

The stoichiometric C-Fe ratio regulates glucose mineralization and stabilization via microbial processes

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1	The stoichiometric	C-Fe ra	atio regulates	glucose	mineralization	and	stabilization	via
2	microbial processes							

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25 Abstract

The association of soil organic matter (SOM) with iron (Fe) oxides by adsorption and/or co-26 precipitation contributes to long term C stabilization in soil. While there is an understanding of 27 the relationship between soil carbon (C) and the biogeochemical cycling of Fe, a lack of 28 information exists on the role of Fe oxides on the accumulation of C in paddy soils. This study 29 aimed to assess the role of Fe (oxyhydr)oxides on mineralization and stabilization processes 30 following amendment of paddy soil with a labile C substrate (99 atom % ¹³C-glucose). The 31 study utilized 4 paddy soils with a total Fe concentration ranging from 13.7 to 55.8 g kg⁻¹. In 32 soils with 42.7 and 55.8 g kg⁻¹ Fe, the addition of glucose resulted in an Fe bound organic C: 33 34 Fe molar ratio (C:Fe molar ratio) \geq 6, suggesting the formation of Fe-OM complexes mainly via co-precipitation. The highest portion of ¹³C (13.8%) protected in Fe-OM complexes was 35 found in soil containing 55.8 g kg⁻¹ of Fe. The stabilization of the added labile C substrate was 36 shown, using random forest analysis, to be controlled by the C: Fe molar ratio, while substrate 37 mineralization was regulated by the core genera Sphingomonas and Devosa (r-strategists) 38 (affiliated to Proteobacteria) and C:N ratio. Substrate mineralization was 47 % lower in soil 39 containing 55.8 g Fe kg⁻¹ compared to 13.7 g Fe kg⁻¹, with a concomitant reduction in SOM 40 priming of 37 %. This reduction in substrate mineralization and the priming effect was likely 41 due to lower C substrate availability via the formation of Fe-OM complexes, thereby protecting 42 the C from mineralization. In conclusion, the Fe concentration in paddy soils plays a central 43 role in the abiotic stabilization of 'new' C through the formation of Fe-OM complexes via co-44

45 precipitation, thereby limiting the availability of this C substrate for microbial mineralization,46 and at the same time modulating the microbial community structure.

47

Keywords: Fe gradient, co-precipitation, Fe organic matter complexes, Bacterial and fungal
DNA amplification and sequencing, Shannon index, random forest analysis, structural equation
modeling

51

52 **1. Introduction**

Paddy soils play an important role in the storage of soil carbon C due to their unique 53 geochemical properties (Ge et al., 2012; Wiesmeier et al., 2019; Wei et al., 2019). Iron (Fe) 54 55 plays an important role in many physicochemical processes, including the stabilization of soil organic matter (SOM) (Kaiser and Guggenberger, 2000; Boyd and Ellwood, 2010; Jeewani et 56 57 al., 2020); as well as biotic processes resulting in Fe adsorption, co-precipitation and mineral transformation (Jones, 1998; Chen et al., 2014; Huang et al., 2018; Wan et al., 2019). Previous 58 studies have coupled the biogeochemical cycling of Fe with SOM (Ponnamperuma, 1972; 59 60 Huang et al., 2018; Wiesmeier et al., 2019; Liu et al., 1029). Unlike C/N/P stoichiometry, only a limited number of studies have evaluated the mechanisms and effect of C:Fe molar ratio on 61 SOM accumulation in paddy soils (Zhou et al., 2009; Song et al., 2016). Therefore, a better 62 understanding of C-Fe cycling and the role of the soil microbial community is needed to 63 optimize C storage in paddy soils. 64

Iron oxides and oxyhydroxides (for simplicity, these are defined as Fe oxides henceforth) constitute a range of pedogenic minerals that are involved in a range of physicochemical processes associated with the stabilization of SOM (Chen et al., 2020; Cornell and Schwertmann, 1996; Li et al., 2020a). Fe oxides, which contain abundant hydroxyl groups

at their surfaces, can form stable organo-mineral complexes by interacting with carboxyl 69 groups of SOM (Kleber et al., 2015), thus stabilising the SOM (Chen et al., 2020). Iron-organic 70 71 matter associations (Fe-OM) play an essential role in C sequestration (Silva et al., 2015; Wang et al., 2017; Wiesmeier et al., 2019). They have been shown to stabilize 21.5% of the SOM in 72 ocean sediments as well as 37.8% of the organic C in forest soils (Lalonde et al., 2012; Zhao 73 et al., 2016; Xue et al., 2020). Importantly, the Fe bound C: Fe molar ratio (C:Fe molar ratio) 74 75 can elucidate the binding mechanism between SOM and Fe oxides in soil (Coward et al., 2018; Chen et al., 2020). When C:Fe molar ratio > 1, SOM and Fe oxides may be associated via co-76 77 precipitation mechanisms (Wagai et al., 2013). In contrast, C:Fe molar ratios < 1 indicate the formation of Fe-OM associations via adsorptive mechanisms (Coward et al., 2018). Fe-OM 78 complexes can occur in paddy soil due to changes in pH or redox potential, where the Fe bound 79 80 C:Fe molar ratios of soil are 0.2-6 (Katoh et al., 2004; Cheng et al., 2010; Henneberry et al., 2012). This is largely due to the sorption capacity of Fe oxides towards dissolved organic C 81 (DOC) (Chen et al., 2014; Chen et al., 2020; Kaiser and Guggenberger, 2000) and the affinity 82 of dissolved Fe phases to form co-precipitates with DOC (Kleber et al., 2015). Co-precipitation 83 can change the surface properties of the minerals (e.g., specific surface area and surface charge) 84 and affect the interaction and stability of Fe-OM associations (Chen et al., 2014; Xue et al., 85 2020). Recently, several studies have evaluated the mechanisms of formation, properties, and 86 reactivity of Fe-OM complexes derived from co-precipitation with different C sources, such as 87 rhizodeposits and forest-floor extracts (Chen et al., 2014; Mikutta et al., 2014; Jeewani et al., 88 2020), humic and fulvic acids (Angelico et al., 2014) and polysaccharides (Mikutta et al., 89 2008). It was shown that glucose could absorb to Fe oxide via hydrogen bonding and co-90 precipitation (Olsson et al., 2011; Chen et al., 2014). 91

