Interactive carbon priming, microbial response and biochar persistence in a Vertisol with varied inputs of biochar and labile organic matter

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Summary

There has been great interest in biochar application to soil for long-term carbon (C) sequestration. However, the interactive priming of organic C mineralization, including shifts in microbial community structure and the persistence of biochar in a clayey soil amended with biochar and labile organic matter (LOM) over a relatively long period (i.e. years) remain poorly understood. A 2-year incubation study was done with ^{13}C-depleted biochars produced from *Eucalyptus saligna* Sm. wood biomass at 450 °C and 550 °C. Each of the biochars (at 2%, w/w) in combination with LOM, such as sugarcane residue at input rates of 0, 1, 2 or 4% w/w, were mixed with a C_4-dominated Vertisol. The interactive effect of biochar and LOM on the structure of the microbial community was analysed by terminal restriction fragment length polymorphism (T-RFLP). Our results showed that at the small LOM rates (0 and 1%), there was a positive priming effect of biochar on organic C in soil (i.e. native soil organic C (SOC) + LOM-C)), which shifted to being negative when the LOM input was increased to 2 or 4%. Over the two years, mineralization of C from the 450 °C biochar (1.2–1.7%) was significantly greater than that for 550 °C biochar (0.6–1.0%), and the positively primed mineralization of biochar-C by LOM was enhanced by the increasing rates of LOM input. The negative priming of native SOC + LOM-C mineralization by biochar was greater at large than small inputs of LOM, which would have been facilitated by greater shifting in fungal communities, while enhancing biochar-C mineralization and possibly soil aggregation. In conclusion, over the long-term, the amount of LOM stabilized by biochar was greater than that of positively primed biochar-C mineralization by LOM, in particular at the large LOM input. Biochar can persist in soil on a centennial scale and decreases the turnover

of native SOC + LOM-C over the long term, whereas LOM input can shift microbial communities, favouring LOM stabilization in the biochar-amended Vertisol.

Key words: *Mineralization, priming, microbial community, fungi, bacteria*

Highlights

• Do C priming, biochar persistence and microbial response change two years after biochar–LOM inputs?

• Examined interactive C priming (stale-C isotope), microbial community structure and biochar MRT

• Priming of SOC ± LOM-C by biochar and *vice-versa* increased with LOM inputs

• Biochar–LOM interaction (2-year) shifted microorganisms, favouring LOM-C stabilization in a Vertisol

Introduction

Biochar, a form of charcoal, is produced intentionally from thermochemical conversion of biomass under an oxygen limited condition. Because of the enrichment of the aromatic carbon (C) structures, biochar has been estimated to persist in soil over decades to centuries (Singh *et al.*, 2012; Fang *et al.*, 2014). Therefore, the application of biochar to soil has been

suggested as a means of long-term C storage in a terrestrial ecosystem to mitigate climate change (Woolf *et al.*, 2010).

The potential for C sequestration in biochar-amended soil is related to the interactions of C mineralization derived from biochar and other combined forms of soil organic matter (SOM), such as relatively stabilized native SOM and labile organic matter (LOM). That is, the C sequestration benefits of biochar would decrease if biochar increases mineralization of native SOC or LOM-C, or both (i.e. positive priming) or *vice-versa* (i.e. negative priming) (Hamer *et al.*, 2004). Further, the mineralization of biochar-C may be positively primed by the presence of LOM-C in soil (Hamer *et al.*, 2004; Luo *et al.*, 2011; Farrell *et al.*, 2013). The amount of positively primed biochar-C mineralization increases with increasing availability of LOM, particularly over a short period of time, and then decreases over time following the depletion of easily available sources of C in biochar (Keith *et al.*, 2011).

The direction and level of priming effect of LOM-C or SOC by biochar may also change over the shorter term (four months) with the rate of addition of LOM to soil (Keith *et al.*, 2011). The negative priming of LOM-C by biochar might result from (i) organo–mineral interactions (Brodowski *et al.*, 2006), (ii) improved aggregate stability (Herath *et al.*, 2013) and (iii) physical entrapment of dissolved organic-C within the porous structure of biochar (Pignatello *et al.*, 2006). However, the adsorption of dissolved organic-C on biochar can decrease over time (with the blockage of pores by organic matter) (Kwon & Pignatello, 2005), which decreases the negative priming effect of LOM-C mineralization by biochar. Biochar is also a habitat for microorganisms and generally increases microbial biomass (Pietikäinen *et al.*, 2000; O'Neill *et al.*, 2009; Lehmann *et al.*, 2011), and it can also adsorb nutrients and water

(Clough *et al.*, 2013). A co-location of organic C, microorganisms and other resources in and around biochar (Pietikäinen *et al.*, 2000; Lehmann *et al.*, 2011) may eventually stimulate mineralization of organic C with time (Fang *et al.*, 2017). This might lead to changes in the contributors (such as microbial activity and structure, organo–mineral interaction, sorption of LOM on biochar) to the interactive priming effects with time. To gain a better understanding of the mechanisms and potential for C sequestration of biochar in soil, there is a need to assess the direction and level of the interactive priming effects of inputs of biochar and LOM to soil over a longer-term period.

Biochar and LOM contain different forms of C for use by various microorganisms; hence, the input of biochar and LOM can influence the microbial community and consequently the interactive priming effects. For example, the easily available C (such as glucose-type C) increases bacterial growth but might decrease fungal growth (Meidute *et al.*, 2008), whereas crop residues increase growth of both bacterial and fungal communities (Meidute *et al.*, 2008; Fang *et al.*, 2018a). Biochar increases the fungal community (Steinbeiss *et al.*, 2009; Farrell *et al.*, 2013) and Gram-positive bacteria in soil (Santos *et al.*, 2012; Farrell *et al.*, 2013), which might be related to the use of labile-C in biochar over the short-term (Farrell *et al.*, 2013). With depletion of the labile-C fraction over time, the increase in microbial community resulting from biochar might change. However, very little is known about changes in microbial community with the combined addition of biochar and LOM to soil over the long term and their likely influence on the interactive priming effects.

In this research, we examined the issues raised above in the context of a clayey soil, i.e. Vertisol, which is one of the key soil types under diverse farming systems in Australia

(Fensham *et al.*, 1999; Hulugalle & Scott, 2008): (i) does the interactive priming of organic C mineralization in a soil with varied biochar and LOM inputs change after a long period? (ii) what is the structure of the shift in microbial community after the combined addition of biochar and LOM, and the link with overall interactive priming effects? and (iii) does the persistence of biochar change after LOM inputs over the long term? To answer these questions, C_3 materials (biochars), C_4-vegetaion derived soil (Vertisol) and C_4 LOM (sugarcane residue) were incubated under controlled laboratory conditions. This approach allowed the mineralization of different C sources (i.e. biochar and native SOC + LOM) and their interactive priming effects to be quantified by a two-pool isotope model. The present study is a continuation of a short-term (4-month) incubation experiment (Keith *et al*., 2011); we monitored the interactive priming over ca. two years and analysed microbial community structure at the end of the experiment.

Materials and methods

Biochar, organic matter and soil incubation experiment

The Vertisol soil (0–10 cm) was sampled from a grassland area where C_4 tussock Mitchell grass (*Astrebla* spp.) had been grown for over 100 years at Toorak Research Station near Julia Creek, Queensland, Australia (21°022493S, 141°782553E). The soil for the incubation experiment and analysis was air-dried, ground to < 2 mm and roots and debris (> 2 mm) were removed. The basic soil properties were: $pH_{1:5H2O}$ 8.10 and organic C 0.45% with predominantly C_4-vegetation organic matter ($\delta^{13}C$ –14.3‰), and it was rich in clay (clay

content 53%) dominated (> 80%) by smectite (Keith *et al.*, 2011). No inorganic C was detected in the soil (Keith *et al.*, 2011).

