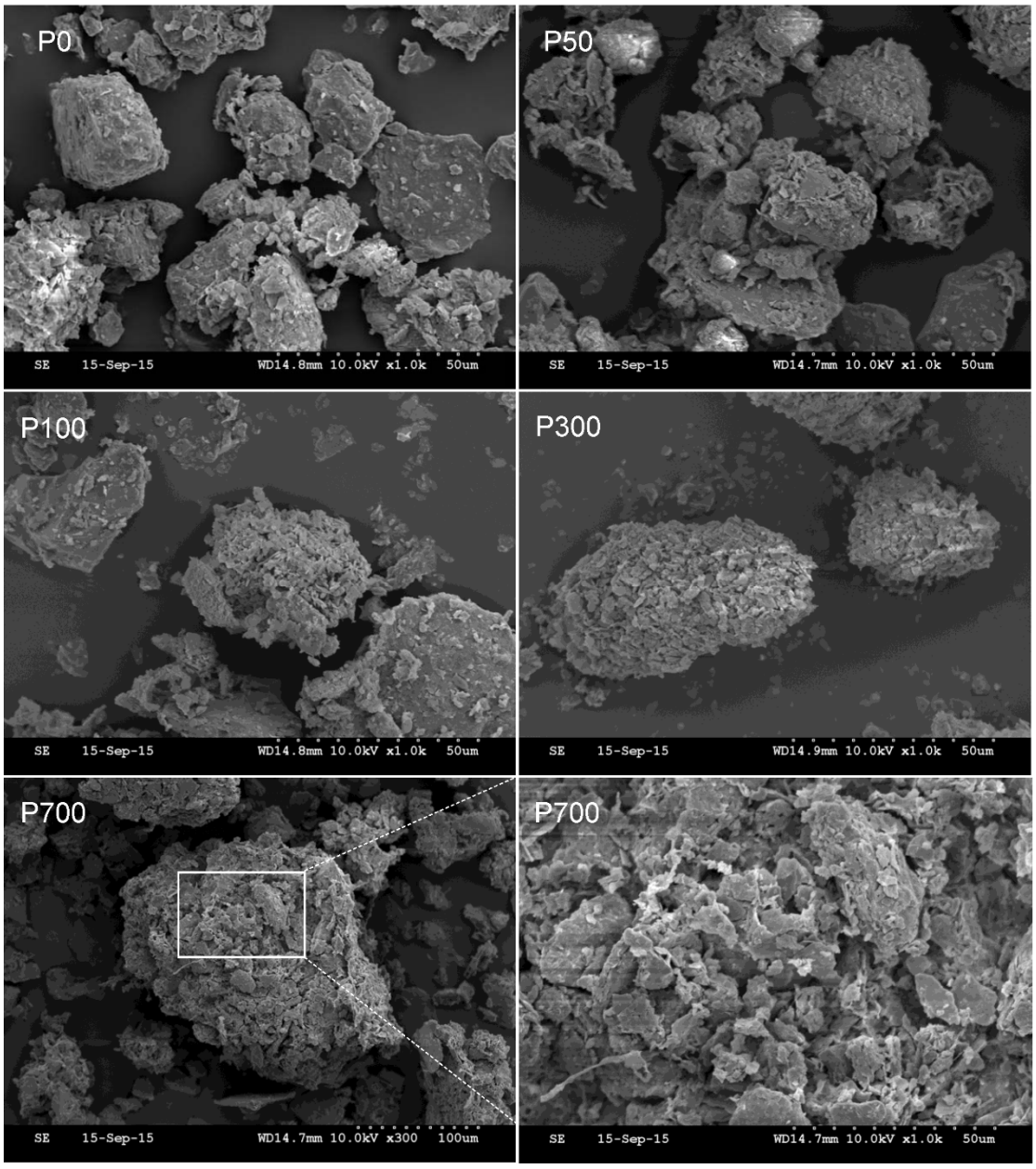
