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Contrasting effects of aged and fresh biochars on glucose-induced priming and microbial activities in paddy soil

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Abstract
Purpose The understanding of soil organic matter (SOM) decomposition to biochar (BC) addition in paddy soil remains limited. This study was to examine the glucose-induced priming effects of paddy soil as affected by aged and/or fresh BCs.
Materials and methods Soil samples were collected from the paddy fields without and with BC applied 1 year before the laboratory incubation. The control without BC, the soil with BC applied in the field (aged BC), aged BC and the additional fresh BC applied (A/F-BC), and the soil with fresh BC (fresh BC), each with glucose (^{14}C) addition or not were incubated under either moist or flooding conditions. Totally, 16 treatments were established.
Results and discussion The aged-BC group treatments had greater carbon dioxide (CO_2) efflux (averagely 26 %) compared to the control group, while CO_2 efflux was reduced following fresh BC addition by 23 and 21 % in the A/F-BC and fresh-BC groups, respectively. In the presence of aged BC, microbial biomass and the potential activities of ß-glucosidase, xylanase, cellobiohydrolase, and chitinase were increased under both moist and flooding conditions, but they were decreased in the fresh-BC group under flooding conditions. For the control group, positive priming of 58 and 102 μg C g^{-1} were observed after glucose addition under moist and flooding conditions, respectively. Positive priming was also observed in the fresh-BC group amounting to 34 and 19 μg C g^{-1} under moist and flooding conditions, respectively. However, negative priming was found in the soil samples with aged BC, especially under moist conditions, which was possibly due to the preferential use of glucose over the more recalcitrant organic C in both SOM and BC and SOC stabilization via either organic matter sorption on BC or the BC-induced organic-mineral interaction.
Conclusions BC effects on CO_2 efflux and microbial activities depend strongly on its age. Aged BC improves soil microbial parameters and also contributes to C sequestration in soil by negative priming. In contrast, fresh BC addition depresses microbial activities but stimulates the mineralization of SOM and itself following glucose addition.

Keywords Carbon cycle · Climate change · Extracellular enzyme activity · Microbial dynamics · Pyrogenic organic matter · Rice field

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1 Introduction

Biochar (BC) is a carbon (C)-rich byproduct generated by pyrolyzing organic residues under high-temperature and oxygen-limited conditions; it is proposed as a novel strategy to aid farming and mitigate climate change (Lehmann et al. 2006). Recent reviews have highlighted the benefits of adding

BC to agricultural soils, such as ameliorating soil properties (Glaser et al. 2002), promoting plant growth (Biederman and Harpole 2013), and reducing major greenhouse gas emissions (Woolf et al. 2010). Because of its highly resistant to biological attack and long mean residence times, BC can represent an important fraction of soil C both in managed and natural ecosystems (Czimczik and Masiello 2007; Cheng et al. 2008; Lehmann et al. 2008). Since approximately 89 % of the technical mitigation potential is due to soil C sequestration in global agriculture (Smith et al. 2007), BC application in soils should be regarded as a sustainable mitigation management (Woolf et al. 2010).

Despite the recalcitrant nature of BC, its stability or longevity in soils has been challenged by growing experimental evidence (e.g., Cheng et al. 2008; Kuzyakov et al. 2009, 2014; Wang et al. 2015). Recent results indicate that BC addition may affect the mineralization of non-BC pools, such as native soil organic matter (SOM) and added labile organic matter (LOM) (Maestrini et al. 2014; Wang et al. 2015). For example, charcoal addition strongly promoted the loss of humus-C in boreal forests over 10 years, where they attributed the enhanced loss of humus-C to charcoal particles acting as foci (Wardle et al. 2008). Other studies also suggest the pronounced native soil organic C (SOC) loss due to BC addition with either short-term (Farrell et al. 2013; Luo et al. 2011) or long-term observations (Singh and Cowie 2014). Conversely, following application of ryegrass-derived BC, sustained suppression in a nutrient-limited loess soil or no significant effect in Haplic Luvisol on native SOM mineralization was found (Kuzyakov et al. 2009). Yet, current understanding of BC addition effects on added LOM mineralization or vice versa is highly limited, and only a few studies offer insight into this issue. In BC-rich Anthrosols, mineralization of added LOM was depressed compared to the BC-poor adjacent soils from the Central Amazon (Liang et al. 2010). Similarly, the depressed LOM mineralization and stimulated BC mineralization both significantly increased with increasing sugar cane residue application rates (Keith et al. 2011). The suppressed mineralization of added LOM is attributed to the caused incorporation of added LOM into aggregates and organo-mineral fractions. In the presence of corn plants, pyrogenic organic matter (maple twigs at 325 °C) counteracted the net C loss by either decreasing SOM decomposition or increasing corn C addition to the soil (Whitman et al. 2014). These divergent results may be attributed to variations in the proportion of labile components in BCs (Luo et al. 2011; Singh and Cowie 2014), the presence or absence of plant-derived LOM input into soil (Keith et al. 2011; Whitman et al. 2014), and, especially, the extent of BC aging in soil or incubation time (Liang et al. 2010; Cross and Sohi 2011; Zimmerman et al. 2011). Consequently, understanding of BC effects on the mineralization of native SOM and added LOM in various soils under different experimental conditions remains incomplete.

Recent studies suggest that the behavior of aged BC differs significantly from fresh BC after it is incorporated into soils with regard to its effects on microorganism habitats (Quilliam et al. 2013), the mineralization of glucose and organic nitrogen (N) (Dempster et al. 2012; Jones et al. 2012), and related greenhouse gas production (Spokas 2013). Moreover, the magnitude and direction of priming effects (PEs) due to BC addition greatly depended on the duration of experiment and, generally, positive priming was observed in the initial period, but negative priming dominated the later phase (Maestrini et al. 2014). Overall, these observations highlight that aging time may play an important role on controlling BC's performance in soils. A better understanding of LOM inputs effect on the PE and itself decomposition as affected by BC in soils is required and thereby will be critical to clarify the SOC dynamics and stabilization in response to climate change.

Cropland management in China is estimated to have a C sequestration potential of 25–37 Tg C $year^{-1}$ (Lal 2002). Between 1979–1982 and 2007–2008, the SOC content in 0–20-cm paddy soils has increased by 7.5 %, which is attributed to the greater increase in crop yields and, thereby, higher plant-derived C inputs into soils (Yan et al. 2011). As developed in a unique management system (i.e., puddling and flooding), paddy soils possess different biogeochemical processes compared to other agricultural soils (Kögel-Knabner et al. 2010). Much information about the PE has been generated through investigations primarily using upland soils (Blagodatskaya and Kuzyakov 2008; Wu et al. 2012). To date, no study has addressed the effect of exogenous substrates on SOM mineralization and microbial dynamics in paddy soils in the presence of BC.