Biochars were produced at 450 and 550 °C by slow pyrolysis from ′^{13}C-depleted *Eucalyptus saligna* Sm. wood biomass, which had been grown for two years under an elevated CO_2 environment (Keith *et al.*, 2011). The biochars were ground to < 2 mm for the experiment. The ′^{13}C of 450 and 550 °C biochars were –36.3 and –36.4‰, respectively, porosity values were 57.2 and 67.5%, respectively, and specific surface areas were 191 and 228 m^2 g^{-1}, respectively (Keith *et al.*, 2011). Sugarcane residue produced from leaves and tops (C_4 biomass) was used as the source of LOM. The dried (60 °C) sugarcane residue was ground to < 2 mm for the incubation experiment. The basic properties of sugarcane residue were: organic C 39.6% and ′^{13}C of –12.7‰. Properties of the soil, biochar and sugarcane residue have been described elsewhere in (Keith *et al.*, 2011; Fang *et al.*, 2014).

The biochars were applied at rates of 0 or 2% (w/w) and the sugarcane residue was added at rates of 0, 1, 2 or 4% (w/w) to 200 g soil (oven-dried basis). Biochar, sugarcane residue and the soil were mixed homogenously together with a nutrient solution plus microbial inoculum, and then incubated in the dark at 20 ± 1 °C in the laboratory after adjusting the soil moisture content to 60% of water holding capacity (WHC). All treatments were replicated three times, in a completely randomized design. The detailed procedures of the incubation experiment have been described in Keith *et al.* (2011). The major nutrients added to the soil were (kg^{-1} soil): 183 mg N, 28 mg P, 220 mg K, 13 mg S, plus some inputs of trace elements (Chapman, 1997). The microbial inoculum was prepared by mixing a range of soils collected from fields under native eucalyptus forests (*Eucalyptus saligna* Sm.), pine plantation (*Pinus radiata*),

maize cropping (*Zea mays* L.) and grazed pastures (*Medicago sativa*, *Phalaris aquatica*, *Trifolium subterraneum* ssp. *yanninicum*), and then extracted by deionized water (50 g soil:100 ml water, sieved to < 100 μm). The soil extract was mixed with the nutrient solution in a ratio of 1:100. The incubation buckets were opened bimonthly for oxygen. The soil moisture was maintained by the addition of deionized water in amounts calculated by weight lost at each sampling time or bimonthly after 240 days.

The CO_2 evolved from the soil and soil–biochar mixtures was trapped continuously in a 2 or 2.5 M 40 ml NaOH solution. The CO_2 traps were replaced after 1, 2, 3, 5, 8, 13, 20, 30, 44, 65, 90, 120, 240, 310, 430, 506, 630 and 758 days. An extra precaution was taken to avoid saturation of the NaOH traps, that is, less than one third of the NaOH trap solution was reacted with emitted CO_2 at different sampling times throughout the incubation period. The statistical analysis for rates of total C and biochar-C mineralization was done throughout the whole incubation period (from 1 to 758 days).

Mineralization of biochar, mean residence time and priming effect

The total C mineralized (mg kg^{-1} soil) in treatments was determined by titrating 1 ml of the CO_2 trap solution against 0.1 M HCl, using phenolphthalein as the indicator. The CO_2-C in the trap solution was precipitated with $SrCl_2$, and an $SrCO_3$ precipitate was obtained for $\delta^{13}C$ analysis. From the $\delta^{13}C$ data, the proportion of CO_2 derived from biochar-C (f_{Biohar} (%)) or native SOC + LOM-C (sugarcane residue) (100 – f_{Biohar} (%)) was determined by a two end-member mixing model (Keith *et al.*, 2011):

$$f_{\mathrm{Elr\,fkdu}} = \frac{\delta_T^t\,{}^{13}\mathrm{CO_2} - \delta_{\mathrm{SLOM}}^t\,{}^{13}\mathrm{CO_2}}{\delta_{\mathrm{B}}\,{}^{13}\mathrm{CO_2} - \delta_{\mathrm{SLOM}}^t\,{}^{13}\mathrm{CO_2}} \times 100\,, \qquad (1)$$

where, $\delta_T^{t\,13}CO_2$ is the δ^{13}C value of total (T) CO_2-C evolved from biochar + LOM amended soil over time (t), $\delta_{SLOM}^{t\,13}CO_2$ is the δ^{13}C value for the CO_2-C evolved from LOM amended (1, 2 and 4% rates of LOM) or non-amended soil (0% LOM) over time, and $'_B{}^{13}C$ is the value of the fresh biochars (−36.3 and −36.4‰ for the 450 and 550 °C biochar, respectively). The amount of biochar-C mineralized was calculated by multiplying $f_{Biochar}$ (%) by total C mineralized.

The amount of native SOC + LOM-C mineralized was calculated by subtracting biochar-C mineralized from total C mineralized. Because of the similar $'^{13}C$ values of soil (−14.3‰) and LOM (−12.7‰), the quantity of C mineralized from native SOC and LOM-C was indistinguishable. We also did a sensitivity analysis to test the effect of using $'_{SLOM}{}^{13}CO_2$ in the presence of biochar as the endmember (Table S1, Supporting Information). Three scenarios are presented with respect to C source partitioning: (i) no effect of the addition of biochar on SOC + LOM-C, (ii) larger positive priming effect of biochar on LOM-C than SOC mineralization and (iii) larger positive priming effect of biochar on SOC than LOM-C mineralization. Assuming 5% variation in the LOM contribution to the C mineralization from SOC + LOM-C ($'_{SLOM}{}^{13}CO_2$) in the presence of biochar, the proportion of biochar-C mineralization (out of total C mineralization) might vary by 3% relative to a scenario where no effect of biochar on the contribution of SOC + LOM-C to $'_{SLOM}{}^{13}CO_2$ was considered (i.e. control) in the two-end member mixing model (see Table S1).

Biochar-C mineralization data were fitted to the two-pool exponential model (Fang *et al.*, 2014) to estimate the MRT of biochar-C in the soil.

The priming effect of biochar-C on the mineralization of native SOC ± LOM-C (PE^{B}_{SLOM}) was calculated as:

$$PE^{B}_{SLOM} = C_{B,SLOM} - C_{SLOM}, \quad (2)$$

where, $C_{B,SLOM}$ is the amount (mg kg^{-1} soil) of native SOC or native SOC + LOM-C mineralized from the biochar-amended soil and C_{SLOM} is the amount (mg kg^{-1} soil) of native SOC or native SOC + LOM-C mineralized from the control soil (without biochar).

The priming effect of LOM on the mineralization of biochar-C (PE^{SLOM}_{B}) was determined as:

$$PE^{SLOM}_{B} = C_{B,SLOM} - C_{B} \quad (3)$$

where $C_{B,SLOM}$ is the amount (mg kg^{-1} soil) of biochar-C mineralized from biochar-amended soil in the presence of LOM and C_B is the amount (mg kg^{-1} soil) of biochar-C mineralized from biochar-amended soil in the absence of LOM.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

DNA extraction. Soil DNA was extracted from 0.25 g soil samples with the MoBio Powersoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) and following the protocol provided by the supplier. Purified soil DNA was stored at −20 °C. The amounts of extracted DNA were assessed qualitatively on a 1.0% agarose gel run at 220 V for 20 minutes and stained with ethidium bromide.

