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Microbially Driven Iron Cycling Facilitates Organic Carbon Accrual in Decadal Biochar-Amended Soil

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ABSTRACT: Soil organic carbon (SOC) is pivotal for both agricultural activities and climate change mitigation, and biochar stands as a promising tool for bolstering SOC and curtailing soil carbon dioxide (CO$_2$) emissions. However, the involvements of biochar in SOC dynamics and the underlying interactions between biochar, soil microbes, minerals (notably Fe oxides), and fresh organic matter (FOM, such as plant debris) remain largely unknown, especially in agricultural soils after long-term biochar amendment. We therefore introduced FOM to soils with and without a decade-long history of biochar amendment, performed soil microcosm incubations, and evaluated carbon and iron cycling as well as microbial properties. Biochar amendment resulted in 2-fold SOC accrual over a decade and attenuated FOM-induced CO$_2$ emissions by approximately 11% during a 56-day incubation through diverse pathways. Notably, biochar facilitated microbially driven iron reduction and subsequent Fenton-like reactions, potentially having enhanced microbial extracellular electron transfer and the carbon utilization efficiency in the long run. Throughout iron cycling processes, physico-chemical protection by minerals could contribute to both microbial carbon accumulation and plant debris preservation, alongside direct adsorption and occlusion of SOC by biochar particles. Furthermore, soil slurry experiments, with sterilization and ferrous iron stimulation controls, confirmed the role of microbes in hydroxyl radical generation and biotic carbon sequestration in biochar-amended soils. Overall, our study sheds light on the intricate biotic and abiotic mechanisms governing carbon dynamics in long-term biochar-amended upland soils.

KEYWORDS: soil organic carbon, biochar, iron cycling, fresh organic matter, biotic–abiotic processes, upland soil

SYNOPSIS: This study elucidated the intricate biotic–abiotic interactions of iron and carbon that underlie SOC accrual in upland soils subjected to decadal biochar amendment.
INTRODUCTION

Terrestrial soils harbor the largest reservoir of active carbon in the Earth system, holding an estimated 1400-1800 Pg of organic carbon within the first meter.\(^1\) Minor alterations in soil organic carbon (SOC) can profoundly influence atmospheric carbon dioxide (\(\text{CO}_2\)) levels and consequently climate change.\(^2, 3\) Over the last 200 years, significant SOC loss from agricultural land has incurred a substantial carbon debt in soils, correlating with the dramatic atmospheric \(\text{CO}_2\) elevation.\(^4, 5\) Plants (e.g., litter debris and root exudates), microbes, and minerals are pivotal in determining SOC dynamics. Recent studies increasingly underscored the dominant role of microbes (e.g., microbial biomass, anabolism, and necromass) in the formation and accumulation of SOC across diverse ecosystems, where microbial growth and activity ultimately drive the soil carbon cycling.\(^6-9\) Meanwhile, these reports also emphasized the importance of mineral-associated organic matter (MAOM) in preserving microbial-derived organic carbon in soil matrix over a long period, owing to a series of physico-chemical reactions between minerals and soil organic matter, and microbes.\(^10, 11\) However, the protective effect minerals have on SOC could be counteracted by root exudates, which stimulate the microbial co-metabolism and the release of organic compounds from MAOM.\(^12\)