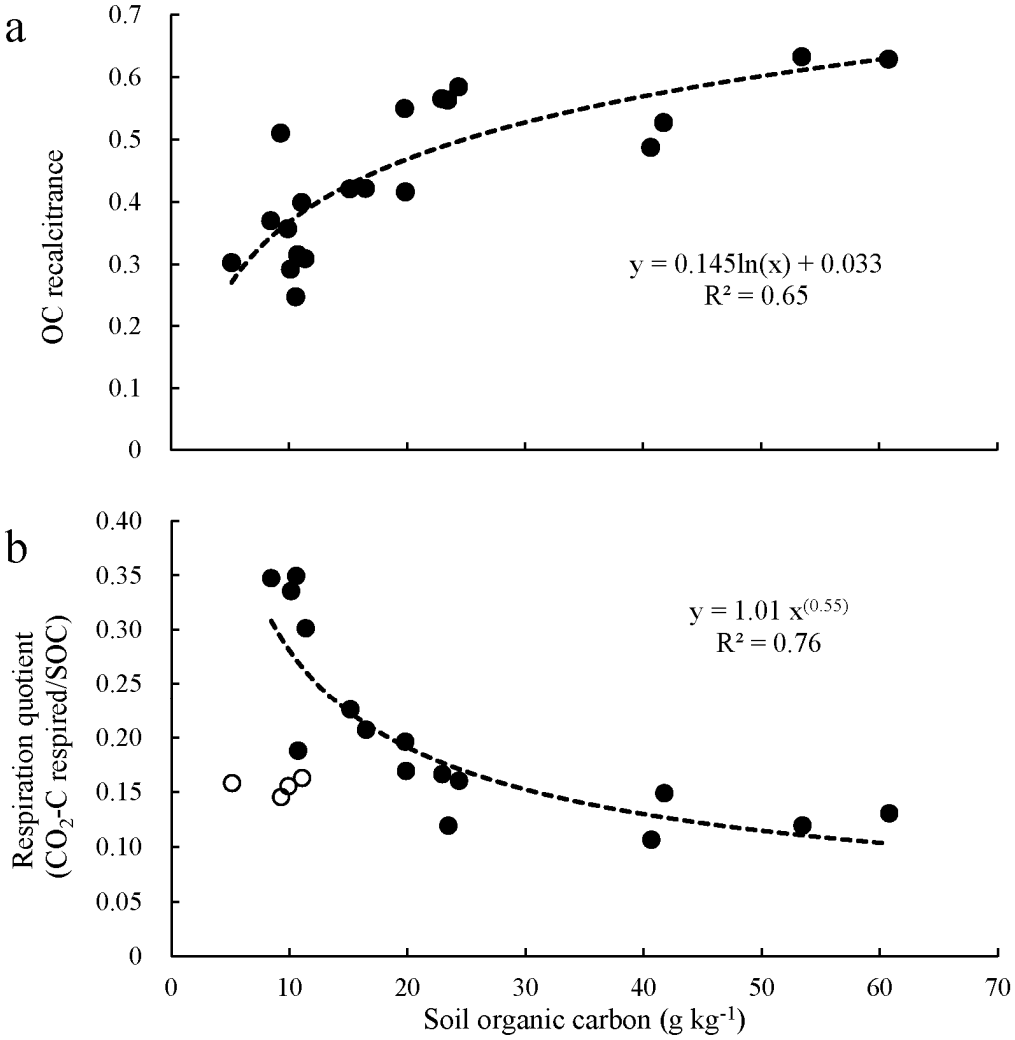