Polymerase chain reaction (PCR) amplification. The T-RFLP analysis of bacterial and fungal communities targeted the 16S rRNA genes and internal transcribed spacer (ITS) genes,

respectively. Fluorescently labelled primers were synthesized by Life Technologies (Carlsbad, CA, USA). For the bacterial communities, T-RFLP was performed with the labelled primers 338F (ACWCCTACGGGWGGCWGC) and 1050R-NED (AYCTCACGRCACGAGCTGAC). The PCR program was run in a total volume of 30 µl, containing 17.8 µl milli-Q water, 6 µl PCR reaction buffer (5 × MyTaq^{TM} Red DNA Polymerase, BIOLINE, London, UK), 1 µl of each primer (10µM), 0.2 µl of *Taq* polymerase and 4 µl of extracted DNA as the template. The thermocycling program included an initial denaturation step at 95 °C for 5 minutes followed by 35 cycles of the denaturation step at 95 °C for 30 s, annealing step at 60 °C for 30 s, elongation step at 72 °C for 1 minute and a final 7 minutes elongation step at 72 °C. The fungal community was detected with the ITS1-VIC (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers under the conditions described above but using a temperature of 47 °C in the annealing step. The size of PCR products was determined by comparing with a size standard on 1.5% agarose gels stained with ethidium bromide (Sigma-Aldrich, Sydney, Australia), using ChemiDoc^{TM} MP (Bio-Rad, Hercules, California, USA). Any extractions that gave insufficient DNA were re-extracted. In the next step, the products (40−55 µl) were purified by ethanol precipitation. In each PCR product, 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol were added, and they were incubated for 30 minutes at room temperature. The supernatant was discarded after centrifugation at 12 000 *g* for 20 minutes and the precipitates washed with 70% ethanol. To dissolve the DNA precipitate, 15 µl of milli-Q water was added. The purified PCR products concentration was between 100 and 200 ng μl^{-1}.

T-RFLP analysis. The purified PCR products were digested with 0.25 µl of the restriction enzyme *Hha* I (bacteria) or *Taq* I (fungi), 0.25µl of BSA, 2.5 µl of 10 × NEB buff 4 and 7 µl of milli-Q water, and then incubated at 37 °C overnight. The restriction enzymes were inactivated with an incubation at 65 °C (*Hha* I) or 80 °C (*Taq* I) for 20 minutes. Bacterial and fungal PCR products were combined and analysed by the Australian Genome Research Facility (AGRF). In the control soil (no biochar and LOM inputs), attempts to PCR-amplify fungal DNA for T-RFLP analysis were unsuccessful (see Figure 5c and d). The labelled, purified PCR products were separated based on their length (base-pair number) by electrophoresis. A typical electropherogram is shown in Figure S1, Supporting Information. More theoretical and analytical information on T-RFLP is available elsewhere (Singh *et al*., 2006; Culman *et al.*, 2008). Details on the data processing are given in the statistical section below.

Statistical analysis

Cumulative total C and biochar-C, MRT, and the interactive priming effects of LOM and biochar were analysed with a two-way (LOM and biochar) analysis of variance performed in GenStat® 14th edition (VSN International, 2011). The effect of LOM (3 DF) was broken into linear (1 DF) and non-linear (2 DF) contrasts. Repeated measures analyses were performed for total C and rates of biochar-C mineralization using a linear mixed model framework in GenStat®. Each analysis consisted of fixed effects of LOM, biochar, time and all associated interactions, and random effects of replicate, and replicate Ï time. To allow for correlation between repeated measures in the same units, a first-order autoregressive model was fitted (giving a larger residual log-likelihood than the power model). Heterogeneity in the residual

variances between time points was included because it was deemed significant with a residual likelihood ratio test.

The T-RFLP data (i.e. electropherograms) were processed to study the microbial community structure and its shifts under the various treatments (LOM rates and biochar type). In an electropherogram, a peak represents a digested PCR fragment. A peak height represents an amount of fluorescence unit. The peak relative abundance was calculated when standardized to the total fluorescence present in a T-TRFLP profile (i.e. in a sample). The T-REX analysis pipeline (Culman *et al.*, 2009) was used to clean the T-RFLP profiles. This step is important to remove the background (fluorescence) noise inherent to this type of analytical technique. Noise filtering was applied to determine true peaks from background noise following the approach developed by Abdo *et al*. (2006). Briefly, true peaks were differentiated from background noise by an iterative approach. Peaks whose height exceeded the standard deviation computed over all peaks (with a standard deviation multiplier of 1.1) were identified as true peaks. These peaks were then removed from the dataset and the process was repeated until no additional true peaks were identified. The peaks were aligned using the T-align algorithm (Smith *et al.*, 2005) with a clustering threshold of 0.5 allowing for more than one peak to be classified as a single T-RF. When concomitant peaks were detected and deemed similar by the algorithm, they were binned into a single category called a T-RF. The same settings were used for both bacteria and fungi T-RFLP data. To decrease further the background noise, the T-RFs present in less than 0.05% of the samples were discarded. The remaining T-RFs comprised 92% (bacteria) and 98% (fungi) of the total cumulative T-RF contribution. This was deemed sufficient. The final dataset contained the T-RF % relative

abundance and was used for statistical analyses (see below). The T-RF % values were Hellinger-transformed, which is usually necessary with this type of data (Nazaries *et al.*, 2018).

A principal component analysis (PCA) was performed on the T-RFLP dataset with Minitab 16 (Minitab Inc., State College, Pennsylvania, PA, USA). A covariance matrix was selected because the variables (T-RF relative abundance) were of the same type (% relative abundance of a T-RF across all T-RFs in a T-RFLP profile). The resulting new axes, the principal components (PCs), contain information based on T-RF relative abundance. These PCs were used to explore shifts in the microbial community structure (see the caption of Figure 5). It is usual to retain only the few few PCs that contain most of the information (Cangelosi & Goriely, 2007). The Supporting Information describes the problem of selecting how many PCs to retain for exploring treatment effects in the present study. In brief, four common 'stopping' rules were tested (see Supporting Information), all deduced from a scree plot (Figure S2, Supporting Information). The choice of the number of PCs to retain was made if at least two of the stopping rules were met. The dimensions selected were the first three PCs for bacteria (Table S2, Supporting Information) and the first two PCs for fungi (Table S3, Supporting Information). Following this, a multivariate ANOVA was run on all selected PCs to evaluate the overall significance of the treatments on the microbial community structure.

Results

Total C mineralization

Rates of total C mineralization (native SOC + biochar-C + LOM-C; mg CO_2-C g^{-1} soil C day^{-1}) decreased with time and they were significantly ($P < 0.001$) affected by the main effects of LOM, biochar, time, and the interactive effects of LOM, biochar and time (Table 2). Rates of total C mineralization decreased significantly ($P < 0.001$) in the presence of biochar (Figure 1): non-biochar (0.03–30.85 mg CO_2-C g^{-1} soil C day^{-1}), > 450 °C biochar (0.01–17.05 mg CO_2-C g^{-1} soil C day^{-1}) and 550 °C biochar (0.01–16.44 mg CO_2-C g^{-1} soil C day^{-1}). On the other hand, the rates of total C mineralization increased significantly ($P < 0.001$) with increasing rates of application of LOM: 0% (0.01–1.55 mg CO_2-C g^{-1} soil C day^{-1}), < 1% (0.02–16.10 mg CO_2-C g^{-1} soil C day^{-1}), < 2% (0.02–23.76 mg CO_2-C g^{-1} soil C day^{-1}) and < 4% (0.05–30.85 mg CO_2-C g^{-1} soil C day^{-1}). However, rates of total C mineralization were not affected by the interactive effects of LOM and biochar (Table 2). Over the 25 months, cumulative total C mineralization (mg CO_2-C g^{-1} total SOC) in each LOM treatment was greatest in the control (no biochar), followed by the 450 °C biochar, and the smallest was in the 550 °C biochar (Figure S3, Table S5, Supporting Information). Further, cumulative total C mineralization was greatest with the largest rates of application of the LOM (Tables 3 and S5). Total C mineralization rate (mg CO_2-C kg^{-1} soil d^{-1}) is also shown in Figure S4 (Supporting Information).