92 The biochemical nature of SOM plays a critical role in mediating the source of energy
93 for microbial metabolism in soil (Baldock et al., 2004; Li et al., 2020b; Wang et al., 2017).

According to Huang et al. (2020), rhizodeposits and plant litter are the primary sources of C 94 for soil microbial activity, thus contributing to soil respiration and formation of SOM. Glucose 95 is a central metabolite and an abundant, available biomolecule that is easily and rapidly 96 mineralized by microorganisms in soils (Canarini et al., 2019; Liu et al., 2019; Van Hees et al., 97 2005). Therefore, knowledge about the pathway of microbial metabolism and mineralization 98 of easily available substances in the soil is crucial to obtain a better understanding of SOM 99 100 formation and mineralization processes. However, few studies have evaluated the contribution 101 of Fe in paddy soils to the formation of Fe-OM complexes with a labile C substrate to mimic 102 rhizodeposition, where plant exudates are key contributors to the DOC pool.

Soil microorganisms are the critical mediators of the biogeochemical cycling of Fe in soil 103 (Whitman et al., 2018; Jeewani et al., 2020). Microbial diversity and community composition 104 strongly affect substrate mineralization (Kamble and Bååth, 2016; Li et al., 2018 Fu et al., 105 2020). Generally, bacteria prefer to mineralize labile compounds and dominate in the initial 106 107 phases of mineralization (Garcia-Pausas and Paterson, 2011). Liang et al. (2014) noted that the dominant bacterial phyla Proteobacteria, Firmicutes, and Actinobacteria play a crucial role in 108 the early mineralization process. Substrate addition to the soil can impact upon native SOM 109 decomposition, a process called the soil priming effect (PE) (Dimassi et al., 2014; Zhang et al., 110 2019; Yu et al., 2018; Zhu et al., 2018; Lu et al., 2019), by modifying microbial activity and 111 community composition (Kuzyakov, 2002; Perveen et al., 2019). Thus there is a need to 112 understand the role of differing Fe concentrations in soil on the accessibility of labile C 113 substrates and the subsequent effect on microbial community composition. 114

115 The objective of the study was to elucidate the role of inherent soil Fe concentration on 116 glucose mineralization, the soil priming effect, and 'new' C stabilization. We hypothesize that: 117 i) substrate mineralization is lower and stabilization of substrate-derived C is higher with 118 increasing soil Fe oxide concentration due to the stabilization of substrate-derived C as Fe-OM 119 complexes, and ii) the increase in Fe oxide concentration will modulate the microbial 120 communities and their substrate utilization pattern associated with the geochemical cycling of 121 C and Fe. To test these hypotheses, we conducted a 56-day laboratory incubation using 4 122 similar paddy soils but with a natural Fe gradient. We supplied ¹³C labeled glucose to represent 123 rhizodeposition, and investigated the mineralization and the stabilization of added substrate, 124 and the interactions with the soil microbial community composition.

125

126 **2. Materials and methods**

127 2.1. Soil sampling and preparation

128 Soils were sampled from the 0–20 cm (plough layer) of long-term rice (*Oryza sativa*) paddies in Hunan province, China. Soil sampling was done at 4 sites along a 200 m transect 129 representing an Fe concentration gradient, with three replicates collected within a 1 m² plot at 130 each sampling location. Ethanol (70%) was used to sterilize equipment during sampling to 131 avoid microbial cross-contamination between soils. The sampling region had a mean annual 132 temperature of 17.7 °C, and a rainfall of 1402 mm (Table 1). The soil was classified as a Gleyic 133 Stagnic Anthrosol (FAO) (Iuss Working Group, 2015). The soils had Fe concentrations of 13.7 134 g Fe kg⁻¹ (referred to as Fe-13), 25.8 g Fe kg⁻¹ (Fe-25), 42.7 g Fe kg⁻¹ (Fe-42) and 55.8 g Fe kg⁻¹ 135 ¹ (Fe-55) (Table 1). The soil samples had visible plant roots manually removed and were sieved 136 at field moisture (<2 mm) before being air-dried. Prior to a 14-day pre-incubation period (at 137 25°C), water holding capacity (WHC) of each soil was determined using a volumetric method 138 (see supplementary information). Soils were then sprayed with deionized water to obtain a 139 water content of 40% that of the WHC. This soil preparation protocol mimics the field situation, 140 where the soil stays mostly dry before rice cultivation and avoids the formation of reducing 141 conditions after addition of substrates during the incubation period. 142