The major objective of this study was to evaluate the PE induced by glucose addition in the presence of aged or/and fresh BC under moist and flooding conditions. Since the C:N ratios differed greatly between soils with and without aged BC, we hypothesized that the PE would be greater in the aged BC-added soil sample than the control soil without BC (H1). Due to the different influences of aged and fresh BCs on soil C and N cycles, we expected that the PE would behave differently between the aged and fresh BC-added soil samples (H2). Lastly, based on the retarded decomposition of SOM under flooding conditions, we expected that the greater positive or negative PE would occur in the soil samples under moist conditions but not flooding conditions for each treatment (H3).

2 Materials and methods

2.1 Soil sampling and preparation

The soil samples were collected after the rice harvest in November 2013 from the surface layer (0–20 cm) of a field under a typical summer rice-winter wheat rotation system in

Nanjing, Southeast China (31° 52 N, 118° 50 E). The soil is classified as *Stagnic Anthrosols* (WRB 2006) with a silt loam texture (Wang et al. 2011). For the soil samples tested herein, two field treatments with three replications were provided without BC application (hereafter, the control, C_{org}=12.8±0.1 g C kg^{-1}; N_{total}=1.4±0.1 g N kg^{-1}; pH(H_2O)=5.67±0.01) and with BC application (aged BC, C_{org}=23.3±0.4 g C kg^{-1}; N_{total}=1.7±0.1 g N kg^{-1}; pH(H_2O)=6.23±0.01) at 40 Mg ha^{-1} (equivalent to 16 g BC kg^{-1} soil), which began in October 2012. Urea was used as N fertilizer and applied at 500 kg N ha^{-1} annually for both treatments in the field following local agronomic practices. The BC (C_{total}=471 g C kg^{-1}, N_{total}=7.7 g N kg^{-1}, pH(H_2O)=10.4, CEC=24.1 cmol kg^{-1}, surface area=8.92 m^2 g^{-1}, and ash content=20.8 %) was purchased from Sanli New Energy Company (Henan, China). Air-dried wheat straw segments were heated in the reactor at 5–10 °C min^{-1} and retained at the highest temperature around 450 °C for 4.5 h. Nitrogen gas was then flushed into the reactor during the cooling-off period to maintain the inert environment. The pyrolysis of wheat straw at 450 °C resulted in 30 % biochar, >3 % bio-oil, and <23 % pyrolytic gas (Pan et al. 2011). BC was ground to particle size ≤2 mm before mixing with the soil samples. The soil samples were stored field fresh in aerated polyethylene bags at 4 °C after sampling. Prior to the experiment, soil samples were sieved (≤2 mm) and homogenized, and fine roots and visible plant debris were carefully removed.

2.2 Experimental design and treatments

The study was designed as a three-factorial experiment, including analyses of the BC application, (2) glucose addition, and (3) water regime. The first factor consisted of four levels: (1a) control: no BC application, (1b) aged BC: soil with BC applied in the field at 40 Mg BC ha^{-1}, (1c) A/F-BC: aged-BC soil mixed with an additional 60 Mg BC ha^{-1}, and (1d) fresh-BC: control soil mixed with 100 Mg BC ha^{-1} (40 g BC kg^{-1} soil). The second factor was glucose addition with two levels (with and without 24.3 μg C g^{-1} soil). Uniformly labeled ^{14}C-glucose (final activity at 329 Bq g^{-1} soil) was added to the unlabeled D (+)-glucose before it was added to the soil. The third factor, the water regime, included two treatments, moist with 60 % water holding capacity (WHC) and flooding. A soil-to-water ratio of 1:1 (*v*/*v*) was used to produce the waterlogging status for the flooding treatment. The control, aged-BC, A/F-BC, and fresh-BC group treatments, each has glucose addition or not under either moist or flooding conditions. In total, the experiments included 16 treatments, each with three replications (Fig. 1 and Fig. S1, Electronic Supplementary Material).

2.3 Incubation and sampling

A two-phase pre-incubation of 35 days was adopted to stabilize the microbial activity, thus avoiding undesired microbial peaks. Fifteen-gram (oven dry weight) soils were weighed and placed into a 100-ml Schott jar. After mixing with BC (only for BC-added treatments), distilled water was added to all soil samples to generate a soil moisture content of 50 % WHC for the first phase of pre-incubation of 2 weeks; they were then incubated in the dark at 22 °C. Small vials with 3 ml of 1 M NaOH were placed in the incubation jars to trap CO_2 production at the same time. After 2 weeks, half of the jars were subjected to the flooding group treatments, while half of the jars were subjected to 50 % WHC for the moist group treatments. Over a 35-day pre-incubation, the vials with NaOH were periodically removed after 2, 6, 14, 21, 30, and 35 days and replaced by vials with a new 3 ml aliquot of 1.0 M NaOH. Six empty jars with or without distilled water were used as controls for the corresponding water regime.

After a 35-day pre-incubation, a glucose solution with the target concentration or distilled water was added, which yielded 60 % WHC soil moisture content for the moist treatment while >100 % WHC for the flooding treatment. The CO_2 measurement for all treatments was performed after 1, 3, 4, 5, 8, 10, 14, 18, 24, 30, 36, 43, 50, 57, 67, 79, and 91 days of incubation. An additional nine jars were prepared for each treatment and destructively sampled, 5, and 15 days to measure microbial biomass and dissolved organic C (DOC), respectively. All treatments were sampled destructively at the end of 91-day incubation.

2.4 Microbial biomass and DOC measurement

The soil microbial biomass was determined by the chloroform fumigation extraction (modified after Vance et al. 1987). Briefly, after destructive sampling or at the end of incubation, the soil was mixed carefully and 5 g of soil were directly extracted using 20 ml of 0.05 M K_2SO_4. An additional 5 g of soil were first fumigated with chloroform for 24 h and then extracted in the same manner. The extracts were frozen until analysis for the total C concentration using a 2100 TOC/TIC analyzer (Analytik Jena, Germany). The total amount of extractable microbial biomass C was determined based on the difference between K_2SO_4-extractable C between fumigated and unfumigated soil samples using the k_{ec} factor 0.45 (Wu et al. 1990). The C content in K_2SO_4 extracts from unfumigated soil samples was accepted as DOC. The amount of glucose-derived C in microbial biomass and DOC were calculated based on ^{14}C activity in microbial biomass and DOC divided by the ^{14}C-specific activity (^{14}C/C) of the added glucose (Chowdhury et al. 2014).