The pH of the soils decreased after 25 months of incubation across all treatments, from pH 8.10 at the beginning (Table 1), which was due to the reaction of CO_2 fluxes with [OH^-] and produced HCO_3^- and CO_3^{2-}. Based on the change in pH (from 8.10 at day 0 to 6.56 at 25 months) and its water content (60% of WHC) in the control soil (without biochar and LOM), about 2.2 mg C kg^{-1} soil could have been absorbed in the soil solution during the incubation

period. Similarly, in biochar and LOM treatments (pH decreased from 8.10 at day 0 to 6.73–7.69 at 25 months); the soil solution might have trapped about 2.5–5.0 mg C kg^{-1} soil across treatments. Yet, the amount of C trapped in the soil was negligible relative to the total CO_2 emitted during the experiment (Figure 1). Moreover, there is no inorganic C in this soil (Vertisol). Therefore, the exchange of HCO_3^- and CO_3^{2-} from the soil would have had negligible effect on the $\delta^{13}C$ values of CO_2 emitted beyond organic C sources in the soil (SOM, biochar or LOM).

Priming effect of biochar on organic C mineralization in soil with or without LOM addition

The ^{13}C values of soil (−14.3‰) and LOM (−12.7‰) were similar; therefore, we could only distinguish the priming effect of biochar on native SOC + LOM-C but not on native SOC alone in the presence of LOM. The direction and level of priming effect of biochar on the mineralization of organic C (native SOC + LOM-C) in the soil were different for the smaller (d 1%) and larger (e 2%) rates of application of LOM. In the 0 and 1% LOM treatments, the biochars generally caused a positive priming effect on the mineralization of native SOC or native SOC + LOM-C over time (Figure 2), that is, 14.67–55.85 mg CO_2-C kg^{-1} soil on 758 days. On the other hand, in the 2 and 4% LOM treatments, the biochar-induced negative priming effect was sustained over the whole incubation period (25 months), ranging from –1.10 to –305.49 mg CO_2-C kg^{-1} soil. The negative priming effect was enhanced when the rate of LOM application was increased from 2 to 4%. Native SOC (0.45%) was small, therefore the negative priming effect of biochar might be attributed mainly to LOM-C mineralization in the soil, in particular at the 4% LOM rate. The magnitude of the negative priming effect of biochar on the mineralization of native SOC or LOM-C, or both, was relatively stable from 4

to 25 months for all treatments, except for the 1% LOM treatment where the negative priming effect changed to positive after 10 months and 18 months in the 450 and 550 °C biochar, respectively. There was no significant difference in the priming effect of the 450 and 550 °C biochars on the mineralization of native SOC and LOM-C (Table 4).

Biochar-C mineralization

Over the 25 months, the proportion of C mineralized (equivalent to mg CO_2-C g^{-1} biochar-C divided by 10) in the 450 and 550 °C biochars in the soil without LOM input (i.e. 0% LOM treatment) was 1.3 and 0.7%, respectively. In the presence of LOM (1, 2 and 4%), between 1.2 and 1.7% (450 °C biochar) and between 0.6 and 1.0% (550 °C biochar) of the added biochar-C was mineralized (Figure 3). Within each LOM treatment, the amount of biochar-C mineralized for the 450 °C biochar was 1.7 to 2.2 times greater than for the 550 °C biochar (Figure 3, Table 4). The rate of biochar-C mineralization decreased with time and it was significantly ($P < 0.001$) affected by the main effects of the LOM, biochar, time and the interactive effects of LOM and time, and biochar and time (Figure S5, Table S4, Supporting Information).

The MRT values of the biochars across different LOM treatments were significantly ($P < 0.001$) smaller for the 450 °C biochar (314−713 years) than 550 °C biochar (567−978 years) (Tables 4, 6 and S6). The MRT of both biochars was significantly ($P < 0.001$) greater in the control soil (i.e. with no LOM addition) than in the LOM treatments. The rates of LOM application ranging from 1 to 4%, however, did not significantly affect the MRT of both biochars.

The $\delta^{13}C$-CO_2 emissions across treatments ranged between –12 and –26‰ (Figure S6, Supporting Information). Of the total C emission over time, the proportion of CO_2 (%) evolved from the biochar decreased with increasing rates of LOM application (Figure S7, Supporting Information), ranging from 17−33, 6−20, 5−15 and 2−8% for 0, 1, 2 and 4% LOM treatments, respectively. For each of the rates of LOM application, the proportion of biochar-derived CO_2 evolved was greater for the 450 °C biochar than the 550 °C biochar.

Priming effect of LOM on biochar-C mineralization

The direction and level of the priming effect of LOM on biochar-C mineralization was relatively stable after 4–6 months (Figure 4, Table S6). In the initial period (i.e. 1 month) of the experiment, the positive priming of biochar-C increased with increasing LOM input: 1% (~11 mg CO_2-C kg^{-1} soil), < 2% (~17 mg CO_2-C kg^{-1} soil) and < 4% (~48 mg CO_2-C kg^{-1} soil). There was a negative priming effect at about 4 months for the 1% LOM treatment (about –6 to –13 mg CO_2-C kg^{-1} soil), which remained negative for both biochars until termination of the experiment at 25 months (with a maximum negative PE value of –21 mg CO_2-C kg^{-1} soil). The positive priming of biochar-C in the 2% LOM treatment decreased to no priming for the 450 and 550 °C biochars at approximately 6 months. After 6 months, the no priming effect of LOM on biochar changed to positive priming for the 450 °C biochar (with a maximum value of 16 mg CO_2-C kg^{-1} soil) and remained close to no priming for the 550 °C biochar. For the 4% LOM treatment, the positive priming of biochar-C that ranged from 4 to 57 mg CO_2-C kg^{-1} soil after 2 days was sustained for the two biochars over the whole incubation period. However, the uncertainty in these measurements was quite large. In

terms of the biochar treatments, there was no significant difference in the priming effect of LOM on biochar-C mineralization between the 450 and 550 °C biochars (Table 5).

Effect of biochar and LOM on microbial community structure

The bacterial community responded to the application of LOM (at 1, 2 or 4% w/w) (see PC1 in Figure 5a; $P < 0.001$), but not to the application of biochar (see PC1 in Figure 5b; $P = 0.125$). The bacterial shift was stronger between 0% rate of LOM application and the other LOM rates (see PC1 in Table S2; $R^2_{adj} = 94\%$), with a non-significant trend of the bacterial community structure forming a gradient with increasing rates of LOM application (Figure 5a). This first three PCs of this analysis explained at least 50% of the total variance in the data (Table S2).

The fungal community also responded to LOM application but with a difference: a significant shift in fungal community structure was only detected with 4% LOM (see PC1 in Figure 5c; $P < 0.001$). This was the strongest effect (see PC1 in Table S3; $R^2_{adj} = 96\%$), Like bacteria, there was a non-significant trend with increasing rates of application of LOM. The application of biochar did not affect the fungal community structure (see PC1 in Figure 5d; $P = 0.954$). The first two PCs of this analysis explained at least 53% of the total variance in the data (Table S3).

Discussion

The interactive priming of C mineralization in the soil amended with biochar and LOM

The present study shows that the priming effects of biochar on organic C mineralization (i.e. native SOC ± LOM-C) and of LOM on biochar-C mineralization, stabilized after 4–6 months.