144 2.2. Experimental design

An incubation experiment (over 56 days) was conducted to examine the priming effects 145 and substrate mineralization using a natural Fe gradient in soil. For each of the 4 soils, there 146 was a control (no glucose amendment) (n=3) and a ¹³C -glucose amendment (n=3), to make a 147 total of 24 incubation jars. Each incubation treatment consisted of a 500 mL glass jar, which 148 had a rubber bung seal. Within each jar, 40 g of oven-dry equivalent soil was contained within 149 a glass vessel along with a CO₂ trap (20 mL 1 M NaOH), which was replaced at several 150 sampling times. To maintain then humidity with in the jars, 10 mL water was maintained in the 151 base of the jar. Labeled glucose (99%-¹³C) (Cambridge isotope laboratories) was applied at 152 500 µg C g⁻¹ soil (1.5-2.9 % total C) soil by evenly mixing with each 40 g of soil. The jars were 153 sealed with a rubber bung and incubated at 25 ⁰C for 56 days. Three blank jars containing only 154 water and CO₂ traps were also included. The incubation jars were opened periodically to 155 maintain aerobic conditions. After the 56 days of incubation, the soil was homogenized by 156 mixing and separated into two uniform batches, one being freeze-dried for the extraction of 157 DNA, and the second was allocated for further analysis. The ¹³C distribution was analyzed in 158 the bulk soil and Fe-OM complexes to estimate the mechanisms of substrate-derived C 159 stabilization along the Fe concentration gradient. 160

161

162 2.3. Soil physical and chemical analyses

Total soil C and N content (air-dried, milled <200 μm) was determined by dry
combustion using a Perkin Elmer EA2400, Shelton, CT, USA. Soil pH was measured at a soil:
water ratio of 1:2.5 (w/w) using a micro-electrode (Seven Compact, Mettler Toledo Inc.,
Switzerland). Microbial biomass C (MBC) was determined using the chloroform fumigation

extraction method (Vance et al., 1987). Fumigated and unfumigated portions of 15 g moist 167 soil were extracted with 30 ml 0.5M K₂SO₄. Organic C in the extracts was measured using a 168 TOC analyser (Multi N/C 3100, Analytik Jena AG, Jena, Germany). Microbial biomass C was 169 calculated as EC/kEC, where EC= (organic carbon extracted from fumigated soils) - (organic 170 carbon extracted from non-fumigated soils) and kEC=0.45 (Wu et al., 1990). DOC was 171 determined from the non-fumigated component of the soil. ¹³C in soil microbial biomass C was 172 determined as described by Luo et al. (2013). The natural abundance of δ^{13} C in soils was 173 measured using air-dried and sieved ($<200 \,\mu$ m) soil, which was accurately weighed (about 0.2 174 175 mg) into tin capsules prior to analysis using an isotope ratio mass spectrometer with IAEA-600 (Caffeine); δ^{13} C=-27.771‰ as standard material (Meng et al., 2013) (Thermo Fisher Scientific, 176 DELTA V plus IRMS, Bremen, Germany) coupled with an elemental analyzer (EA NA1500 -177 EA 1110 device, Carlo Erba and Thermo Fisher Scientific, Bremen, Germany). 178

The total Fe concentration of the soil sample was determined after digestion with a 179 concentrated acid mixture of HF, HClO₄, and HNO₃ (Zhou et al., 2007). Dithionite-citrate-180 bicarbonate (DCB) extraction method was used to quantify the total extractable soil iron oxide 181 (Fe_d) concentration by determining the Fe concentration in the solution (Lalonde et al., 2012; 182 Wang et al., 2017; Wan et al., 2019). Freeze-dried soil (0.50 g) was mixed with 30 mL of buffer 183 solution (0.27 M trisodium citrate and 0.11 M sodium bicarbonate, pH 7.3) in 50 mL centrifuge 184 185 tubes, which was then placed in a water bath (80 \degree C). Sodium dithionite (0.50 g) was added to the mixture, and it was maintained at 80 °C for 15 min. Soils were extracted by sodium chloride 186 (NaCl) instead of trisodium citrate and sodium dithionite, at an equivalent ionic strength as the 187 control under the same conditions. The suspension was then separated by centrifugation at 4000 188 x G for 10 min. The residue was thrice rinsed with ultra-pure H₂O (5 mL), centrifuged, and the 189 supernatant was carefully removed. The precipitate was freeze-dried overnight. Organically 190 complexed Fe oxides (Fe_p) and soil amorphous Fe oxides (Fe_p) were extracted with sodium 191

pyrophosphate and ammonium oxalate, respectively (Keiluweit et al., 2015; Wan et al., 2019). 192 The TC and TN in the extracted residue was analyzed using an elemental analyzer, and the 193 δ^{13} C isotopic composition of the residue was obtained by an isotope ratio mass spectrometer 194 (Thermo Fisher Scientific, DELTA V plus IRMS, Bremen, Germany) coupled with an 195 elemental analyzer (EA NA1500 - EA 1110 device, Carlo Erba and Thermo Fisher Scientific, 196 Bremen, Germany). For the analysis of the soluble Fe concentration, washings and supernatants 197 198 were combined and acidified to pH 2.0 before being filtered using a 0.45-µm PTFE membrane filter. The Fe concentration in solution was determined using ICP-MS. The calculations are 199 200 found in the supplementary information.