P0
SE 15-Sep-15 WD14.8mm 10.0kV x1.0k 50um
P50
SE 15-Sep-15 WD14.7mm 10.0kV x1.0k 50um
P100
SE 15-Sep-15 WD14.8mm 10.0kV x1.0k 50um
P300
SE 15-Sep-15 WD14.9mm 10.0kV x1.0k 50um
P700
SE 15-Sep-15 WD14.7mm 10.0kV x300 100um
P700
SE 15-Sep-15 WD14.7mm 10.0kV x1.0k 50um

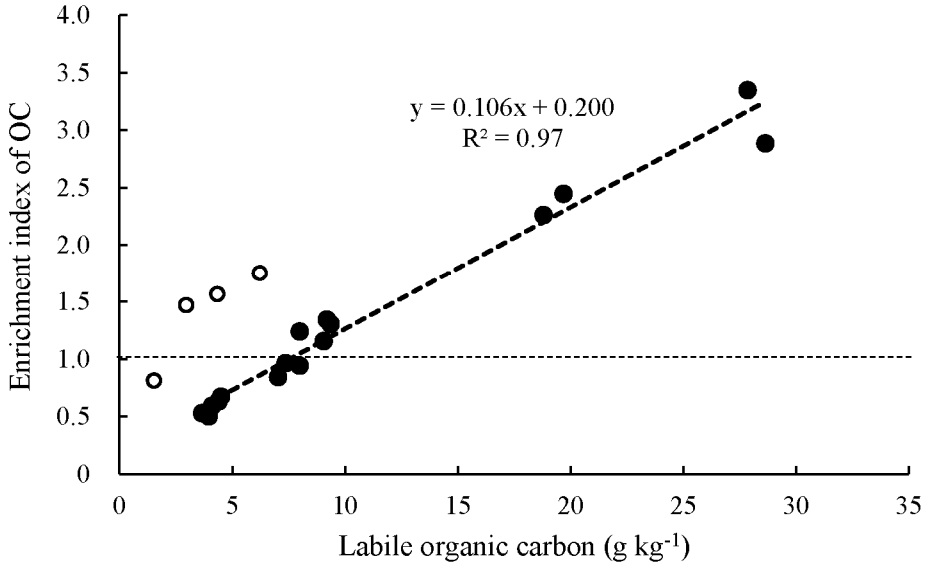
**Fig. 2**

1209




1210

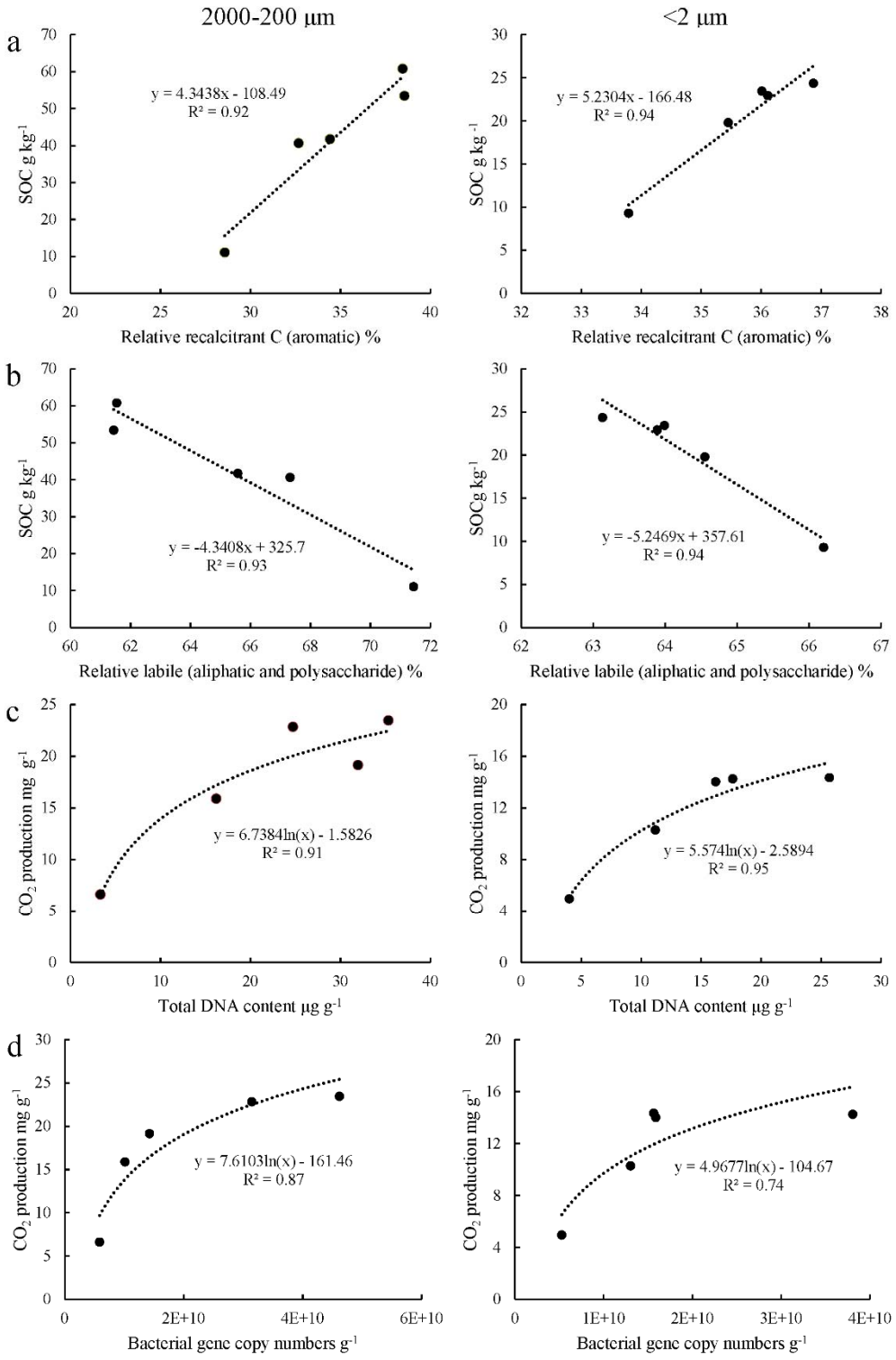