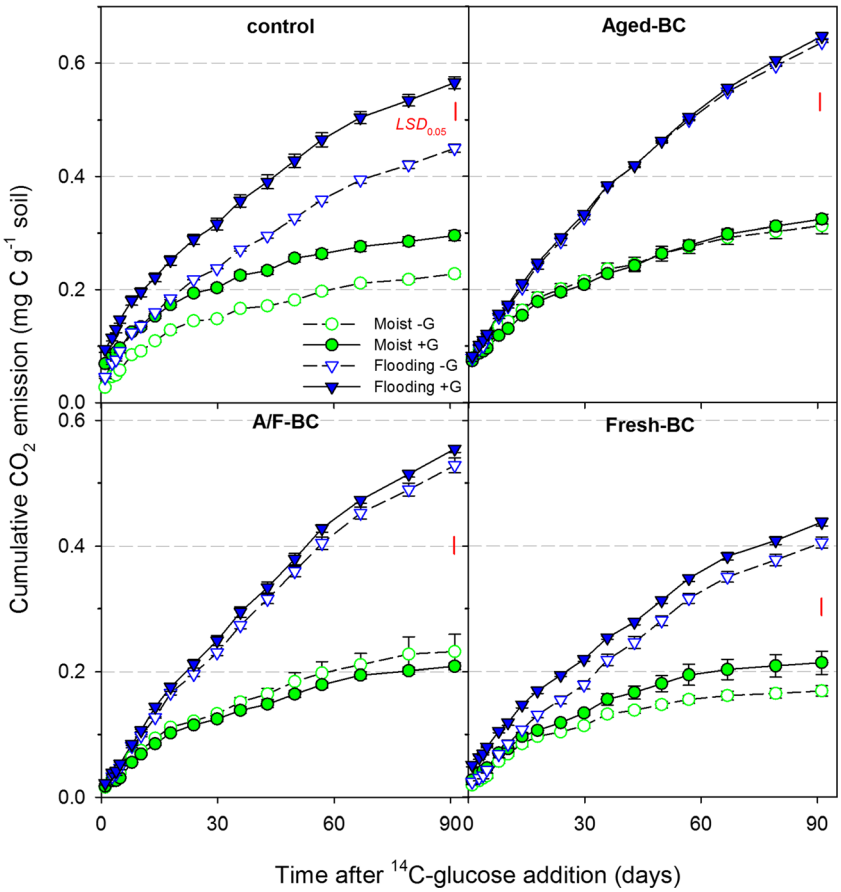

Fig. 1 Cumulative CO_2 emissions from the control, aged-BC, A/F-BC, and fresh-BC group treatments, each has glucose addition or not under either moist or flooding conditions. The values are the means±standard errors of means (SEM) (n=3). P values from the three-way ANOVA are as follows: biochar (BC), <0.001; water regime (W), <0.001; glucose (G), <0.001; BC×W, <0.001; BC×G, <0.001; W×G, *ns*; and BC×W×G, *ns*. *ns* indicates no significant difference between treatments. The *vertical lines* show the least significant differences at P<0.05 ($LSD_{0.05}$)

2.5 Enzyme assays

At the end of incubation, the soil samples were used to measure the activities of the four hydrolytic enzymes according to the fluorimetric protocol in Saiya-Cork et al. (2002) with the modification in German et al. (2012). Four types of artificial fluorogenic substrates were used: 4-methylumbelliferyl-β-D-glucopyranoside (MUF-G, EC 3.2.1.21) to detect β-glucosidase activity, 4-methylumbelliferyl-β-D-cellobioside (MUF-C, EC 3.2.1) to detect cellobiohydrolase activity, 4-methylumbelliferyl-β-D-xylopyranoside (MUF-X, EC 3.2.1) to detect xylanase activity, and 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide dehydrate (MUF-NAG, EC 3.2.1.14) to detect chitinase activity. Briefly, a buffer solution composed of 0.1 M MES ($C_6H_{13}NO_4SNa_{0.5}$) was prepared for MUF substrates. The substrates were dissolved in dimethyl sulfoxide (DMSO) and further diluted with sterile distilled water as well as MUF buffer for the desired concentrations. A half-gram of soil (dry weight equivalent) was homogenized in 50 ml of sterilized distilled water and dispersed for 2 min by an ultrasonic probe at 50 J s^{-1}. To determine the background fluorescence or quenching effects, the soil suspensions and buffer solution were also mixed with eight different volumes (0–120 μl) of 10 μM MUF standards. The solutions were pipetted into deep-well plates using a 50-μl soil suspension, 50 μl MES, and 100 μl of substrate solution with a series of concentrations from 0 to 100 μM for all substrates. We used a Victor^3 1420-050 Multilabel Counter (PerkinElmer, USA) at 365 nm excitation and 450 nm emission to measure fluorescence. The enzyme activities were expressed as MUF release in nanomoles per gram dry soil per hour. The maximum potential rates of activity (V_{max}) were calculated for each soil composite by fitting the Michaelis-Menten equation to the enzyme activity data with nonlinear regression using SigmaPlot 12.0 (Systat Software Inc., Chicago, IL, USA).

2.6 Chemical and isotopic analyses

Carbon dioxide trapped in 1 ml aliquot of NaOH solution was precipitated using a 0.5 M $BaCl_2$ solution, and the NaOH was then titrated with 0.1 M HCl against the indicator phenolphthalein (Zibilske 1994). The CO_2 production was calculated according to the method used in Chowdhury et al. (2014). An additional 1 ml aliquot of NaOH solution was mixed thoroughly with 2 ml of the scintillation cocktail Rotiszint Eco Plus (Carl Roth Company Germany) to measure ^{14}C activity using a Beckmann LS 6500 Liquid Scintillation Counter (Beckmann Counter Inc., USA). The ^{14}C counting efficiency was 87 %, and the ^{14}C activity measurement error did not exceed 2 % (Kuzyakov et al. 2009).

2.7 Calculations and statistical analysis

In our study, three C sources including SOM, BC, and glucose are presented in soil CO_2 evolution. The contribution of glucose-derived C to CO_2 efflux can be determined based on the current ^{14}C radioactivity and the amount of glucose added (Kuzyakov et al. 2009; Chowdhury et al. 2014). Because two different isotopic labels (such as ^{14}C and ^{13}C) are necessary to partition CO_2 in the presence of three sources (Kuzyakov 2010), it was not possible to distinguish C sources between SOM and BC. Therefore, PE (μg C g^{-1} soil) can be evaluated only for glucose not for SOM and/or BC as follows:

$$PE = T_{CO2} - G_{CO2} - S_{CO2}$$

where T_{CO2} (μg C g^{-1} soil)=total CO_2 from the treatment with glucose added, G_{CO2} (μg C g^{-1} soil)=CO_2 originated from glucose, S_{CO2} (μg C g^{-1} soil)=CO_2 derived from the treatment without glucose added.