The result of stabilization of the priming effects after ~6 months accords with previous findings from laboratory incubation experiments that examined similar types of biochars in different soil types, including Inceptisol, Entisol, Oxisol and Vertisol (Fang *et al.*, 2015; Fang *et al.*, 2017) or different types of biochar in the same Vertisol (Singh & Cowie, 2014). This could be due to several reasons. First, the stabilization of priming effects in such a time period (beyond 4–6 months) was possibly related to a decrease in microbial growth and microbial use of C (Fang *et al.*, 2018b); easily available C sources in the soil might have depleted within a few months (Fang *et al.*, 2017). Second, the labile organic compounds might have been stabilized because of organo–mineral associations, i.e. in the first four months (Liang *et al.*, 2010; Fang *et al.*, 2018c). Third, the adsorption–desorption of labile-C on biochar might have reached an equilibrium after 4–6 months (Smebye *et al.*, 2016). Fourth, the stabilization of native SOC and LOM through direct sorption of dissolved SOM and microbial enzymes on surfaces and within pore spaces of biochar might reach a maximum over time because of clogging of the biochar's pore network (Pignatello *et al.*, 2006). The interactive priming of biochar and native SOC ± LOM-C mineralization was not significantly affected by biochars produced at the two pyrolysis temperatures (Tables 4 and 5), which could be due to some similar surface properties, such as specific surface area and pore volume, of the biochars (Keith *et al.*, 2011).

Shift of microbial community structure and priming effect

In the present study, biochar exerted no influence on soil bacterial and fungal communities at 25 months after incubation, which contrasted with previous findings from short-term studies where biochar addition induced a shift in soil microbial communities (O'Neill *et al.*, 2009;

Santos *et al.*, 2012; Farrell *et al.*, 2013). The labile biochar-C component (~1%; Table 6) was depleted over the 25 months, possibly resulting in the disappearance of any short-term changes in the microbial community from the addition of biochar. Moreover, observed changes in the microbial community in some biochar studies (O'Neill *et al.*, 2009; Santos *et al.*, 2012; Farrell *et al.*, 2013) could be partly attributed to the increased pH after the addition of biochar in acidic soils (pH: 4.2−5.8). However, the soil in the present study had an alkaline pH (8.1) and was amended with alkaline biochars (pH 8.6–9.9) that might not have induced the shift of microbial community as expected or observed in acidic soils.

The greater shift in the fungal communities at the 4% rate of LOM of application (cf. 1 and 2%), suggested that fungal communities might have shifted to a structure that can degrade relatively recalcitrant C forms, thus causing the positive priming of biochar-C at 4% LOM (Figure 5). Further, the results of no effect of biochar on bacterial and fungal communities suggest that the shift of microbial communities might not be linked directly to the negative priming of LOM-C mineralization by biochar at the larger rates of application of LOM (2 and 4%). However, the shift of fungal community might indirectly relate to the increasing negative priming of LOM by biochar. That is, the larger rates of addition of LOM in the biochar-amended soil might have facilitated fungal growth which may have enhanced soil aggregation and aggregate stability (Tisdall & Oades, 1982).

The persistence of biochar in soil

The proportion of wood biochar-C mineralized over 25 months (0.6−1.7%) was within the range (0.4–1.5%) from previous studies that also incubated plant-based biochars at similar lengths of time in similar or different soils (Singh *et al.*, 2012; Fang *et al.*, 2015). The smaller

degree of mineralization of biochar-C from the 550 °C biochar (0.6–1.0%) than the 450 °C biochar (1.2–1.7%) was attributed to the small labile C pool (Table 6) and large aromaticity of the high-temperature biochar (Singh *et al.*, 2012). The longer MRT of biochar in the control (0% LOM) than in the LOM treatments (1, 2 and 4%) (Table 6) indicated that the input of plant-derived C, which could be expected under natural field conditions, had decreased the longevity of biochar-C. Hence, these realistic conditions should be considered when estimating the MRT of biochars. Duration of the incubation period also influences the estimates of biochar MRT. This was indicated by a much longer MRT of both biochars (314−713 years for the 450 °C biochar and 567−978 years for the 550 °C biochar) in the present study (25 months) compared to the estimates obtained from the short-term biochar-C mineralization results reported earlier (62−112 years for the 450 °C biochar and 100−248 years for the 550 °C biochar) (Keith *et al.*, 2011). The MRT values estimated from data with shorter incubation times are likely to be biased towards labile biochar-C components, whereas data from longer incubation experiments include more data points that might represent the recalcitrant C components of biochar better (Singh *et al.*, 2012). Thus, the inclusion of additional data points with a longer incubation time, plus the above-mentioned realistic field conditions (such as the input of plant litter) is likely to provide more robust estimates of biochar MRT than those estimated from shorter incubation periods (Keith *et al.*, 2011).

Conclusions

A negative priming effect of biochar on LOM-C mineralization occurred with large rates of application of LOM (2 to 4% w/w), which stabilized after 4–6 months. This suggested further enhancement of the sequestration potential of biochar-C over the long term in a clayey soil receiving organic matter inputs. The addition of biochar did not change the microbial community structure in the Vertisol after the long-term incubation period. However, the addition of LOM caused changes in the structure of the microbial (particularly fungal) communities in the soil, which might have contributed to the negative priming effect of biochar on LOM-C mineralization indirectly, in addition to enhanced biochar-C mineralization with the largest addition of LOM. Our results on biochar MRTs and the negative priming effects indicated that biochars produced through slow pyrolysis at 450 and 550 °C can provide long-term C storage benefits, even in the presence of LOM. However, further research is warranted to elucidate indirect mechanisms of LOM-C stabilization by biochar, and to evaluate specific fungal community structures and their role in the degradation of biochar in the presence of LOM to underpin the C storage dynamics in the clayey soil.

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Table 1 Mean soil pH (n = 3) after the 25-month of incubation period.

Treatments		pH
LOM /%	**Biochar type /°C**	
0	Nil	6.56
	450	6.73
	550	6.88
1	Nil	7.07
	450	7.16
	550	7.30
2	Nil	7.37
	450	7.45
	550	7.55
4	Nil	7.56
	450	7.62
	550	7.69

Table 2 Wald *F* statistics for the effects of time, labile organic matter (LOM) and biochar and their interactions on the total carbon mineralization rate **(**repeated measures ANOVA).

Source	Degrees of freedom, df	Wald *F* statistic, df	Denominator degrees of freedom, ddf	*P* value
TC_{min} rate /mg CO_2-C g^{-1} soil C day^{-1}				
Time (t)	17	1795.3	49.4	<0.001
LOM (L)	3	331.9	60.5	<0.001
Biochar (B)	2	211.4	60.5	<0.001
t Ï L	51	209.7	205.2	<0.001
t Ï B	34	136.0	179.2	<0.001
L Ï B	6	1.84	60.5	0.107
t Ï L Ï B	102	13.23	229.3	<0.001

TC_{min}, total carbon mineralization rate.

Table 3 Analysis of variance (ANOVA) of the effects of labile organic matter (LOM) and biochar, and their interactions, on the cumulative total C mineralized (two-way ANOVA).

Source	Degrees of freedom, df	Mean square	*F* ratio	*P* value
CTC /mg CO_2-C g^{-1} soil C				
LOM (L)	3	73 409.9	1946.1	<0.001
Linear (l)	1	195 642.2	5186.4	<0.001
Non-linear (nl)	2	12 293.7	325.9	<0.001
Biochar (B)	2	64 959.5	1722.1	<0.001
None vs. some (the average of B450 and B550, B_1)	1	129 477.7	3432.4	<0.001
B450 vs. B550 (B_2)	1	441.2	11.7	<0.001
L Ï B	6	2839.2	75.3	<0.001
Ll Ï B_1	1	6766.7	179.4	<0.001
Lnl Ï B_1	2	5104.2	135.3	<0.001
Ll Ï B_2	1	54.3	1.4	0.242
Lnl Ï B_2	2	2.9	0.08	0.926
Residuals	24	37.72		
Total	35			

CTC, Cumulative total C mineralized (Figure 1).