201

202 2.4. Analysis of $\delta^{13}C$ -CO₂

The CO₂ traps were collected and changed after 1, 3, 7, 14, 28, and 56 days for the 203 determination of C mineralization. At sampling, a 5 mL aliquot of trap solution was diluted 204 with 10 mL water and titrated against standardized 0.5 M HCl using an Easy Plus auto titrator 205 (Mettler Toledo, Greifensee, Switzerland) to determine the total CO₂ evolved (Tinsley et al., 206 1951). To determine the δ^{13} C-CO₂ from the trapped CO₂, an 8 mL aliquot of the trap solution 207 was added to 8 mL of 1.5 M BaCl₂ into 50 mL falcon tubes and incubated at 25 °C for 0.5 h to 208 allow precipitation. The resulting BaCO₃ precipitate was then filtered, rinsed three times with 209 210 deionized water, and freeze-dried overnight in a falcon tube (Luo et al., 2011; Luo et al., 2017). The precipitates were scraped from the falcon tube, accurately weighed (about 0.2 mg) into tin 211 capsules, and analyzed using a stable isotope ratio mass spectrometer (IRMS, Thermo Fisher, 212 213 USA) (Aoyama et al., 2000).

214

215 $2.5 \,\delta^{13}C$ calculations

217

The mineralization of the substrate was distinguished from SOM mineralization based on the changes of stable isotopic composition (δ^{13} C) over time as follows.

218
$$\delta^{13}C(\%_0) = \left[\left(\frac{\text{Rsample}}{\text{R}_{\text{VPDB}}}\right) - 1\right] \times 1000$$
 (1)

R_{sample} is the mass ratio of ¹³C to ¹²C of the sample, and R_{PDB} is the mass ratio of ¹³C to ¹²C of the Vienna Pee Dee belemnite (VPDB) standard (0.0112372). The labeled ¹³C (%) was then estimated from:

222
$$CO_2 \stackrel{13}{\Box}C(\mu g g^{-1} \text{soil}) = CO_2 \stackrel{13}{\Box}C(\%) \times \text{Total evolved } CO_2C(\mu g g^{-1} \text{soil})/100$$
 (2)

223
$$CO_{2} \overset{13}{\square} C(\%) = (\delta^{13}_{\square} C_{\text{treatment}} - \delta^{13}_{\square} C_{\text{original}}) / \delta^{13}_{\square} C_{\text{substrate}} - \delta^{13}_{\square} C_{\text{original}}) * 100$$
(3)

Where $CO_2^{13}C$ (%) is the proportion of evolved ¹³C in CO_2 from the added substrate (glucose) $\delta^{13}C_{\text{treatment}}$ is the $\delta^{13}C$ (‰) of evolved CO_2 from substrate added to the soil, $\delta^{13}C_{\text{control}}$ is the $\delta^{13}C$ (‰) of original soil C without substrate was added. $\delta^{13}C_{\text{substrate}}$ is the $\delta^{13}C$ (‰) from the added substrate. Thus, the $CO_2^{13}C$ resulting from the mineralization of the substrate (glucose) during the incubation (substrate-derived C) was calculated.

The primed soil CO₂C, resulting from enhanced decomposition of SOM with the addition ofglucose was calculated from:

Priming soil
$$CO_2C(\mu g C g^{-1} soil) = CO_2C_{treatment-1} - CO_2C_{control-1}$$
 (4)

Where, $CO_2C_{treatment-1}$ is the non-isotopically labeled C in CO_2 evolved from substrate amended soil, and CO_2 - $C_{control-1}$ is the C in total CO_2 evolved from the control.

- Substrate-derived C represents the CO_2 evolved from glucose (based on ${}^{13}CO_2$ measurements).
- 235 Primed soil C signifies CO₂ evolved from native soil, primed by added glucose. Basal soil-

236 derived C accounts for the total CO₂ evolved from the respective control soil without glucose237 addition.

238

239 2.6. DNA extractions and sequencing

DNA of 0.50 g of freeze-dried soil was extracted using a Fast DNA Spin Kit (MP 240 Biomedicals, Santa Ana, CA, USA). The extracted DNA was dissolved in 50 µL of TE 241 buffer, and the concentrations of DNA were quantified using a Nanodrop 2000 (Thermo 242 243 Scientific, Willmington, USA). The bacterial 16S rRNA gene fragments were amplified using primer sets targeting the V4-V5 variable region. The forward primer is 515F (5'-244 GTGCCAGCMGCCGCGGTAA-3') linked with a specific-sample 5-bp barcode sequence at 245 246 the 5'end of primer, and 806R (5'- GGACTACHVGGG TWTCTAAT -3') was used as the reverse primer (Bates et al., 2011). The ITS1 region was amplified by PCR for fungal genes 247 using the 5'- CTTGGTCATTTAGAGGAAAAGTAA-3' forward primer and 5'-248 GCTGCGTTCTTCATCGATGC-3' reverse primer (Bates et al., 2011). Samples were 249 amplified in three replicates, and the three reaction products were pooled and purified using 250 251 Agincourt Ampure XP beads (Indianapolis, USA). All amplicons were pooled across all samples at equimolar concentrations (20 ng μ l⁻¹) into a representative sample, and the index 252 sequencing of paired-end 250 bp was performed on an Illumina HiSeq 2000 platform. Bacterial 253 254 and fungal DNA amplification and sequencing were performed by Major Bio, Inc. (Shanghai, 255 China).