An analysis of variance (ANOVA) was used to examine the factorial effects on the cumulative CO_2 efflux rates and primed CO_2 among all treatments. Repeated-measures ANOVA was applied to determine the effects of BC, water regime, and glucose addition (only for microbial biomass content and DOC) on microbial biomass and DOC as well as the recovery amounts of ^{14}C-glucose from microbial biomass and DOC in all treatments at three destructive samplings. Fisher's LSD post hoc test was used to test the differences between different treatments ($P<0.05$). Correlations (r) between CO_2 emissions and the values of microbial biomass and DOC at day 15 and the potential activities of four hydrolytic enzymes in soils were analyzed by Pearson's correlation method (Table 1). All statistical analyses were performed using STATISTICA v.10 (StatSoft Inc., USA).

3 Results

3.1 Soil CO_2 efflux

The highest CO_2 efflux rates were recorded for the first sampling (after 1 day) across all treatments (Fig. 1), in which the control and aged-BC group treatments exhibited higher CO_2 efflux rates compared with the fresh-BC group treatments ($P<0.001$). Thereafter, the CO_2 evolution rates decreased sharply at day 3 and became generally stable across the subsequent incubation period. Overall, the cumulative CO_2 amounts varied greatly from 0.17 mg C g^{-1} to 0.65 mg C g^{-1} (Fig. 1). On average, the aged-BC group treatments had averagely 26 % greater total CO_2 emissions than the control group (Fig. 1, top), while the significant depressed effect was found under treatments with fresh BC addition (Fig. 1, bottom). The main effects of water regime (flooding) and glucose addition increased total CO_2 emissions by an average of 116 and 14 %, respectively ($P<0.001$). These effects varied significantly between BC treatments ($P<0.001$), such that glucose effect on total CO_2 efflux was significant in both the control and fresh-BC group treatments, but no significant difference was found in the aged BC group treatments.

3.2 Glucose decomposition

Similar to the temporal dynamics of soil CO_2 efflux, the maximum mineralization rate for glucose occurred in the first day for all treatments (Fig. 2). Mineralized amounts ranged from 1.34 μg C g^{-1} in the fresh-BC treatment under flooding conditions to 4.38 μg C g^{-1} under the control group. An initial rapid phase (*ca.* 1–9 days) of $^{14}CO_2$ evolution and then followed by a secondary slower phase was observed (Fig. 2). On the first day, the $^{14}CO_2$ efflux after adding ^{14}C-glucose was much lower in the treatments with fresh BC under flooding conditions (1.3–1.8 μg C g^{-1}) than that in the other treatments (3.8–4.4 μg C g^{-1}; $P<0.001$). This was due to lower values of the rate constant of respired C pool in the fresh BC-added treatments (0.15–0.16 day^{-1}; Table S1) than those of other treatments (0.36–0.91 day^{-1}). These differences gradually decreased after 9–12 days of incubation (Fig. 2, bottom). Overall, ANOVA results indicated that there had been a significantly interactive effect of BC and water regime on glucose decomposition (measured as $^{14}CO_2$ efflux) throughout the incubation ($P=0.003$). Generally, the higher $^{14}CO_2$ effluxes were observed in aged BC-added treatment under flooding conditions.

Table 1 The Pearson correlations (r) between CO_2 emissions and the values of microbial biomass and dissolved organic carbon (DOC) at day 15 and the potential activities of four hydrolytic enzymes in soils amended with or without biochar (BC)

	Microbial biomass	DOC	β-glucosidase	Xylanase	Cellobiohydrolase	Chitinase
Cumulative CO_2	0.852**	0.822**	0.643**	0.594**	0.728**	0.763**
SOM with or without BC-derived CO_2	0.859**	0.818**	0.636**	0.589**	0.720**	0.763**
Glucose-derived CO_2	0.887**	0.821**	0.468*	0.528**	0.476*	0.687**
Priming effect (PE)	0.465	0.265	–0.334	–0.179	–0.176	–0.146

*$P<0.05$; **$P<0.01$

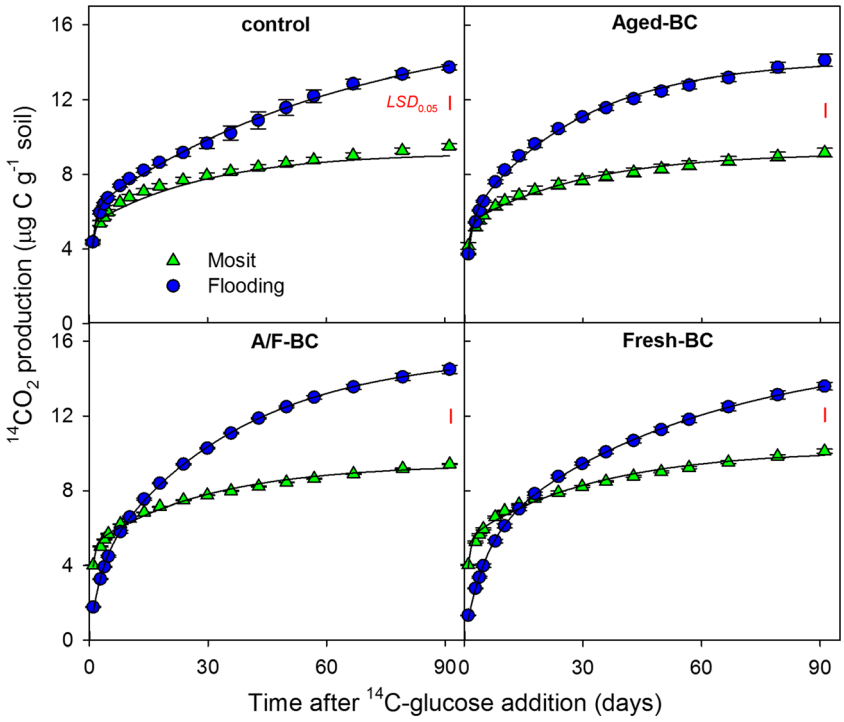

Fig. 2 The amount of $^{14}CO_2$ production in the control, aged-BC, A/F-BC, and fresh-BC group treatments plotted against time after ^{14}C-glucose addition. The values are the means±SEM (n=3). The modeled lines are fits of the double exponential kinetic equation to the experimental data. The *vertical lines* show the least significant differences at $P<0.05$ ($LSD_{0.05}$)

3.3 Microbial biomass and DOC

Microbial biomass significantly increased during the early period (from days 5 to 15), and then it decreased dramatically at the end of incubation (Table S2, Electronic Supplementary Material). Flooding strongly stimulated microbial biomass across all treatments relative to the moist conditions. Across all sampling periods, the presence of aged BC in soil samples increased microbial biomass with an average of 25 % (–1 to 65 %) compared to the control group (Fig. 3, top). In contrast, microbial biomass in the fresh-BC group treatments clearly decreased, particularly under flooding conditions compared to the control group. Similarly, DOC content gradually increased with time in most cases and significantly elevated following flooding (Table S2, Electronic Supplementary Material). BC addition contributed to increase the DOC content in soil samples, especially under flooding conditions (Fig. 3, bottom). The correlations between CO_2 emissions and the values of microbial biomass and DOC at day 15 were significant with the exception of PE (Table 1).