1211 **Fig. 3**




1212

1213 **Fig. 4**

1214




1215

1216 **Fig. 5**

1217

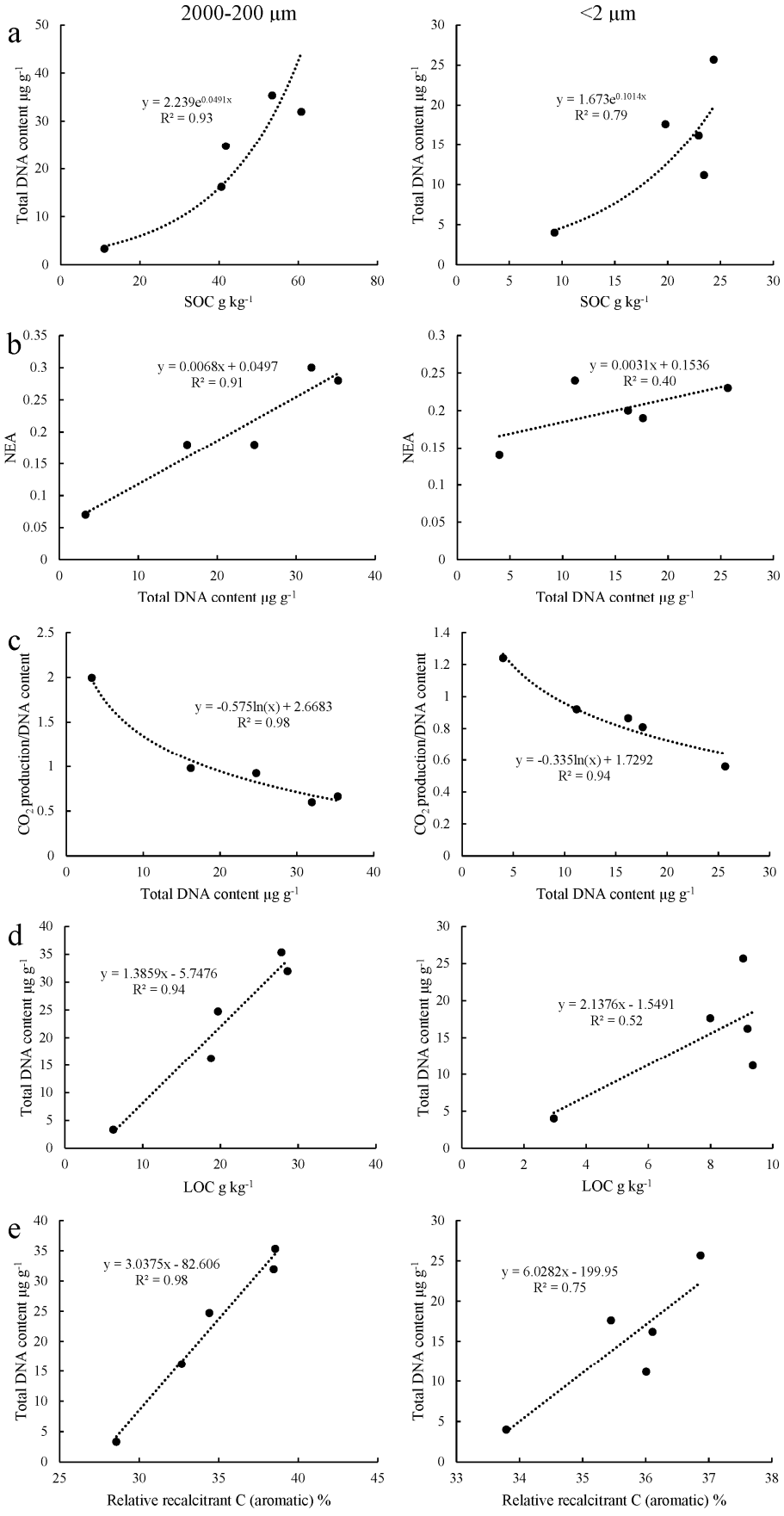
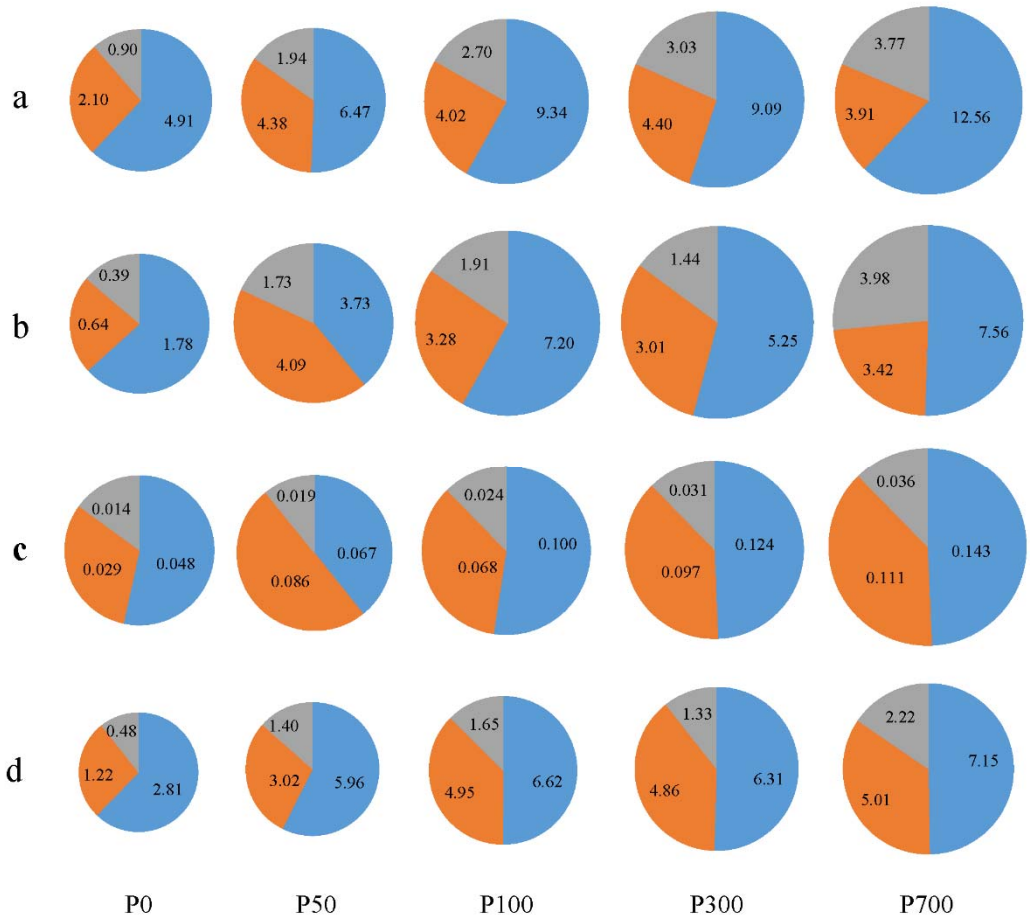