Biochar, the naturally buried pyrogenic carbon, or solid carbonaceous residue derived from pyrolyzed or hydrolyzed organic materials under low oxygen conditions, shows promise in building soil carbon pools and mitigating climate change by reducing greenhouse gas emissions.\(^13, 14\) The application of biochar is commonly proposed as a means to decrease soil \(\text{CO}_2\) emissions, signifying a negative priming effect, through direct physical protection of SOC and indirect alteration of soil physiochemical and microbial properties.\(^15-18\) Notably, an increase in MAOM around biochar and plant roots has been documented in a previous study, where applying biochar to the ryegrass field over a decade stabilized both newly formed and native SOC.\(^19\) However, several studies have suggested that the improved habitats and additional substrates provided by biochar could spur excessive microbial respiration, potentially causing an increase in SOC loss, indicative of a positive priming effect, mainly in the short run.\(^20-22\) The varied impacts of biochar on SOC dynamics are attributed to the characteristics of biochar and soils, as well as to the co-occurrence of other organic matter and the duration of amendment, which can shape the microbial community and their survival strategies.\(^23, 24\) Additionally, increasing studies discussed the critical role of biochar as electron transfer catalysts (i.e. electron shuttle) in redox reactions in soil systems.\(^25-27\) While numerous studies have documented alterations in the microbial diversity, specific communities, and the fungi-to-bacteria ratio within biochar amended soils,\(^28\) fewer studies have combined microbial physiological traits (e.g. \(q\text{CO}_2\), CUE) with microbial communities in conjunction with pivotal biogeochemical processes such as iron cycling. Moreover, the long-term impacts on SOC especially in the presence of fresh organic matter (FOM) remains largely unresolved. These
necessitate further investigation on the direction and magnitude of the priming effect, as well as the transformation and activity of soil microbes in naturally biochar-amended soils.\textsuperscript{23, 29}

Iron (Fe) is the most prevalent redox-active metal in subsurface environments, and its biogeochemical cycle, particularly the redox reactions between Fe(II) and Fe(III) species, is closely interconnected with SOC turnover.\textsuperscript{30, 31} Recent studies showed that Fe does not inherently protect organic matter in soils,\textsuperscript{32} especially in environments with frequent redox fluctuation and heterogeneous aerobic–anaerobic microsites.\textsuperscript{33, 34} The reductive dissolution of Fe oxides associated with organic matter, a typical component of the MAOM, can release previously protected SOC.\textsuperscript{32, 35} The oxidation of aqueous Fe(II) species can lead to the precipitation of Fe oxides exhibiting high affinities for organic carbon, as well as the coprecipitation of Fe oxides and organic matter.\textsuperscript{36, 37} However, reactive oxygen species (ROS), especially the hydroxyl radical (•OH), have been found to significantly contribute to SOC mobilization during Fe(II) oxidation via Fenton and Fenton-like reactions.\textsuperscript{32, 38-41} Actually, ROS have both beneficial and detrimental effects on microbial survival and growth,\textsuperscript{42} thus influencing biotic processes related to SOC turnover besides its direct chemical oxidation towards organic matter.\textsuperscript{43, 44} Furthermore, the notable electron transfer capability of biochar suggests its potential involvement in Fe redox cycles and other biogeochemical reactions.\textsuperscript{45} Hence, we hypothesized that biochar would facilitate the reactive iron cycling in biochar-amended soils, which might be further stimulated by the introduction of FOM. Given the intricate roles of Fe and ROS species, there remains a gap in our comprehension of coupled carbon and iron dynamics, especially in field sites after long-term biochar amendment.

In this study, we introduced freshly prepared plant debris to soils that had been consistently amended with biochar for a decade and then performed a 56-day microcosms incubation (1) to depict the carbon dynamics in decadal biochar-amended soils upon the introduction of FOM and determine the role of biochar in SOC accrual, (2) to investigate the Fe species and Fenton(-like) reactions in biochar-amended soils from uplands, and (3) to elucidate the biotic and abiotic processes involved in iron-coupled carbon cycling under the influence of biochar. To achieve these objectives, the dynamics of various carbon pools, reactive iron species, and the generation of •OH were examined. Subsequent investigations into the biotic–abiotic mechanisms of soil iron-carbon cycling were carried out through microbial community analysis and correlation studies. Furthermore, we conducted slurry experiments with different treatments to verify the significance of microbially driven Fenton(-like) reactions. This study aims to shed light on the intricate biogeochemical mechanisms governing soil carbon turnover in long-lasting biochar-amended field soils.