256

257 2.7. Processing of sequencing data and related statistical analysis

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by 258 Trimmomatic, and connected by FLASH using the following steps. Firstly, the 250 bp reads 259 260 were cut-off at the site, receiving an average quality score of less than 20 over a 50 bp sliding window, and the cut-off reads shorter than 50 bp with the reads containing mismatch characters. 261 Secondly, sequences that were longer than ten bp were assembled according to their overlapped 262 sequence. The maximum mismatch ratio of the overlap region is 0.2, and those mismatched 263 264 reads were discarded. Thirdly, samples were differentiated according to the barcode and primers. Finally, sequence direction adjustment and barcode matching was undertaken. 265 266 Between 4,682 and 51,150, valid sequences were obtained per sample for all experimental samples. UPARSE (version7.1, http://drive5.com/uparse) was used to cluster the operational 267 taxonomic units (OTUs) with 97% similarity cut-off. Chimeric sequences were removed. The 268 269 RDP classifier (http://rdp.cme.msu.edu/) was used to examine the taxonomy of each OTU representative sequence against the 16S rRNA database using a confidence threshold of 0.7 270 (Chen et al., 2017). Total datasets were rarefied to 39,000 sequences per sample for the 271 bacterial α - and β -diversity analyses. α -diversity of bacterial and fungal communities was 272 calculated using the alpha diversity index (herein referred to as the Shannon index) that 273 represents both the richness and the evenness of the community. Principal coordinates analyses 274 (PCoA) were used to analyze the β -diversity analysis. Relative effects of soil variables such as 275 TC, TN, C:N ratio, C:Fe molar ratio, DOC, Fe-OM, and pH on soil bacteria and fungi 276 277 communities were analyzed by distance-based linear model multivariate analysis (distLM) using the distLM forward3 software (Anderson, 2003). SIMCA-P 14 (Version 14.1.0.2047) 278 software used to determine the two-way orthogonal partial least squares (O2PLS) analysis to 279 280 correlate the microbial taxa to the priming, substrate mineralization, and substrate-derived C stabilization. The Y-matrix was designed as the C dynamics datasets, and the X-matrix was 281 intended as the microbial community datasets (Trygg and Wold, 2003). The relative 282

importance of edaphic factors on the substrate mineralization and stabilization was quantified 283 using random forest analysis (Liaw and Wiener, 2002). Soil physicochemical variables (C:N 284 ratio, Fe concentration, C: Fe molar ratio, DOC, pH, TN, TC, and TP; total phosphorus) and 285 biological variables (bacterial diversity, fungal diversity, Actinobacteria, Proteobacteria, 286 Ascomycota and Basidiomycota) were used in the random forest analysis to assess their relative 287 contributions to the substrate derived C mineralization and stabilization. The pathways and 288 289 drivers of stabilization and mineralization of substrate in the soils were investigated by structural equation modelling (SEM), which can determine the direction, magnitude, and effect 290 291 relationships. SEM was conducted using AMOS 21.0 to confirm possible causal relationships between abiotic variables and the biotic community on C dynamics. In the SEM, chi-square 292 was used to evaluate model fitting, while a non-chi-squared test (P>0.05) indicates a good fit 293 294 of the model to the data. The analysis of correlation metrics calculated the coefficients of each path. The path in this model was considered significant with a P < 0.05. SPSS software version 295 20.0 for Windows (SPSS, Chicago, IL, USA) was used for statistical tests via a one-way 296 analysis of variance (ANOVA) on the soil biogeochemical data to assess the effect of substrate 297 (glucose) addition. Residues were analyzed for normal distribution and homogeneity by 298 Shapiro-Wilk and Levene's tests. If conditions were met, the Tukey Post-hoc test was 299 performed to determine the differences between the treatments. PCoA of the microbial 300 community based on the sequencing data was performed using SPSS 20.0 for Windows and 301 was visualized using Origin Pro 9.1 (Origin Lab, Northampton, MA, USA). 302

303

304 3. Results

305 3.1. Sources of CO₂ efflux

The respired CO₂ during the first 0-7 days was partitioned into substrate derived, basal soil-derived, and primed soil CO₂, compared across the gradient of soil Fe concentrations and glucose amendment (Fig 1). Total CO₂ efflux in the Fe-13 and Fe-25 soil was greater than that of the Fe-42 and Fe-55 soil. There was a general trend with the glucose addition that higher Fe concentrations resulted in lower CO₂ efflux. The highest primed soil C and substrate-derived C efflux with glucose amendment was reported for Fe-13, and lowest substrate-derived C and primed soil C was from the Fe-55 soil (Fig. 1).

313

314 *3.2. Fe induced distribution pattern of substrate-derived C in the soil after incubation*

Retention of ¹³C in soil increased (58%) with the increase of Fe concentrations from Fe-13 to Fe-55, relative to the total ¹³C content (Fig. 2A). The ¹³C distribution was analyzed to quantify the ¹³C stabilization within the Fe-OM complexes. The ¹³C accumulation in the Fe-OM complexes was controlled by the Fe concentrations in the soil, with the higher Fe concentrations having higher retention of ¹³C (Fig. 2A). The glucose addition resulted in a ¹³C content in Fe-OM of 3.8 mg kg⁻¹ in Fe-55, which was 4.2-fold greater than in the Fe-13 soil.

The ratio of Fe_p :Fed increased with the increase in soil Fe concentration (Fig. 2D), suggesting enhanced Fe-OM complexation with increasing Fe concentration. The Fe bound organic C:Fe ratio (C:Fe molar ratio) of Fe-OM complexes of the four soils varied between 3.4-6.5 (Figs. 2B and 2C) suggesting that co-precipitation (C:Fe > 6) (Chen et al., 2014; Wang et al., 2017) was the key process in the Fe-42, and Fe-55 soil.