2000-200 µm
<2 µm
a
Total DNA content µg g-1
y = 2.239e0.0491x
R² = 0.93
y = 1.673e0.1014x
R² = 0.79
SOC g kg-1
b
NEA
y = 0.0068x + 0.0497
R² = 0.91
y = 0.0031x + 0.1536
R² = 0.40
Total DNA content µg g-1
Total DNA contnet µg g-1
c
CO2 production/DNA content
y = -0.575ln(x) + 2.6683
R² = 0.98
y = -0.335ln(x) + 1.7292
R² = 0.94
Total DNA content µg g-1
d
y = 1.3859x - 5.7476
R² = 0.94
y = 2.1376x - 1.5491
R² = 0.52
LOC g kg-1
e
y = 3.0375x - 82.606
R² = 0.98
y = 6.0282x - 199.95
R² = 0.75
Relative recalcitrant C (aromatic) %

**Fig. 6**




1220

1221 **Fig. 7**

1222

1223 **Table 1** Basic properties of the studied soils of the chronosequence (Mean ± SD,

1224 $n = 3$)

| Soil | pH ($H_2O$) | Total OC ($g\ kg^{-1}$) | Total N ($g\ kg^{-1}$) | BD ($g\ cm^{-3}$) | CEC ($cmol\ kg^{-1}$) | Fed ($g\ kg^{-1}$) |
|---|---|---|---|---|---|---|
| P0 | 8.62±0.07 | 6.32±0.58 | 0.79±0.02 | 1.31±0.05 | 6.32±0.34 | 1.76±0.02 |
| P50 | 7.84±0.04 | 15.96±0.66 | 1.81±0.06 | 1.13±0.03 | 12.82±0.06 | 1.96±0.01 |
| P10 | 6.39±0.05 | 17.07±0.49 | 2.06±0.09 | 1.06±0.04 | 12.54±0.12 | 2.04±0.04 |
| P30 | 6.40±0.03 | 17.97±0.81 | 2.09±0.08 | 1.07±0.07 | 13.78±0.26 | 2.08±0.05 |
| P70 | 6.65±0.08 | 21.07±1.21 | 2.14±0.06 | 1.06±0.05 | 12.97±0.27 | 1.71±0.02 |

1225 Note: BD, bulk density; CEC, cation exchange capacity; Fed: dithionate extractable

1226 iron oxyhydrates.

1227

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

| 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 |

1233 **Table 3** Total OC, total N and LOC in g $kg^{-1}$ and SMBC in mg $kg^{-1}$ of the size
1234 fractions of the size fractions (PSFs) of the chronosequence soils. Different capital
1235 and low case letters indicate a significant ($p$<0.05) difference respectively between
1236 fractions of a single soil, and between soils for a single fraction, in a single column.

| PSF | Soil | Total OC | Total N | LOC | SMBC |
|---|---|---|---|---|---|
| Coarse sand | P0 | 11.07±1.20Ad | 1.04±0.11Ad | 6.22±0.18Ac | not determined |
| | P50 | 53.44±1.09Ab | 4.15±0.49Aa | 27.85±1.61Aa | 794.7±47.0Ac |
| | P100 | 41.74±1.31Ac | 3.37±0.38Ab | 19.69±1.16Ab | 1051.8±73.7Ab |
| | P300 | 40.64±1.57Ac | 2.72±0.12Ac | 18.80±1.45Ab | 1385.5±88.1Aa |
| | P700 | 60.79±1.88Aa | 4.43±0.22Aa | 28.64±1.90Aa | 1479.9±166.2Aa |
| Fine sand | P0 | 9.90±0.43Ac | 1.01±0.14Ac | 4.34±0.14Bb | 188.0±8.0Ac |
| | P50 | 8.45±0.27Cc | 0.73±0.11Dd | 3.66±0.57Cb | 309.2±16.5Bb |
| | P100 | 16.48±0.41Cb | 1.57±0.14Cb | 7.36±0.32Ca | 441.1±13.4Ba |
| | P300 | 15.16±1.45Cb | 1.51±0.13Bb | 7.03±0.30Ca | 445.9±28.2Ba |
| | P700 | 19.86±1.11Ca | 1.81±0.12Ca | 7.99±0.65Ba | 449.9±25.9Ba |
| Silt | P0 | 5.13±0.19Bb | 0.52±0.14Bd | 1.53±0.13Db | 166.7±4.5Ad |
| | P50 | 10.73±0.55Ba | 1.20±0.11Cb | 4.50±0.13Ca | 296.2±15.0Bc |
| | P100 | 10.13±0.44Da | 1.15±0.09Cc | 4.10±0.26Da | 287.0±2.7Cc |
| | P300 | 11.37±0.58Da | 1.33±0.11Ba | 4.39±0.29Da | 392.1±15.0Ba |
| | P700 | 10.57±0.43Da | 1.11±0.08Dc | 3.95±0.69Ca | 348.3±10.5Cb |
| Clay | P0 | 9.29±0.29Ac | 1.17±0.15Ad | 2.96±0.27Cc | 155.6±18.1Ac |
| | P50 | 19.80±1.47Bb | 2.27±0.14Bc | 7.99±0.28Bb | 284.9±19.7Bb |
| | P100 | 22.94±1.43Ba | 2.70±0.12Bb | 9.19±0.35Ba | 279.4±5.0Cb |
| | P300 | 23.45±1.46Ba | 2.92±0.12Aa | 9.36±0.40Ba | 324.8±13.1Ca |
| | P700 | 24.36±1.65Ba | 2.73±0.16Bb | 9.05±0.47Ba | 325.7±8.1Ca |