**MATERIALS AND METHODS**

**Soils and Materials.** Soil samples were collected from the field site in Huantai, Shandong, a representative
Soils in this area are categorized as Fluvic Cambisols, displaying a sandy loam texture composed of 73.5% sand, 12.5% silt, and 14.0% clay. Additional details on the climate, cropping practices, and management have been described in a previous study. Two types of soil were used in the current study. Biochar-amended (BC) soils have been continuously treated with corn straw-derived biochar at an amount of 9.0 t ha\(^{-1}\) y\(^{-1}\) since 2007. Control (CK) soils underwent the same treatment processes as BC soils but did not receive any biochar addition. The applied biochar was produced from a 24-hour anoxic thermal cracking of crushed corn straw at 360 °C and ground to a size of < 1 mm (Jinfu Biochar Company, Liaoning Province). Soils were collected from the 0–20 cm depth in late March 2019 and then subjected to air-drying, debris removal, and sieving (< 2 mm) to ensure consistency across samples. For the soil incubation experiments, wheat and soybean straws were chosen as representative fresh organic matter in the field site, which is the common planning of the cropland in the northern area of China and other regions. Straws (Lianyungang Surui Straw Processing Company, Jiangsu) were oven-dried, crushed, and sieved to a particle size range of 0.9–2 mm before use. Elemental compositions of soil and straw particles were measured with an elemental analyzer (Elementar Vario EL cube, Germany). The surface properties of biochar picked from the BC soil and the prepared straw particles were analyzed using Fourier transform infrared spectroscopy (FTIR, Thermo Scientific Nicolet iS20, USA). Detailed physiochemical properties of soils, biochar, and straws are shown in Supporting Information (SI, Tables S1-3, and Figure S1).

**Soil Microcosms Incubation and Analysis.** CK and BC soils were divided into treatments with or without the addition of 2% (w/w) FOM (wheat or soybean straw) (Table S4): CK soils without FOM input (C), CK soil with wheat straw (C-W), CK soil with soybean straw (C-S), BC soils without FOM input (B), BC soil with wheat straw (B-W), BC soil with soybean straw (B-S). There were 24 bottles for each treatment. Among these, 18 bottles were settled for sacrificial sampling to extract carbon and Fe-related components, compromising 3 replicate bottles at 6 time-points (days 3, 7, 21, 28, 42, and 56). An additional 6 bottles were reserved for microbial community and solid-phase analysis on days 28 and 56.

At first, air-dried and sieved soils (< 2 mm, every 300 g) were placed into 1000-mL bottles and supplemented with ultrapure water (18.2 MΩ cm\(^{-1}\)) to achieve a water content of 13%, for a 14-day preincubation aiming for soil microbial community resuscitation. Subsequently, 30 g of the pre-incubated soil (equivalent to 22.7 g dry weight) was placed into 125 mL glass bottles, followed by either 0% or 2% (w/w) FOM addition. Ultrapure water was added to achieve a soil water content of 32% (equivalent to 60% water holding capacity). The mixture was thoroughly homogenized, and the total weight of each container was recorded to compensate for potential water loss every 2–3
days. All bottles were sealed with microporous sealing films to maintain the oxygen supply for aeration condition and covered with aluminum foils to protect from light exposure. All soils were incubated at room temperature (~24°C, Figure S2a) in the dark.

Throughout the incubation, the three of day-56 bottles were designated for non-destructive gas sampling on days 1, 3, 7, 14, 21, 28, 35, 42, 49, and 56. Detailed gas sampling and the calculation for the cumulative CO₂ emissions were presented in Text S1. Sacrificed soil samples were divided into three subsets for different analyses. One subset was subjected to immediate analysis for their physiochemistry properties including carbon and Fe-related indices. Microbial biomass carbon (MBC) was determined using the chloroform fumigation-extraction method. Dissolved organic carbon (DOC) was obtained using the same extraction procedure excluding the fumigation step. Since previous studies demonstrated that only highly reactive iron species (extracted with 0.5 M HCl) positively correlated to bio-reduced Fe(II) and ‘OH yield, therefore, Fe₅HCl extracted with 0.5 M HCl was used as highly reactive iron species to understand its critical role in reactive soil iron cycling. Detailed extraction processes and the determination of iron species through ferrozine method were presented in Text S2. The potential for ‘OH formation was evaluated by employing 10 mM sodium benzoate as a probe for a 10-hour reaction. Details of these analyses are presented in Texts S2 and S3. Soil pH measured at a soil-to-water ratio of 1:5 was recorded in Figure S2b. Another subset was preserved in a –80 °C freezer for DNA extraction, which was subjected to 16S rRNA and ITS rRNA amplification and Illumina sequencing for microbial community analysis as described in Text S4, with a specific focus on enumerating Fe-reducing bacteria (FeRB) referring to previous reports. The remaining soils were stored in a –20 °C freezer before being freeze-dried and grinned for solid morphology and chemical composition analysis by scanning electron microscope with an energy dispersive spectrometer (SEM–EDS, ZEISS Sigma 300, Germany).