The recovery amount of ^{14}C-glucose in microbial biomass varied significantly with BC and its interaction with water regime (Table S2, Electronic Supplementary Material; Fig. 4, top). The recovery amounts of ^{14}C-glucose in microbial biomass were significantly increased in the aged BC-added treatment but decreased in the fresh-BC treatment under flooding conditions. The main effects of BC and water regime on the recovery amounts of ^{14}C-glucose in DOC were significant (Table S2, Electronic Supplementary Material; Fig. 4, bottom). Fresh BC addition contributed to more ^{14}C-glucose recovered in DOC, and conversely, the aged-BC treatment exhibited a moderate response.

3.4 Enzyme activity (V_{max})

The V_{max} values of all enzymes increased significantly from all treatments in the presence of aged BC (Table S3, Electronic Supplementary Material; Fig. 5). A significant interaction between BC and water regime indicated that the responses of V_{max} values to BC addition varied with water regime. When computed as the difference between BC present treatments and the control group, for example, greater V_{max} increases were observed in the aged- and A/F-BC group treatments under flooding conditions across all cases. However, flooding tended to significantly decrease the V_{max} for all enzymes in the fresh-BC treatment. In addition, the main effect of adding glucose on V_{max} values was significant in both xylanase and cellobiohydrolase (Table S3, Electronic Supplementary Material). Overall, the activities of four hydrolytic enzymes were significantly correlated with CO_2 emissions with the exception of PE (Table 1).

3.5 PEs

Glucose addition triggered an immediate increase in PE in both the control and fresh-BC group treatments, and thereby, on day 1, the primed CO_2 amounted up to 4.0–45.2 μg C g^{-1} (Fig. 6). However, small negative PEs were observed for treatments with aged BC with primed CO_2 amounts of −9.0–0.3 μg C g^{-1} after 1 day of incubation. Over 3 months of incubation,

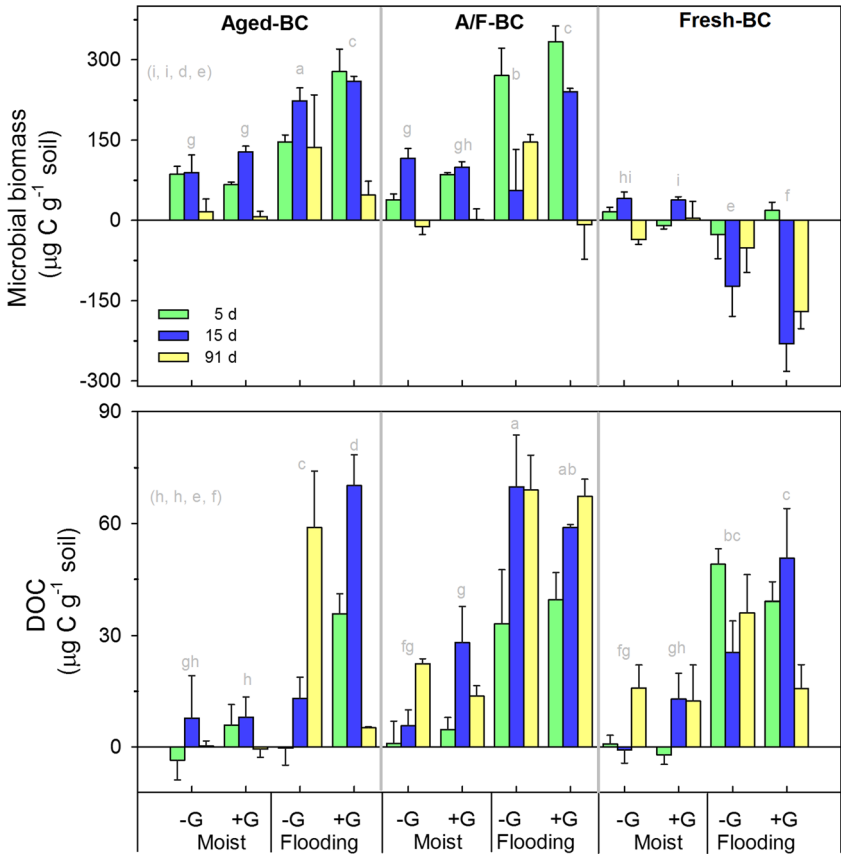

Fig. 3 Changes in microbial biomass (*top*) and DOC content (*bottom*) from BC-added group treatments related to the control during three destructive samplings. The values are the means±SEM (n=3). In each panel, *groups of three bars topped by the same lowercase letters* are not significantly different at $P<0.05$ (results for the control are inserted in the *parentheses*). The repeated-measures ANOVA results are in Table S2 (Electronic Supplementary Material)

the temporal dynamics of the primed CO_2 clearly showed a negative PE due to the presence of aged BC under moist conditions. In comparison, soil samples under flooding conditions behaved quite differently between the aged- and A/F-BC treatments, especially during the later incubation phase. The obvious suppression of PEs due to fresh BC addition was found in the fresh-BC group treatment compared to the control group. Overall, both the main and interactive effects of BC and water regime significantly influenced cumulative primed CO_2 amounts for each treatment and, thus, a significant difference in the primed CO_2 amounts due to water regime was observed both in the control and A/F-BC group treatments.

4 Discussion

4.1 Effect of BC addition on the total CO_2 efflux

Fresh BC addition alone reduced the total CO_2 efflux by 21 % on average compared to the control group, irrespective of water conditions and glucose addition (Fig. 1). These results are consistent with previous results from numerous studies on either short-term or long-term scales (e.g., Kuzyakov et al. 2009; Keith et al. 2011). Similar response was also determined in the aged BC-added soil samples following fresh BC addition (Fig. 1). In contrast, comparable but stimulated CO_2 evolution in the same soil after rice husk BC addition was reported under aerobic conditions, suggesting that the effect of BC on soil respiration is feedstock-specific and should be explored further (Luo et al. 2011; Wang et al. 2012). In agreement with our results, the repeated BC application can reduce the total CO_2 evolution by 27 % in the field (Kimetu and Lehmann 2010). Thus, the reduced CO_2 effluxes are mainly attributed to sorption of DOC on fresh BC, which thereby reduced the microbial and enzyme activities (Table 1; Figs. 3, 4, and 5). On the other hand, the significantly increased soil pH due to fresh BC addition may play an important role on the suppression of CO_2 emission (Fig. S1, Electronic Supplementary Material; Sahrawat 2003). In addition, the presence of aged BC in soil samples yielded higher total C mineralization compared with the control group (Fig. 1), which is inconsistent with the lower C mineralization observed in BC-rich Anthrosols relative to their respective BC-poor adjacent soils (Liang et al. 2010). Therefore, the enhanced decomposition of SOM and BC in aged BC-added soil samples can be explained by the stimulated microbial and enzyme activities (Table 1; Figs. 3 and 5).