326

327 3.3. Linkages between the bacterial and fungal diversity and composition with substrate328 addition

Microbial community composition at the completion of the incubation shows that 329 Proteobacteria (varied between 30-40%) and Ascomycota (varied between 40-80%) were most 330 331 abundant in all four soils, irrespective of treatment (Figs. 3A and 3B). Actinobacteria increased by 7% and Basidiomycota by 50% in the soil with highest Fe concentration (Fe-55 soil). Fungal 332 diversity strongly increased along with the Fe concentrations from Fe-13 to Fe 55 (Fig. 4B). 333 Compositional dissimilarities of microbes between treatments were assessed by PCoA 334 335 analysis, and it showed the loadings of PC1 26.5% and PC2 19.2% for bacteria, and PC1 36.4% and PC2 14.2% for fungi (Figs. 4C and 4D). Considerable variation in microbial communities 336 337 were observed with increasing Fe concentration, as indicated by the separation along the PC1. 338

220

339 *3.4. Correlations between C dynamics and microbial communities related to edaphic variables*

The distLM analysis was used to analyze the contributions of edaphic variables, 340 including the C:Fe ratio, TC, C:N, DOC, pH, TN, and Fe-OM, to the microbial community 341 (Anderson and Legendre, 1999). The soil bacterial community was affected by TC (16%), C:Fe 342 343 molar ratio (14%), C:N (18%), and DOC (13%). The variables related to C and N dynamics (TC, TN, C:N, and DOC) accounted for 61% of the total variation in the bacterial community. 344 The fungal community was influenced by TC (26%), C:Fe molar ratio (18%), DOC (6%), and 345 TN (6%). The C: Fe molar ratio was the second most important factor contributing the soil 346 microbial community after TC in Fe rich soils (14% for bacteria and 18% fungi) (Table 2). Fe 347 concentration in soil exerted a contribution of 10% to bacteria and 12% for fungi (Table 2). 348

O2PLS analysis was used to identify the microbial community associated with substrate-derived C mineralization and the PE. The following conditions were considered for analysis: (i) variable influence projection (VIP) value ≥ 1.6 ; (ii) correlation coefficient (*P*<0.05); (iii) the number of microbial taxa being highly correlated ($r \geq 0.7$). Based on those

criteria, six genera were selected as key genera related to the PE and substrate mineralization 353 (Table 3). Most importantly, Proteobacteria positively correlated with a high VIP value for 354 355 substrate mineralization. The genera affiliated to the phyla Actinobacteria, Ascomycota, and Basidiomycota were positively correlated with the PE. The substrate-derived C mineralization 356 was positively correlated with the genera including Sphingomonas and Devosia (affiliated to 357 phylum Proteobacteria), while Sordariomycetes (affiliated to phylum Ascomycota), 358 359 Cryptococcus (affiliated to the phylum Basidiomycota), Gaiella and Streptomyces (affiliated to the phylum Actinobacteria) were highly correlated with the PE (Table 3). 360

Proteobacteria (Sphingomonas and Devosia) was positively correlated (P>0.001 and 361 P>0.01, respectively) with a high VIP value for substrate mineralization. Random forest 362 analysis showed that substrate mineralization was mainly governed by the involvement of soil 363 biological factors such as Proteobacteria and bacterial diversity. Whereas substrate-derived C 364 stabilization was largely regulated by physicochemical properties such as Fe, C:Fe molar ratio, 365 followed by the Fe concentration and TC (Fig. 5). While substrate stabilization was strongly 366 influenced by the C:Fe ratio (Figs. 5 and 6), it was negatively associated with microbial 367 composition, and positively associated with bacterial diversity (Fig. 6). Variations in Fe 368 concentration in soil positively influenced priming and substrate mineralization via fungal 369 diversity, with a lesser influence from bacterial diversity. The effect of the C:N ratio on 370 371 substrate mineralization was positively influenced by the involvement of Proteobacteria. Whereas, the PE was largely determined by fungal diversity, mainly associated with 372 Ascomycota. 373

374

375 **4. Discussion**

376 *4.1. Effect of C:Fe molar ratio on soil C stabilization*

The accumulation of ¹³C from the added glucose was higher in soils containing high 377 Fe concentration (i.e. Fe-55) (Fig. 2A), with a concommitant increase in the C:Fe molar ratio 378 (Fig. 2B). The C:Fe molar ratio has recently been considered to reflect the binding mechanisms 379 between SOM and Fe oxides (Coward et al., 2018). Sodano et al. (2017) emphasized that the 380 complexation of organic carbon with Fe, forming Fe-OM complexes, is an important C 381 sequestration mechanism in paddy soils. More specifically, when the C:Fe >6, the formation 382 383 of Fe-OM is via co-precipitation or aggregation (Wagai et al., 2013; Atere et al., 2020). In contrast, C:Fe ratios <1 represent the formation of Fe-OM associations via inner-sphere 384 385 adsorptive processes (Coward et al., 2018). In our study, the C:Fe molar ratio in the Fe-13 and Fe-25 soils was between 3.4 and 6.5 whereas the Fe-42 and Fe-55 soils had ratios >6 (Fig. 2B). 386 These results support the observations of previous research where a greater retention of forest 387 floor-derived DOC, and plant root-derived rhizodeposits was through co-precipitation rather 388 than by adsorption, where the initial C:Fe molar ratio >1 (Wan et al., 2019; Jeewani et al., 389 2020). Similarly, Wissing et al. (2014) reported direct interactions between DOC-Fe(III) 390 compounds and chemical protection via the Fe-OM complexes. Although co-precipitation led 391 to robust and selective retention of aromatic constituents, the initial complexation of Fe²⁺ by 392 aliphatic carboxylic compounds and co-precipitation as C-rich Fe-OM associations contributed 393 to the overall retention of C in soil, particularly at higher solution C:Fe ratios (Chen et al., 394 2014). As such, co-precipitation represents the main process in DOC retention in Fe oxide rich 395 396 soils and is of particular interest in understanding SOM stabilization and turnover mechanisms, in particular, their role in controlling the C source and sink of paddy soils. 397