Statistical Analysis. To access the differences in Fe₅HCl contents, and ‘OH formation potentials across various microcosms, as well as the variations in the relative abundance of FeRB and microbial alpha diversity indices, we employed a one-way analysis of variance (ANOVA) followed by a post-hoc Tukey’s test using SPSS (version 26.0) at the confidential level of \( P < 0.05 \). To discern the relationships between various physiochemical indicators, we conducted Spearman correlation analysis using SPSS. Further linear regression for specific indicators was calculated and graphed by OriginPro (student version 10.0).

RESULTS AND DISCUSSION

Soil Carbon Dynamics. As no significant differences were revealed between soils with soybean and wheat straw
inputs, the following discussions would mostly focus on the overall impacts of FOM inputs. In the absence of FOM, a comparison of the cumulative soil CO$_2$ emissions (CCE) from biochar-amended soil (B) to the control soil (C) showed no discernible positive or negative priming effect resulting from biochar amendment (Figure 1a,b). By the end of incubation, CCE level of soils with FOM input (~200 mg C kg$^{-1}$ soil) was about 3 times higher than that of soils without FOM input (~55 mg C kg$^{-1}$ soil), which indicated a strong positive priming effect induced by plant debris. The introduction of wheat and soybean straw as FOM could provide bioavailable organic compounds, acting as energy sources for microbial co-metabolism. This mechanism could be reflected by generally higher contents of DOC (Figure 1c) and MBC (Figure 1d) in soils with FOM input, leading to intensified respiration. However, after a 56-day incubation, biochar-amended soils with FOM input (B-W, 192.70 mg C kg$^{-1}$ soil; B-S, 218.68 mg C kg$^{-1}$ soil) displayed CCE values ~11% lower than control soils (C-W, 216.91 mg C kg$^{-1}$ soil; C-S, 246.66 mg C kg$^{-1}$ soil), indicating that biochar amendment can notably attenuate the positive priming effect initiated by the straw addition.

Such findings coincide with a previous study on a 9.5-year biochar-amended field, which indicated that long-term biochar amendment diminished soil rhizosphere priming and enhanced underground SOC sequestration of new plant-derived carbon by 20% relative to the control field. In contrast, another study with soils after a 3.5-year biochar amendment, reported a negligible suppression in the priming effect induced by cane sucrose and beet sugar. Different from the low molecular weight organic carbon compounds that are readily utilized by microbes, the FOM used in the current study is relatively complex original plant debris. Nonetheless, the presence of biochar aids in the preservation of newly added plant debris in this study, possibly through physical adsorption and occlusion of decomposed FOM particles and FOM-derived DOC molecules, which could be supported by the dynamics of DOC, MBC, and SOC.

Dynamic trends in DOC concentrations during the incubation period indicate the role of external factors and biochar amendments. Throughout the incubation, DOC contents in all groups initially declined over the first 10 days, only to rise between day 21 and day 28, eventually settling between 40–100 mg kg$^{-1}$ (Figure 1c). The sharp incline of DOC for CK soils on day 28 might be correlated to the raised room temperature (~26 °C) (Figure S2a), and we would only focus on the overall trends of different treatments at the moment. BC soils displayed an overall lower DOC content with relatively fewer variations than CK soils. Interestingly, a legacy effect in BC soils was observed, with the incline of DOC content delayed until days 30–40. These subtle differences in fluctuations hinted that biochar might hinder the release of DOC derived from FOM and microbes, potentially altering the degradation pattern of FOM.