Table 4 Analysis of variance (ANOVA) of the effects of labile organic matter (LOM) and biochar, and the interactions of LOM and biochar, on the variables (two-way ANOVA).

Source	Degrees of freedom, df	Mean square	*F* ratio	*P* value
Primed SOC + LOM-C /mg CO_2-C kg^{-1} soil				
LOM (L)	3	84 801.5	3.71	0.034
Linear	1	194 880.4	8.520	0.010
Non-Linear	2	29 762.1	1.300	0.299
Biochar (B)	1	729.6	0.03	0.860
L Ï B	3	4 552.5	0.20	0.895
Linear	1	8 975.5	0.393	0.540
Non-Linear	2	2 340.9	0.102	0.903
Residuals	16	22 866.0		
Total	23			
CBC /%				
LOM (L)	3	0.22	3.14	0.054
Linear	1	0.53	7.61	0.014
Non-Linear	2	0.06	0.911	0.422
Biochar (B)	1	2.58	36.78	<0.001
L Ï B	3	0.01	0.04	0.988
Linear	1	0.01	0.06	0.803
Non-Linear	2	0.002	0.03	0.971
Residuals	16	0.07		
Total	23			
MRT /years				
LOM (L)	3	22 6281.3	9.46	0.001
Linear	1	372 676.5	15.58	0.001
Non-Linear	2	153 083.7	6.40	0.009
Biochar (B)	1	45 5257.5	19.04	<0.001
L Ï B	3	1379.9	0.06	0.981
Linear	1	770.6	0.03	0.860
Non-Linear	2	1 684.5	0.07	0.932
Residuals	16	23 915.6		
Total	23			

Primed SOC + LOM-C, Priming of biochar on soil organic carbon (SOC) and labile organic matter (Figure 2); CBC, Percent of cumulative biochar-C mineralized (Figure 3); MRT, mean residence time of biochar (Table 6).

Table 5 Analysis of variance (ANOVA) of the effects of labile organic matter (LOM) and biochar, and their interactions on the primed biochar by LOM (two-way ANOVA).

Source	Degrees of freedom, df	Mean square	*F* ratio	*P* value
Primed biochar /mg CO_2-C kg^{-1} soil				
LOM (L)	2	6160.4	3.46	0.065
Linear	1	12 263.8	6.887	0.022
Non-Linear	1	57.0	0.032	0.861
Biochar (B)	1	770.1	0.43	0.523
L Ï B	2	15.0	0.01	0.992
Linear	1	23.7	0.013	0.910
Non-Linear	1	6.4	0.004	0.953
Residuals	12	1780.6		
Total	17			

Primed biochar, Priming of LOM on biochar-C (Figure 4).

Table 6 The mean residence time of the labile and recalcitrant pools of biochar-C estimated with a two-pool exponential model fitted to the biochar-C mineralization data over the 25-month of incubation period (n = 3). The standard error of the predicted mean for each combination of mean residence time of recalcitrant pools was 89 years.

Treatments		Mean residence time		Labile pool /%
Biochar type /°C	LOM /%	Labile /days	Resistant /years	
450	0	1	713	0.97
450	1	2	322	0.63
450	2	1	321	0.77
450	4	1	314	1.03
550	0	2	978	0.45
550	1	4	643	0.28
550	2	3	585	0.35
550	4	2	567	0.62

Figure captions

Figure 1 Rate of total C mineralization (mg CO_2-C g^{-1} total SOC day^{-1}) from soil mixed with the LOM (0, 1, 2 or 4 %) and biochars (450 or 550 °C) during the 25-month incubation period. Square, triangle and circle represent the control (i.e. no biochar), 450 and 550 °C biochar treatments, respectively. Note the change of scale after the break on the x-axis (i.e. duration of incubation). The insets show the rate of total C mineralization from day 120 with an expanded *y*-axis scale. Red thick bars show the standard error of the predicted mean for each combination of biochar and LOM at different time-points.

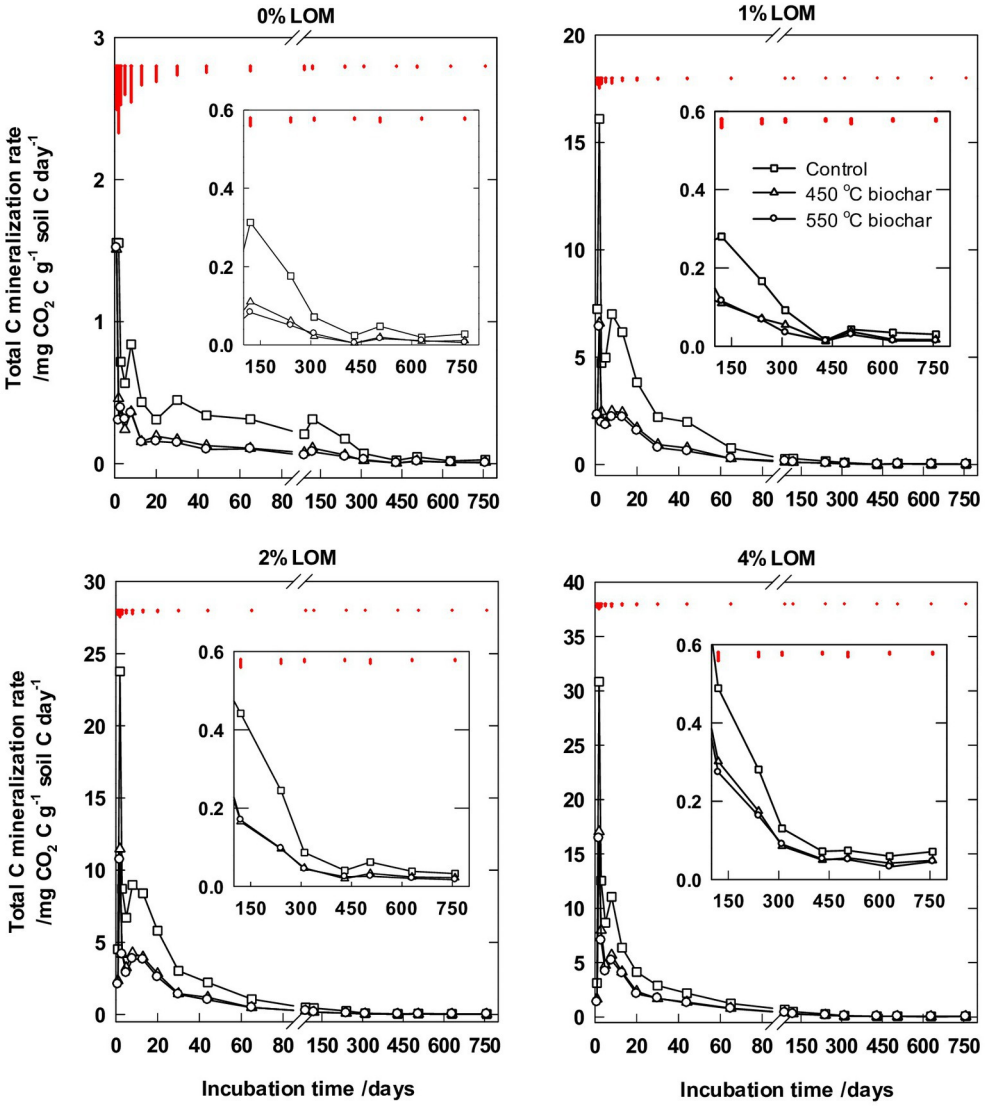
Figure 2 The cumulative priming effect of biochars on the rate of mineralization of native soil carbon (SOC), or native SOC plus labile organic matter (LOM)-C, during the 25-month incubation period. Triangle and circle represent 450 and 550 °C biochar treatments, respectively. Black bars show the standard error of the predicted mean for each combination of biochar and LOM at the end of incubation.