Importantly, our data has revealed the mechanisms in which glucose is stabilized in Fe-OM complexes. The higher C retention obtained in Fe-42 and Fe-55 soils (Figs. 2A and 2B) by co-precipitation was most probably due to the complexation of Fe compounds with lowmolecular-weight organic compounds, followed by co-precipitation as Fe-OM complexes

(Sodano et al., 2017; Wan et al., 2019). It was reported that glucose could adsorb to Fe oxides by 402 hydrogen bonding (Olsson et al., 2011), or associate via co-precipitation processes (Eusterhues 403 et al., 2011; Henneberry et al., 2012; Wan et al., 2019). Kleber et al. (2015) showed that the 404 association of labile-C molecules with Fe minerals via adsorption or co-precipitation, or the 405 formation of multi-layer organic bonds with mineral stabilized organic compounds, can 406 significantly lower the availability of labile C to microbes. Our results on the accumulation of 407 408 C from glucose in Fe-OM complexes support evidence for the role of Fe oxides in paddy soils in stabilizing labile C (e.g., rhizodeposits) under unsaturated conditions. 409

410

411 4.2. Microbial community assembly is associated with substrate availability in Fe rich paddy
412 soils

The soil microbial community structure can be modified by the complex 413 414 physicochemical characteristics of soil (Fig. 3). The microbial community composition is 415 displayed by the relative abundance (Figs. 3A and 3B) as a function of the Fe concentration. In soils with a greater Fe concentration, Actinobacteria dominated, while Proteobacteria and 416 Bacteroidetes had a lower abundance (Fig. 3A). These are recognized as copiotrophs (Fierer et 417 al., 2007), which grow rapidly and are usually more prevalent in soils with a high C availability. 418 Further, it was confirmed that Gaiella and Streptomyces (affiliated with phylum Actinobacteria) 419 420 were core genera responsible for the PE (Table 3). Actinobacteria has a broad metabolic diversity and is thus better able to adapt to resource-poor oligotrophic conditions, when the 421 bioavailability of labile C is limited (McCarthy and Williams, 1992; Holmalahti et al., 1994; 422 423 Wei et al., 2020). It can therefore be concluded that decreasing the bioavailability of labile C in soil, as a result of the protection mechanisms (e.g., Fe-OM) lowering glucose availability, 424 425 modified the bacterial community to favour the phylum Actinobacteria.

It is well known that filamentous fungi belong to the phyla Ascomycota and Basidiomycota, and are well adapted to degrade SOM. Thus it is not surprising that fungal diversity increased with increasing Fe concentrations (Fig. 4B), where the availability of labile C decreased. Further, SEM and O2PLS analysis supported the notion that Ascomycota and fungal diversity directly influence the PE (Fig. 6 and Table 3). It was reported that sequences assigned to Ascomycota were dominant at different stages of decomposition, revealing that they are key drivers responsible for residue degradation (Lundell et al., 2014).

The distLM analysis revealed that TC, C:Fe ratio, C: N ratio, DOC and Fe concentration 433 were major edaphic factors contributing to the bacterial and fungal communities (Table 2), 434 confirming the role of C in the community assembly. Specifically, the limited glucose C 435 availability in the soil rich in Fe oxides modulated the community that was dominated by K-436 strategists, including Gaiella, Streptomyces (affiliated to the phylum Actinobacteria), 437 Sordariomycetes (affiliated to Ascomycota) and Cryptococcus (affiliated to Basidiomycota) 438 (Figs. 3A and 3B). The limitation of C and nutrients favored K-strategists that possess the 439 ability to explore the soil for C, being able to survive in both copiotrophic and oligotrophic 440 soils. Therefore, as Basidiomycota, Ascomycota, and Actinobacteria exhibit higher substrate 441 versatility and metabolic diversity, they can therefore survive in both copiotrophic and 442 oligotrophic conditions. Their ability to prime SOM provides a competitive advantage (Cui et 443 444 al., 2020; Dini-Andreote et al., 2015; De la Cruz-Barrón et al., 2017).

445

446 4.3. Glucose mineralization and the priming effect is regulated by Fe concentration and447 associated core microbes

Glucose had higher mineralization in the soils with lower Fe concentration (Fig. 1).Blagodatskaya et al. (2009) demonstrated that the input of small amounts of readily available

substrates (i.e., glucose or root exudates) significantly increased the growth rates of soil 450 microorganisms by between 13-20 %. Similarly, Wang et al. (2016) demonstrated that glucose-451 stimulated microbial activity with a rapid shift from K-strategists and towards r-strategies. Our 452 random forest analysis showed that Proteobacteria (e.g., Sphingomonas; r-strategist) and 453 bacterial diversity had the greatest influence on substrate-derived C mineralization (Fig. 5 and 454 Table 3). As supported by SEM, substrate-derived C mineralization was mainly governed by 455 456 the C:N ratio with the involvement of Proteobacteria and bacterial diversity (Figs. 5 and 6). Therefore, the higher substrate-derived C efflux reported in soils with the lower Fe 457 458 concentration was due to the stimulation of r-strategists resulting from the access to labile C (i.e., glucose). 459