The MBC contents generally showed a reverse trend to DOC contents, which declined as DOC increased
This might signify an initial rapid uptake of DOC for microbial metabolism, followed by an increased decay and lysis of microbes in subsequent phases, indicating interactions among the active carbon pools. Except for day 56, B-W and B-S consistently exhibited the highest MBC contents, which plateaued after an initial surge within the first 7 days. This elevation could be evoked by the readily available carbon from FOM and the beneficial conditions from the longstanding biochar amendment. Regarding SOC contents, BC soils contained significantly elevated SOC contents (B, 31.9 ± 0.53 mg C kg\(^{-1}\) soil) than CK soils (C, 12.9 ± 0.2 mg C kg\(^{-1}\) soil) (Figure S3). After a 56-day incubation with wheat straw, the B-W group exhibited a more pronounced increase of SOC (8.5 ± 0.87 mg C kg\(^{-1}\) soil) than the C-W group (4.0 ± 0.32 mg C kg\(^{-1}\) soil). These results reaffirm that decadal biochar amendment can augment SOC reserves in fields receiving natural organic matter inputs.

When considering both MBC contents and CO\(_2\) emissions, BC soils were found to have a lower microbial metabolic quotient (\(q_{\text{CO}_2}\)) than CK soils (Figure S4). As \(q_{\text{CO}_2}\) can serve as an indicator of microbial carbon utilization efficiency (CUE),\(^{61}\) our results indicated an enhanced microbial CUE in the soil amended with biochar over a decade. A similar increase in microbial CUE was reported in another study, where the addition of fresh biochar and corncob residues to alkaline soils led to a substantial increase in MBC, but a decrease in \(q_{\text{CO}_2}\).\(^{62}\) These observations highlight long-lasting effects of biochar in soil ecosystems, which may have manipulated soil carbon dynamics through the pools of DOC, MBC and total SOC.

**Responses of soil microbial communities.** Decadal biochar amendments have a notable influence on soil microbial community composition and underlying carbon turnover. Overall, principal coordinates analysis (PCoA) of soil microbial communities revealed significant separation among different treatments (Figure S5), indicating the profound impact of biochar and FOM on soil bacterial and fungal communities. BC soils exhibited a lower bacterial α-diversity (Figure S6a) but a higher fungal α-diversity (Figure S6b) compared to CK soils, linking a potentially diverse microbial functional characteristics function in the two types of soils.

Specifically, the relative abundance of Actinobacteria and Firmicutes phyla (Figure 2a) was higher in BC soils than in CK soils. These phyla are known for their proficient capability in aerobically degradation of various labile and complex organic molecules, particularly *Streptomyces* and *bacillus* genera,\(^{63}\) which also exhibited increased abundance in BC soils (Figure 2b). This suggests that biochar may facilitate the thriving of potent decomposers in BC soils with limited bioavailable carbon, owing to biochar’s adsorption and occlusion functions as well as its inherent recalcitrance. Additionally, Proteobacteria and Actinobacteria (copiotroph, \(r\)-strategists) thrive better in nutrient-rich habitats than Acidobacteria (copiotrophs, \(K\)-strategists).\(^{64,65}\) Such shift in taxonomic composition could
reflect the change in soil nutrients conditions in BC soils. Moreover, the elevated Proteobacteria while decreased relative abundance of Acidobacteria in BC soils, both predominant as illustrated in Figure 2a, might be a potential reason for the lower $q_{CO_2}$ observed in microbial carbon mineralization (Figure S4). These microbial and physiological results, together with genome-scale models predicting a higher potential CUE of Proteobacteria compared to Acidobacteria, could further underscore the enhanced microbial CUE in current biochar-amended soils.