4.2 Effect of BC addition on glucose mineralization

The temporal pattern of glucose mineralization was independent of the tested soil samples. However, a lag phase (days 1 to

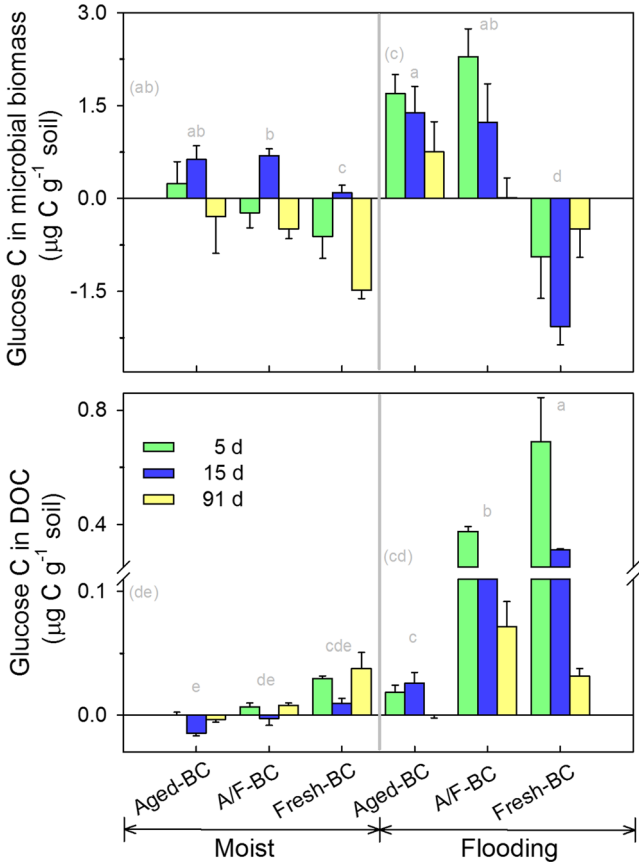

Fig. 4 Changes in ^{14}C-glucose in microbial biomass (*top*) and DOC (*bottom*) from the BC-added group treatments related to the control during three destructive sampling periods. The values are the means±SEM (n=3). In each panel, *groups of three bars topped by the same lowercase letters* are not significantly different at P<0.05 (results for the control are inserted in the *parentheses*). The repeated-measures ANOVA results are in Table S2 (Electronic Supplementary Material)

14) in treatments with fresh BC under flooding conditions was observed (Fig. 2, bottom). This finding is inconsistent with the absence of a lag phase for immediate glucose mineralization in the mixture of BC and C-free sand (Hamer et al. 2004) and residue decomposition following BC addition (Keith et al. 2011). The difference is likely due to glucose sorption on fresh BC particles (Brodowski et al. 2006; Lehmann et al. 2011; Jones et al. 2012), thereby leading to the observed transient lag phase. Considering microbial uptake and use of organic materials outcompetes all physicochemical processes in soils (Fischer et al. 2010), the total glucose mineralization following BC addition did not differ significantly at the end of incubation under the corresponding water conditions. Currently, conflicting results of inhibited (Liang et al. 2010; Keith et al. 2011) and stimulated labile C substrate mineralization (Hamer et al. 2004; Wardle et al. 2008; Jones et al. 2011) response to BC addition have both been reported in various soils.

BC addition did not increase glucose mineralization which may result from at least two reasons. First, the more complex organic compositions of added plant materials behave differently from the simple sugar used herein. Based on the double exponential decay model results, the glucose-derived C in both the respired and immobilized C pools exhibited no significant difference among the treatments under each water condition (Table S1, Electronic Supplementary Material), suggesting that BC addition has minor effect on glucose mineralization, as reported by Jones et al. (2012). Second, the discrepancy is based on the well-recognized principle that labile organic substrate is a more accessible energy source compared to native SOM because of typical limitation of available C to microorganisms in soils (Fontaine et al. 2003). Considering the enhanced degradation of added glucose under flooding

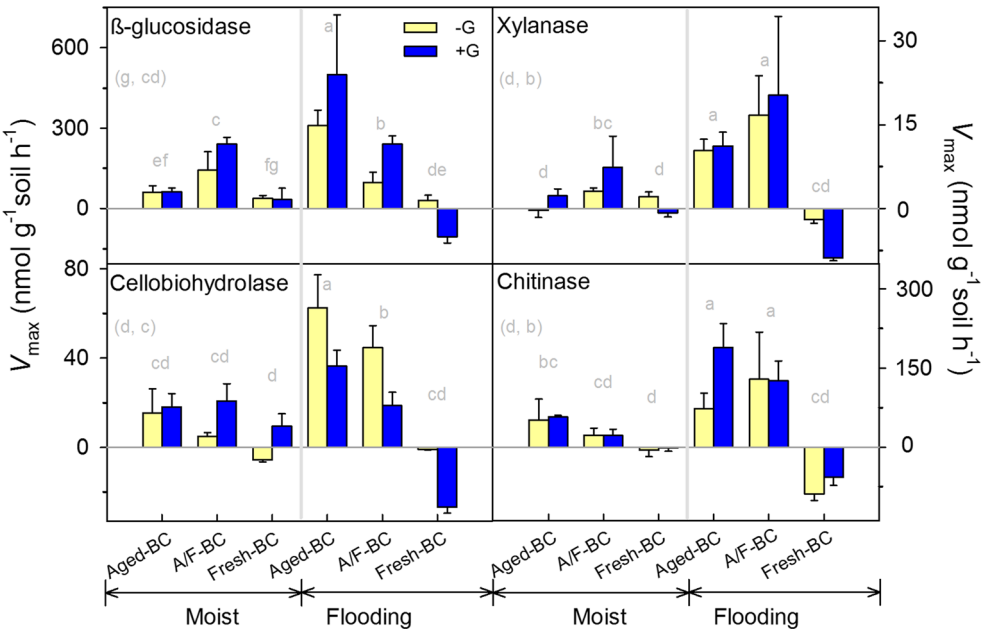

Fig. 5 Changes in the Michaelis-Menten kinetics parameters of maximal biochemical activity (V_{max}, nmol g^{-1} soil h^{-1}) for β-glucosidase, xylanase, cellobiohydrolase, and chitinase from BC-added group treatments related to the control. The values are the means±SEM (n=3). In each panel, *groups of two bars topped by the same lowercase letters* are not significantly different at P<0.05 (results for the control are inserted in the *parentheses*). The ANOVA results are in Table S3 (Electronic Supplementary Material)

conditions relative to moist conditions, we conclude that soil water status is critical in controlling glucose mineralization rather than the presence of BC in our tested soil.