A higher positive PE was found in paddy soil with the lowest Fe concentration (i.e., Fe-460 13), with the PE abating with higher Fe concentration (Fig. 1). Ascomycota (K-strategist) and 461 Actinobacteria (r-strategist) were the dominant phyla in the F-13 soil. The corresponding 462 463 genera (Sordariomycetes, Gaiella, and Streptomyces) are likely to have responded rapidly to the added glucose, resulting in further microbial co-metabolism by increasing the 464 mineralization of native SOM (i.e., positive PE), especially in the soil with a low Fe 465 concentration. In contrast, in soil with the higher Fe concentrations (i.e., Fe-42 and Fe-55), the 466 glucose-derived C was directly co-precipitated by Fe-OM phases with the involvement of 467 468 physicochemical factors such as the C:Fe ratio and Fe concentration (Figs. 5 and 6), thereby lowering the microbial availability of glucose. Thus, mineralization of glucose and priming of 469 native SOM were strongly controlled by the Fe concentration of soil by limiting the 470 accessibility of C by co-precipitation. 471

472

473 **5.** Conclusion

This study has established the importance of Fe concentration in paddy soils in 474 stabilizing C. The development of Fe-OM complexes, the main C stabilization mechanism, 475 increased in soils with higher Fe concentration. Paddy soil with an Fe concentration of 55.8 g 476 kg⁻¹ had 44.7% lower mineralization of glucose compared to soil with an Fe concentration of 477 13.7 g kg⁻¹ at day 1 of the incubation. At the same time, the priming of SOM was 37.4% lower 478 in soil with the highest Fe concentration. The addition of glucose resulted in the proliferation of the 479 480 r-strategists Sphingomonas and Devosa, while positive priming of SOM was mostly caused by genus Gaiella, Sordariomycetes, and Cryptococcus (K strategists). We conclude that inherent Fe 481 concentration exceeding around 42 g kg⁻¹, have a molar C:Fe ratio of >6 and promote Fe-OM 482 complexes via co-precipitation. This limits the availability of the substrate for microbial 483 mineralization, thus allowing the accumulation of C in paddy soils. 484

485

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490 7. References

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720 Figure and Table captions

Fig. 1. Cumulative basal soil-derived (A), substrate-derived (B) and primed soil (C) CO₂ during first 7 days of incubation following the addition of glucose. 'Basal soil-derived C' signifies the total CO₂ evolved from the native soil (non labeled C) from the glucose added treatment. 'Substrate-derived' signifies the CO₂ evolved from added labeled glucose (based on 13 CO₂ measurements). 'Primed soil' C signifies CO₂ evolved from the mineralization of soil organic matter. Error bars represent standard deviation of the means (n= 3).

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Fig. 2. Content of ¹³C derived from the addition of labile C substrate in bulk soil and in the Fe-OM complexes (A) and corresponding molar ratio of Fe-bound OC to dithionite-extractable iron (Fe_d) (B). Fe-bound soil organic carbon (Fe-bound OC) content (C) and ratio of Fe_p:Fe_d indicating degree of Fe complexation with organic matter (D) at the completion of the 56 day incubation. Values show means (n=3) \pm standard deviation.

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Fig. 3. Relative abundance plots showing the relative distribution of various bacterial (A) andfungal (B) phyla.

Fig. 4. Shannon index of soil bacterial (A) and fungal (B) alpha diversity and PCoA plots
representing beta diversity based on Bray-Curtis dissimilarity of bacterial (C) and fungal (D)
communities.

740

Fig. 5. Random forest analysis represents relative importance of soil physicochemical (C:N
ratio, Fe, C:Fe ratio, DOC, pH, TC, TN and TP) and biological variables (Bacterial diversity,
Fungal diversity, and composition of Actinobacteria, Proteobacteria, Ascomycota and
Basidiomycota) for substrate (labeled glucose) mineralization (A) and stabilization (B).

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Fig. 6. Path analysis showing the underlying relationships between soil physicochemical factors and microbial community diversity and composition for substrate stabilization, priming effect and substrate mineralization in the soil. All the values present were statistically significant (***P<0.001, **P<0.01 and *P<0.05). Thickness of lines are proportionate to regression weights. Red lines represent negative values; blue lines represent positive values. The bacterial and fungal diversities are represented by the Shannon index. Standardized path coefficients are listed beside each path. (Model fit :Chi-square value=64.72, *df*=13, *P*=0.052).

753

754 **Table 1**.

Table 1. Properties of four paddy soils. Soil with total Fe concentration of 13.7 g kg⁻¹ is referred to as (Fe-13), soil with total Fe concentration 25.8 g kg⁻¹ (Fe-25), soil with total Fe concentration 42.7 g kg⁻¹ (Fe-42), soil with total Fe concentration 55.8 g kg⁻¹ (Fe-55).

758	Abbreviations:	TC; total	carbon,	TN; tota	l nitrogen,	TP; 1	total	phosphorous,	WHC;	water
759	holding capacity	y, MC; mo	oisture co	ontent						

Table 2. Contributions of edaphic variables to the bacterial and fungal community as analyzed
by distance-based linear modeling (distLM) analysis

Table 3. Two-way orthogonal partial least squares analysis to reveal the core functional genera
(with variable influence projection (VIP) >1.6) involved in C dynamics including priming
effect (PE) and substrate mineralization