In addition to bacteria, soil fungi are adept at degrading aromatic carbon in biochar-amended soils, especially Ascomycota and Basidiomycota phyla that also dominate in the present study (Figure S7a), which may stem from their well-developed hyphae and extracellular polymeric substances that could penetrate biochar and promote aggregation. Besides, fungi usually comprise a large portion of microbial biomass and exhibit lower $q_{CO_2}$ than bacteria, thus mightly contributing to the increased MBC while decreased $q_{CO_2}$ in BC soils. To sum up, long-term biochar application may enhance biotic degradation and assimilation of newly inputted plant debris through the transformation of bacterial and fungal community structure, as well as the microbial metabolic traits.

**Iron Redox Cycling and Hydroxyl Radical Production.** The contents of highly reactive iron ($Fe_{HCl}$), extracted with 0.5 M HCl, were notably lower in BC soils (~1200–2100 mg kg$^{-1}$) than in CK soils (~2000–2800 mg kg$^{-1}$) (Figure 3a). This suggests that biochar plays a role in the sequestration of Fe in soils, either through adsorption of $Fe^{II/III}$ species on biochar or due to aggregation of Fe oxides, as previously reported interactions between biochar and minerals. Intriguingly, B-W and B-S exhibited higher ratios of $Fe^{II}_{HCl}$ to $Fe^{III}_{HCl}$ at median values of 0.19 and 0.20 (Figure 3b), despite having relatively lower absolute amounts of $Fe^{II}_{HCl}$ compared to the C-W and C-S (Figure S8b). Considering that ferrous iron can be readily re-oxidized and transformed to amorphous Fe oxides in neutral and oxic conditions, these results point to a higher extent of iron reduction in B-W and B-S groups, which further imply an intensified *in situ* iron cycling near plant debris and biochar. This is similar to previous study showing increased Fe(II) content and Fenton reactions at the straw-soil interface, possibly influenced by microbial activities, and with studies reporting that the conductivity of biochar facilitated to transfer electrons for iron species in solution systems. Additionally, through SEM–EDS mapping, a stronger signal of Fe in soils with the addition of FOM and/or biochar were detected (Figure S9). These findings could also indicate the formation of MAOM around the FOM and biochar, with a prevalence of particles smaller than 50 μm in BC soils (Figure S10). Besides, a previous study has shown the accelerated formation of organo–mineral coating on biochar surfaces and pores in the decadal biochar-amended grasslands. Hence these increases in MAOM could be ascribed not only to physical adsorption and complexation, but also to the promoted cryptic $Fe_{HCl}$ cycling, which concurrently facilitated the coprecipitation
of iron-rich MAOM in biochar-amended soils.

To assess the presence of ROS that likely formed through Fenton(-like) reactions in the soil, sodium benzoate was used as a probe to examine the cumulative •OH concentration over 10 hours, defined as the •OH formation potentials. In C and B groups where no FOM was added, the •OH formation potentials did not exceed 10 μmol kg⁻¹ soil, exhibiting minimal variance between the two types of soils. However, upon the introduction of FOM, the •OH formation potentials were significantly enhanced in C-W, C-S, B-W, and B-S groups, experiencing an increase of approximately 3–6 times compared to the C and B groups (Figure 3c). Remarkably, BC soils with FOM input (B-W and B-S) displayed the highest •OH formation potentials, indicating a synergetic effect of biochar and FOM in boosting •OH generation. Similarly, Du et al. reported an iron-dependent burst of hydroxyl radicals at the straw–soil interface without biochar amendment, while Wang et al. found a biochar-initiated •OH generation from sulfide oxidation in biochar suspensions under dark oxic conditions. Therefore, in the current study, we speculated that FOM and biochar synergistically boost Fenton(-like) reaction hotspots at the redox interface of soil aerobic–anaerobic microsites. The input of FOM might act as a triggering factor, with the presence of biochar amplifying iron redox reactions and •OH production, which would be further discussed in the next section.