Figure 3 The proportion (%) of applied biochar carbon (C) mineralized from different combinations of treatments of labile organic matter (LOM) addition (at 0, 1, 2 or 4%, w/w) and biochars (450 or 550 °C) at the end of the 25-month incubation. Yellow and purple bars represent the 450 and 550 °C biochar, respectively. Error bars represent the standard errors of the predicted mean for the combination of biochar and LOM.

Figure 4 The cumulative priming effect of labile organic matter (LOM) on the mineralization of biochar-C during the 25-month period. Triangle and circle represent 450 and 550 °C

biochar, respectively. The black bars show the standard error of the predicted mean for each combination of biochar and LOM at the end of incubation.

Figure 5 The soil microbial community structure (bacteria: panels a and b; and fungi: panels c and d) under the labile organic matter (LOM) and biochar treatments in the Vertisol. A principal component analysis (PCA) was applied to the T-RF % relative abundance of the T-RFLP profiles (covariance matrix). The symbols on the graph represent PC score of the individual samples plotted in the plane of the first two components (PC1 and PC2). The proportion of variance in the data accounted for by each PC is given on the axes. Additional numerical information is given in Table S2 for bacteria and Table S3 for fungi. Panels (a) and (c) show the LOM treatments (at 0, 1, 2 or 4% w/w rates of application), and panels (b) and (d) show the interactive effect of the rate of LOM application with type of biochar (nil, 450 or 550 °C biochar at 2%, w/w) on the soil microbial community structure. The *P* values show the overall treatment effects as tested with a multivariate ANOVA (Tables S2 and S3). The symbol ” is the cumulative variance accounted for by all PCs selected (three PCs for bacteria and two PCs for fungi, Supporting Information). Note that in 0% LOM treatments, the fungal community was not detected.



LOM ● Biochar ● Time (*F* statistics: *P* < 0.001)

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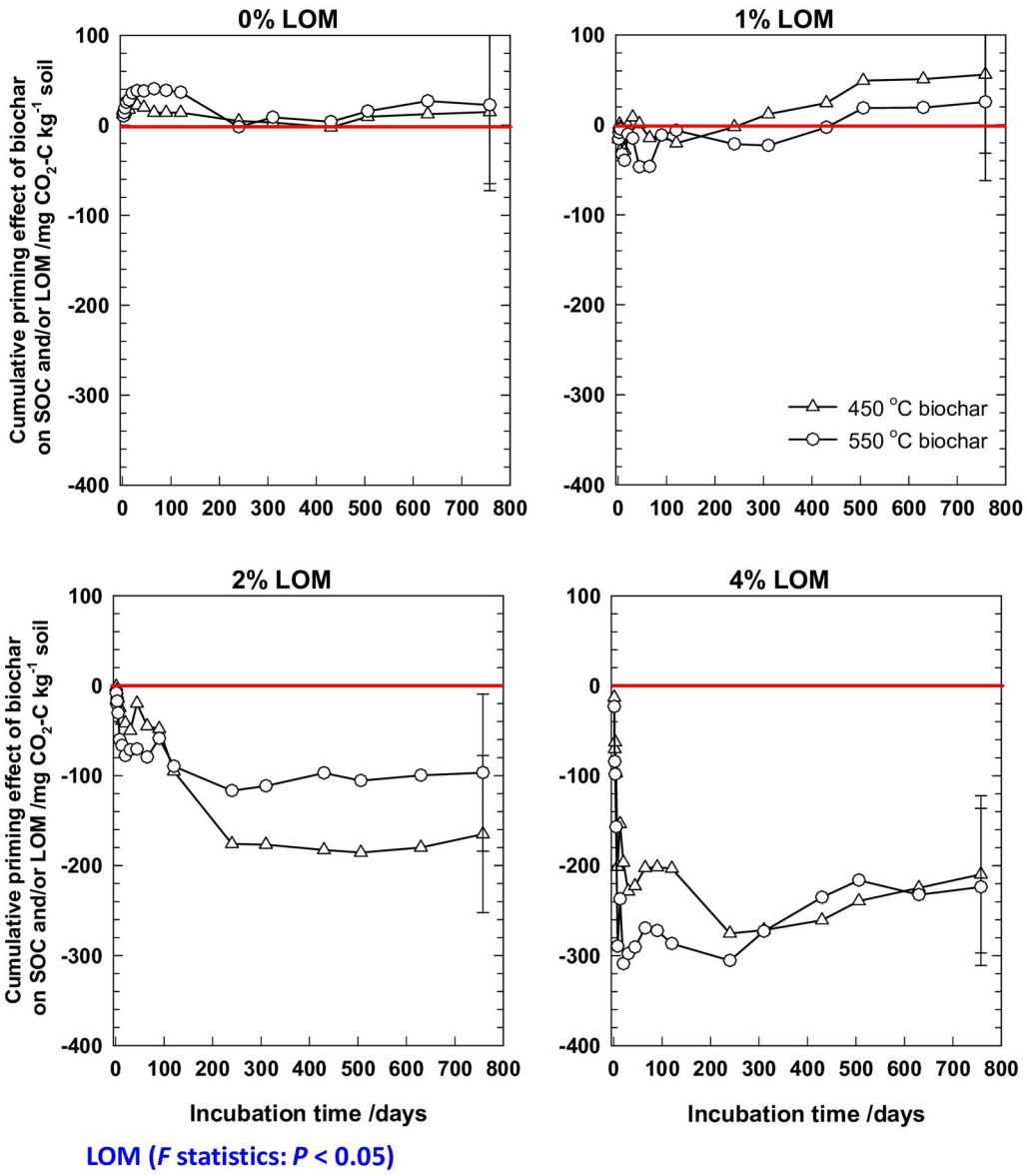

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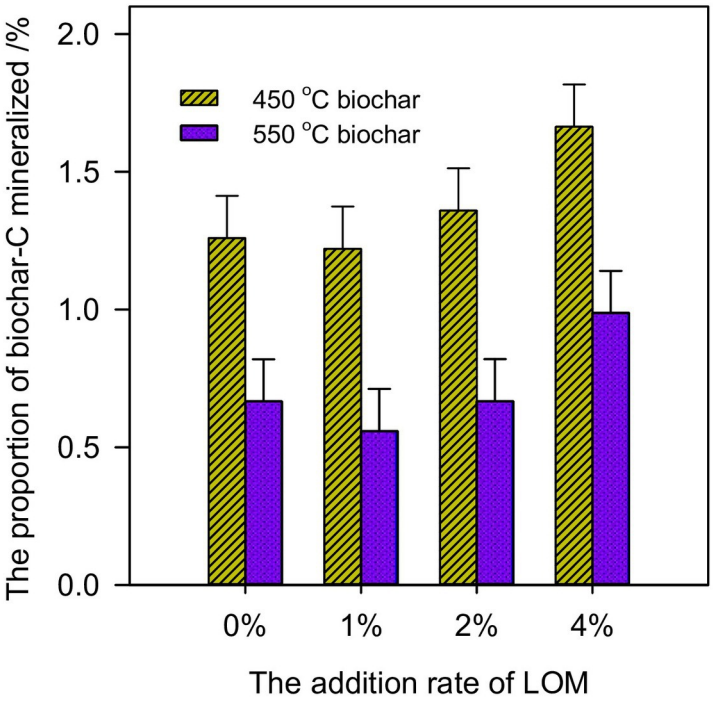

LOM (*F* statistics: *P* < 0.10)
Biochar (*F* statistics: *P* < 0.001)