1237 Table 4 Relative proportion (%) of carbon chemical groups and carbon recalcitrance
1238 (ratio of aromatic to polysaccharide carbon) in size fractions by FTIR analysis.
1239 Different capital and low case letters indicate a significant ($p$ <0.05) difference
1240 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±1.41Bc | 0.03±0.00Ac | 71.41±5.76ABa |
| | P50 | 38.55±5.73Aab | 0.50±0.09Aa | 60.94±2.54Cb |
| | P100 | 34.43±3.78ABab | 0.27±0.03Ab | 65.31±4.72Bab |
| | P300 | 32.67±0.78ABb | 0.28±0.04Ab | 67.04±4.66BCab |
| | P700 | 38.47±1.59Aa | 0.37±0.03Ab | 61.17±4.30Cb |
| Fine sand | P0 | 26.30±1.57Ba | 0.05±0.01Ab | 73.64±4.83ABa |
| | P50 | 26.98±1.15Ba | 0.04±0.00Bb | 72.98±4.43ABa |
| | P100 | 29.62±1.07Ba | 0.13±0.03Ba | 70.24±3.47ABa |
| | P300 | 29.60±1.42Ba | 0.07±0.02Bb | 70.32±4.60ABa |
| | P700 | 29.33±1.28Ba | 0.17±0.02Ba | 70.51±4.09Ba |
| Silt | P0 | 23.22±1.27Ca | 0.01±0.00Ba | 76.76±3.81Aa |
| | P50 | 23.98±1.50Ca | 0.01±0.00Ca | 76.02±4.29Aa |
| | P100 | 22.61±1.32Ca | 0.00±0.00Db | 77.37±4.73Aa |
| | P300 | 23.61±1.14Ca | 0.00±0.00Db | 76.39±4.21Aa |
| | P700 | 19.87±0.83Cb | 0.00±0.00Db | 80.14±3.87Aa |
| Clay | P0 | 33.78±1.69Aa | 0.00±0.00Bb | 66.20±3.2B2a |
| | P50 | 35.46±1.36Aa | 0.03±0.00Ba | 64.52±4.23Ba |
| | P100 | 36.10±1.74Aa | 0.04±0.01Ca | 63.85±4.57Ba |
| | P300 | 36.02±1.72Aa | 0.03±0.01Ca | 63.96±4.65Ca |
| | P700 | 36.86±1.88Aa | 0.05±0.01Ca | 63.08±3.73Ca |

1241

**Table 5** DNA content (μg $g^{-1}$), copy numbers of bacterial (BA, copies×$10^9 g^{-1}$), fungi (FA, copies×$10^7 g^{-1}$) and archaeal (ArA, copies×$10^8 g^{-1}$) of the size fractions. Different capital and low case 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±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 |
| Clay | 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 |
| | 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 |

**Table 6** Normalized enzyme activity (NEA) and soil respiration (mg $CO_2$ $kg^{-1}$) of the chronosequence soils. Different capital and low case 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 | P0 | 0.07±0.01Bc | 662±66Ac |
| | P50 | 0.28±0.03Aa | 2345±805Aab |
| | P100 | 0.18±0.01Ab | 2283±506Aab |
| | P300 | 0.18±0.01Bb | 1588±309Ab |
| | P700 | 0.30±0.05Aa | 2914±190Aa |
| Fine sand | P0 | 0.10±0.01Bc | 565±153ABb |
| | P50 | 0.12±0.03Cc | 1076±139Ba |
| | P100 | 0.21±0.03Ab | 1252±103Ba |
| | P300 | 0.27±0.03Aa | 1256±096Aa |
| | P700 | 0.30±0.02Aa | 1234±143Ba |
| Silt | P0 | 0.07±0.01Bd | 298±053Cc |
| | P50 | 0.21±0.02Bb | 740±258Bb |
| | P100 | 0.17±0.01Ac | 1246±063Ba |
| | P300 | 0.25±0.02Ab | 1256±071Aa |
| | P700 | 0.30±0.02Aa | 1354±095Ba |
| Clay | P0 | 0.14±0.01Ac | 496±053Bb |
| | P50 | 0.19±0.02Bb | 1425±430Aa |
| | P100 | 0.20±0.02Aab | 1401±289Aa |
| | P300 | 0.24±0.02Aa | 1028±226Aa |
| | P700 | 0.23±0.01Ba | 1434±196Ba |