Furthermore, our findings underline an underestimated capability of •OH generation in upland soils upon the introduction of FOM and biochar. Direct comparison of •OH yields across various systems is inherently challenging. However, based on the 10-hour cumulative concentration of •OH generated, the •OH generation in our system (0.1–1 μM) is comparable to that in rice rhizosphere during the daytime (0.1–1 μM). It is notably lower than yields reported in anoxic paddy soils (~100 μM) and underground sediments (1–10 μM) encountering oxic conditions, but exceeds those in anoxic soil slurries (0.1 μM) and static-oxic soil microcosms (0.01 μM). Thus, the yield of •OH in fields similar to our studied soils could rise even higher under varying conditions, such as during periods of redox fluctuations due to precipitation and irrigation, which merits further investigation.

**Microbially Driven Fenton and Fenton-like Reactions.** To understand the underlying mechanisms of the hypothesized Fenton(-like) reactions, microbial analysis, and correlation analysis among potential factors were conducted first. Statistical analysis demonstrated strong positive correlations between •OH and Fe(II)HCl (0.739, P<0.01), and between •OH and MBC (0.692, P<0.01) for all treatments (Figure S11), suggesting the possibility of both abiotic Fenton(-like) reactions and microbial ROS formation in our system, triggered by the introduction of FOM. Meanwhile, the insignificant correlation between FeHCl and Fe(II)HCl, despite an identical determination method, underscores the potential critical role of reactive iron cycling along with soil incubation. The abiotic ROS
formation is primarily driven by reduced substances (e.g., Fe(II) species and humic substance), which is dominant in the environments experiencing redox fluctuations. As regards the biotic ROS formation, Han et al. have illustrated both exogenetic iron-dependent and iron-independent \( \cdot \)OH formation during the microbially mediated redox transformation of Fe oxides. During the exogenetic iron-dependent \( \cdot \)OH formation, the microbial excretions, such as extracellular ROS and enzymes, would also play a role in Fe(II) formation and subsequent abiotic Fenton(-like) reactions. Given the static aerobic conditions during our soil incubation, the abiotic process might not fully explain the substantial \( \cdot \)OH formation observed. Thus, microbial activities and microbially initiated abiotic Fenton(-like) reactions could predominantly account for \( \cdot \)OH formation in our soils.

When comparing the data for CK and BC soils, distinct linear relationships emerged with noticeably different Pearson’ \( r \) values all higher than 0.5 (Figure 4), indicating strong positive correlations across variables. Specifically, the correlation between \( ' \)OH formation potential and Fe(II)\textsubscript{HCl} was stronger in CK soils (0.827, \( P<0.001 \)) than in BC soils (0.547, \( P<0.001 \)), with a lower slope in CK soils (Figure 4d). Meanwhile, BC soils demonstrated a stronger positive relationship between \( ' \)OH formation potential and MBC content (0.835, \( P<0.001 \)) than CK soils (0.571, \( P<0.001 \)) (Figure 4c). These findings indicate underlying differences in \( ' \)OH formation between CK and BC soils.

While biochar could act as an electron shuttle or conductor to activate pre-existing ROS (i.e., microbial-derived ROS, and abiotic ROS from Fenton(-like) reactions) to yield more \( ' \)OH, it might also initially foster the generation of microbial-derived Fe(II) and \( ' \)OH in BC soils. Consequently, the elevated \( ' \)OH formation potentials along with higher Fe(II)\textsubscript{HCl} and MBC contents would be attributed a lot to microbially mediated Fenton(-like) reactions, particularly in the biochar-amended soil.