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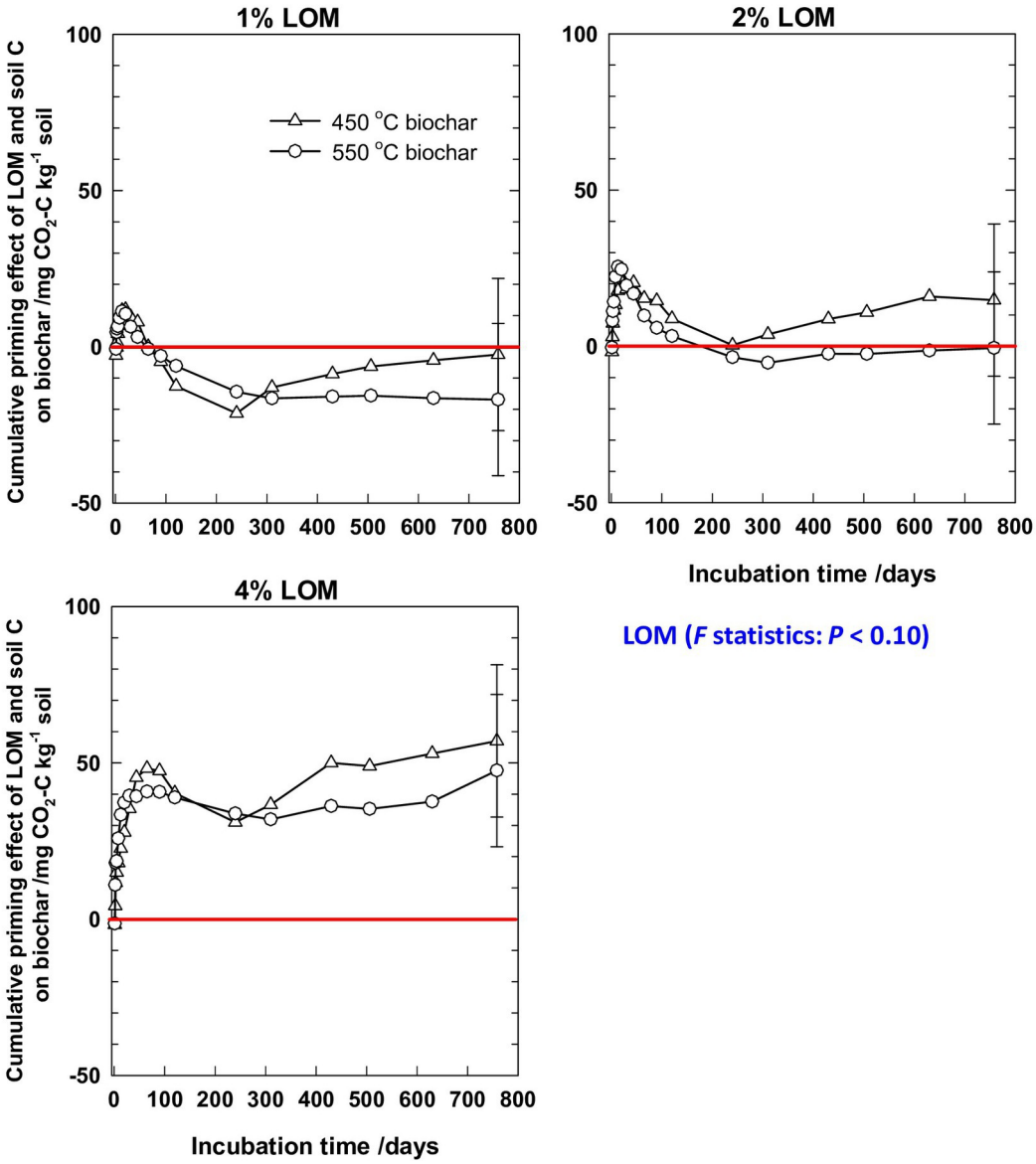

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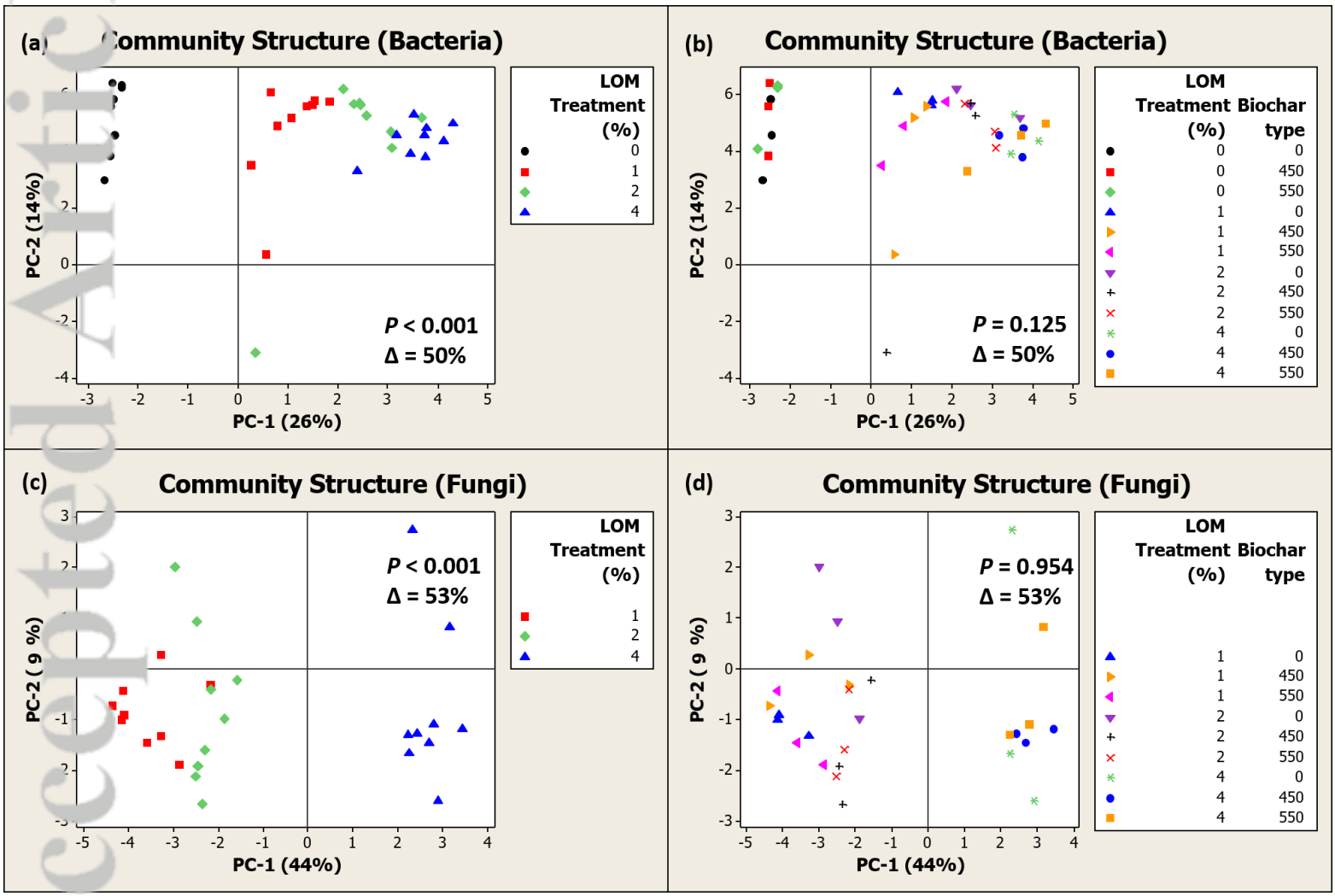

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