4.3 Effects of BC addition on microbial biomass

Consistent with the higher CO_2 efflux observed in aged BC-added soil samples, more microbial biomass were observed in the aged BC-added treatments, with higher amount under flooding conditions (Fig. 3, top). Our results are in line with the greater microbial biomass found in BC-rich Anthrosols from the Central Amazon (Liang et al. 2010) and in the 2-year field BC-added soil (Jones et al. 2012) compared to the controls without BC. Conversely, fresh BC addition alone decreased microbial biomass (Fig. 3, top), which is supported by Liu et al. (2011). They reported that no change or pronounced decrease in microbial biomass was observed following fresh BC addition in a paddy soil under waterlogging incubation conditions.

These conflicting responses in microbial biomass, especially during the early incubation phase, may result from (i) microbial colonization of aged BC after 1 year of burial in the field (Wardle et al. 2008), (ii) "charosphere" formation as proposed by Quilliam et al. (2013), and/or (iii) labile SOM sorption on BC particles, thus decreasing its bioavailability (Cross and Sohi 2011; Ahmad et al. 2014). Consistent stimulation of ^{14}C-glucose incorporation into microbial biomass in aged BC-added soil samples (Fig. 4, top) further implies that microorganisms may behave differently with fresh BC addition alone in soils. As described above, however, whether a suitable habitat or favorable microsite conditions following BC addition or both contributing to the enhanced microbial biomass remains unclear (Lehmann et al. 2011). Considering the low amount of microbial BC colonization after 3 years of burial in the field (Quilliam et al. 2013), the "charosphere" hypothesis seems to explain the enhanced microbial biomass herein.

4.4 Effects of BC addition on enzyme activities

At the end of incubation, a fluorometric assay as a robust means was adopted to measure the potential activities of four hydrolytic enzymes (V_{max}) in BC-added soil samples (Bailey et al. 2011). The V_{max} values for all enzymes significantly increased in the soil samples with aged BC under flooding conditions, regardless of glucose addition (Fig. 5). In contrast, the V_{max} clearly decreased for all enzymes in the soil samples with only fresh BC added. These findings are consistent with the study in Jones et al. (2012), which showed that glucose mineralization was strongly stimulated in the aged BC (after 3 years of burial in the field) but depressed in the fresh BC. They also suggested the increased denitrification enzyme activity in soil samples treated with BC in the field (Jones et al. 2012). However, in most cases, the V_{max} values of all enzymes from soil samples with added BC under moist conditions exhibited no significant difference compared with the control. Supporting this finding, few effects were observed from BCs on the β-glucosidase, xylanase, and chitinase activities in both forest and arable soils (Bamminger et al. 2014) or on the activities of β-glucosidase, cellobiohydrolase, and chitinase in two contrasting soils without substrate added (Awad et al. 2012) under aerobic conditions. Inconsistent with the increased β-glucosidase activity in aged BC soil samples herein, significantly lower β-glucosidase activity was observed in a paddy soil treated with BC in the field (Chen et al. 2013). We assume that this discrepancy is likely due to the weakness of the colorimetric assay for soils with BC used in the later study compared with the fluorometric assay used herein (Bailey et al. 2011).

Considering that extracellular enzyme activity can be interpreted as an indicator of microbial activity, soil moisture as a limiting factor for microorganisms in our tested soil may have more fundamental effect on microbial activity than the different BCs used. We attribute the stimulated enzyme activities to the increased microbial biomass and high DOC availability. These observations are similar to that the stimulated enzyme activity is expected in a soil with high C content and in the presence of an exogenous substrate (Awad et al. 2012). Regarding the inhibitory effect of fresh BC on enzyme activity, this is likely attributed to DOC sorption on fresh BC particles (Fig. 3), which in turn inhibits the enzyme-substrate reaction by blocking the reaction sites (Bailey et al. 2011; Zimmerman et al. 2011). On the other hand, sorption of enzymes that are exposed to BC may also be related to the lower enzyme activities (Lammirato et al. 2011; Nannipieri et al. 2012; Ahmad et al. 2014). Therefore, we expect that the lower activities due to fresh BC addition will disappear with extensive observations, and all enzyme activities studied herein would be stimulated under optimal conditions, such as flooding in the presence of aged BC. Because conflicting results have emerged (Bailey et al. 2011; Awad et al. 2012; Bamminger et al. 2014), future studies are necessary to understand the underlying mechanism of extracellular enzyme activity upon BC addition through long-term field investigations.

4.5 Effects of BC addition on PEs

Adding glucose to the soil accelerated native SOM mineralization, which produced a positive PE on the control amounting up to 58±8.5 μg C g^{-1} and 102±10.1 μg C g^{-1} under moist and flooding conditions, respectively (Fig. 6). These primed C amounts fell within the ranges summarized by Blagodatskaya and Kuzyakov (2008) when the quantities of added substrate were less than 15 % of microbial biomass. However, the values are much higher than that of a subtropical

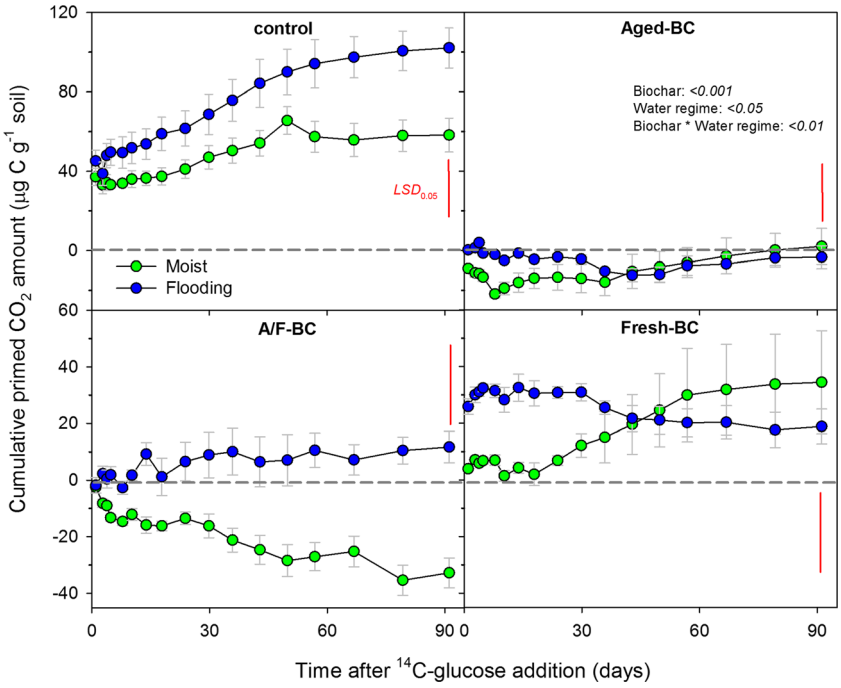

Fig. 6 Cumulative primed CO_2 released from soil samples of control, aged-BC, A/F-BC, and fresh-BC group treatments. The values are the means±SEM (n=3). The ANOVA results with the P values are inserted. The *vertical lines* show the least significant differences at P<0.05 ($LSD_{0.05}$)

paddy soil after glucose addition (Wu et al. 2012). The difference is likely due to a pool substitution with increased turnover of microbial biomass from the high amounts of glucose added to the soils (500 μg C g^{-1}; Wu et al. 2012) rather than the stimulation of native SOM mineralization (Blagodatskaya and Kuzyakov 2008).

To our knowledge, this is the first report on a PE induced by glucose addition from the paddy soil in the presence of aged or/and fresh BC. Notably, the triggered BC degradation is expected due to cometabolic decomposition following addition of the primers (glucose), but researchers have assumed that this effect would strongly decrease over time (Kuzyakov et al. 2009; Keith et al. 2011; Singh and Cowie 2014; Wang et al. 2015). A growing body of evidence suggests that only a small fraction of initial BC-C could be incorporated into microbial biomass, and hence, BC is a negligible C source for microorganisms (Kuzyakov et al. 2014; Wang et al. 2015). Based on these findings, the different behaviors of native SOM mineralization in the presence of aged BC and/or fresh BC are expected. In contrast to our first hypothesis (H1), the control soil with a lower C:N ratio exhibited a greater positive PE than the soil samples with aged BC, which exhibited a negative PE (Fig. 6), suggesting that factors other than N availability affect microbial growth in aged BC-added soil samples (Kuzyakov 2002). This result probably attributed to aged BC soil that provide more favorable conditions for microbial growth due to its great surface area and porosity than the control soil, and thus, they switch from using native SOM to glucose (Zimmerman et al. 2011; Maestrini et al. 2014). Supporting this explanation, soil samples with aged BC exhibited a higher microbial biomass and ^{14}C-glucose recovery in microbial biomass throughout incubation period compared with the control (Figs. 3 and 4). Additionally, these observations may be related to the microbial communities that are altered due to BC buried in a field for longer than 1 year (Jones et al. 2012).

Consistent with our result in aged-BC treatment, two out of three BC-rich Anthrosols exhibited negative PEs after sugar cane residue was added, despite the different primers used (Liang et al. 2010). The suppressed mineralization of SOM and BC is also consistent with the conclusions in Maestrini et al. (2014), which were drawn from a meta-analysis of multiple studies, suggesting that PEs triggered by BC addition on native SOM switch from transient stimulation in the initial period to potential protection during the later period. Based on current understanding of negative priming by various substrates, we attribute the suppressed mineralization of SOM and BC in the presence of aged BC (i) to the preferential use of glucose over the more recalcitrant organic C in SOM and BC (Blagodatskaya and Kuzyakov 2008) and/or (ii) to SOC stabilization due to either organic matter sorption on BC or the BC-induced organic-mineral interaction (Liang et al. 2010; Jones et al. 2011; Ahmad et al. 2014).

In support of our second hypothesis (H2) and elsewhere reported (Cross and Sohi 2011; Keith et al. 2011), a positive PE was observed in the soils with fresh BC added. Because easily available substrate (glucose) was added, our current findings can be explained by (i) stimulated the native SOM decomposition (Kuzyakov et al. 2000) and/or (ii) the promotion of fresh BC degradation due to co-metabolism (Kuzyakov et al. 2009; Hilscher and Knicker 2011; Luo et al. 2011). In addition, fresh BC addition reduced the magnitude of PE

compared with the control (Fig. 6), which is likely due to lower microbial activities from sorption of both native DOC and glucose on the fresh BC surfaces due to its great surface area and porosity (Figs. 3 and 4; Maestrini et al. 2014). From the C sequestration perspective, the fresh BC-added soil exhibited lower C sequestration potential compared to the soil samples with aged BC. Nonetheless, the positive PE induced by glucose addition in the fresh BC-added soil samples (i) will disappear due to exhaustion of labile organic components in the fresh BC via the BC-SOM interaction with prolonged observations and (ii) is far compensated for by the increased soil C from refractory BC-C input. Modeling results also indicate that the potential effect of increased LOM decomposition on the long-term soil organic C stock is negligible after adding BC (Woolf and Lehmann 2012).

4.6 Effects of water regime on the total CO_2 efflux and PE

In contrast with our third hypothesis (H3), flooding increased the amounts of both total C mineralization and primed C in most cases (Figs. 1 and 6). Flooding is a unique feature of paddy soils and plays a crucial role in regulating the DOC concentrations and fluxes, which in turn control the dynamics of CO_2 evolution and SOC storage (Kögel-Knabner et al. 2010). It is recognized that rates of organic matter decomposition in paddy soils are generally considered slower under anaerobic than aerobic conditions, which yields a relatively greater tendency to accumulate organic matter (Sahrawat 2003). This discrepancy is attributable to the significantly enhanced activities of microorganisms and enzymes in the soils under flooding conditions (Figs. 3 and 5). Supporting our findings, through incubating the ^{14}C-labeled dissolved organic matter in a paddy soil, the amounts of CO_2 evolution as the largest recovered pool was enhanced with increasing levels of soil moisture (Chen et al. 2014). Moreover, there is evidence that the high inputs of organic matter contributed to most SOC accumulation in paddy soils rather than retarded decomposition (Yang et al. 2005; Yan et al. 2011). Hence, it remains to be evaluated in detail whether this is due to high organic matter inputs or to low decomposition rates under anoxic conditions (Kögel-Knabner et al. 2010). Considering that all cases showed more responsive to flooding compared to the moist treatment (Figs. 1, 2, 3, 4, 5, and 6), we do not discuss the water effect in detail in this study.

5 Conclusions

The presence of aged and fresh BCs in the paddy soil exhibited contrasting effects on CO_2 efflux over 3 months of incubation. Aged BC improved soil microbial parameters and also contributed to C sequestration in soils by negative priming. In contrast, fresh BC application decreased microbial respiration and enzyme activities, but stimulated positive priming. These findings are attributed to increased microbial biomass in the aged BC soil and the lower microbial biomass in the soil with fresh BC added, especially under flooding conditions. Our results suggest that the preferential use of glucose relative to native SOM and BC by microorganisms and/or the presence of aged BC that stabilizes SOM may contribute to the suppressed mineralization of SOM and BC. Fewer but positive priming were observed in the soil samples with fresh BC alone and may be related to either co-metabolism of fresh BC or stimulated native SOM decomposition or both in response to glucose addition. Our results indicate that BC application would aid long-term C sequestration in our tested soil, despite the positive priming observed in the fresh BC-added soil over a short-term period.

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