To investigate the involvement of microbes in the generation of Fe(II) species, iron-reducing bacteria (FeRB), which have a remarkable ability for extracellular electron transfer, were enumerated separately from microbial communities. While the correlation between Fe(II)\textsubscript{HCl} content and MBC content was not outstanding (CK, 0.471, \( P<0.001 \); BC, 0.380, \( P<0.01 \)), a significant relationship between the relative abundance of FeRB and Fe(II)\textsubscript{HCl} content (CK, 0.951, \( P<0.0001 \); BC, 0.843, \( P<0.01 \)) (Figure S12) underscores the potential role of FeRB in the generation of Fe(II)\textsubscript{HCl} and \( ' \)OH detected in our system. Specifically, soils treated with FOM (C-W, B-W) exhibited a higher relative abundance of FeRB than those without FOM input (C, B) (Figure 2c). These distinctions illustrate that the microbially mediated Fenton(-like) reactions occurred dramatically in the upland soils when FOM was introduced. Among the FeRB, Bacillus, which belongs to the Firmicutes phylum, was dominant in both CK and BC soils. Recognized as a facultatively anaerobic bacterium, Bacillus commonly exists in aerobic agricultural environments. Other FeRB identified in our soils typically prefer anaerobic conditions and use Fe(III) species for anaerobic respiration.
relative abundance was greater in CK soils than in BC soils, suggesting that biochar may improve the aeration of BC soils due to the high porosity structures. Fungi (e.g., Ascomycota, Basidiomycota) are also known for their important roles in iron cycling, ROS production, and plant debris degradation. With abundant Ascomycota and Basidiomycota fungi in BC and CK soils (Figures S6b and S7a), fungi could also play a role in Fenton(-like) reactions in upland soils.

Overall, the relatively higher abundance of FeRB, stronger correlation between Fe(II)$_{\text{HCl}}$ and FeRB, and between \( ^{\cdot} \text{OH} \) and MBC in BC soils compared to CK soils collectively indicate the improved electron transfer ability of the microbial community under long-term biochar amendment. This was further supported by PICRUSt2 function prediction, which revealed that BC soils exhibited a heightened abundance of cytochrome-c oxidase (Figure S13), a typical enzyme facilitating electron transfer through microbial cells. As illustrated by the FTIR spectrum (Figure S1), biochar particles picked from BC soils exhibited redox functional groups (ketones, aromatic carbon, and carboxyl groups at around 1700 and 1600 cm\(^{-1}\)) and highly condensed aromatic sheets (overall high ratio of aromatic C−H to aromatic C=C) favoring electron transfer, similar with previous studies. Moreover, the electron conductivity of current soils showed a higher electron transfer capacity of BC soils (Table S5), determined by the conductivity meter (Leici DZS-706). As previous studies reported that biochar can enhance microbial activity and soil bioelectricity by electrochemical assessments, our observations in microbial analysis and soil properties reaffirm the acclimated extracellular electron transfer capacity in the field-collected BC soils. The facilitated biotic–abiotic electron transfer, consequently, may have contributed to the intensified hotspots of microbially mediated Fenton(-like) reactions in the biochar-amended soil.

**Microbial assimilation and Physical Association for SOC Accrual in Biochar Soil.** The substantial presence of \( ^{\cdot} \text{OH} \) in FOM-containing soils, coupled with enhanced soil CO$_2$ emissions (Figures 1a, 3c, and 4b), implies that Fenton(-like) reactions likely contributed to the abiotic mineralization of organic matter. Nevertheless, BC soils (B-W, B-S) showed higher Fe(II)$_{\text{HCl}}$ to Fe$_{\text{HCl}}$ ratios and \( ^{\cdot} \text{OH} \) formation potentials than CK soils (C-W, C-S), yet without an equivalent increase in CO$_2$ emissions (Figures 1a, 3b, 3c, and 4b). This indicates that \( ^{\cdot} \text{OH} \) produced in BC soils may not contribute to abiotic SOC mineralization as effectively as that in CK soils. Previous studies suggested that \( ^{\cdot} \text{OH} \) can break down recalcitrant organic matter, liberating bioavailable carbon with essential nutrients for soil microbial growth. Therefore, in BC soils, the surplus organic carbon released as a result of iron cycling and \( ^{\cdot} \text{OH} \) attacking, was likely effectively assimilated by microbes and subsequently incorporated into MAOM, instead of being mineralized thoroughly. Due to the short lifespan of \( ^{\cdot} \text{OH} \) (half-life <10$^{-9}$ s) that limits diffusion to microbial
cells and the protection effect of MAOM on microbes, the detrimental effect of $^\cdot$OH on microbes\textsuperscript{42, 90} might be negligible or obscured in BC soils with higher MBC contents (Figure 1d). Upon closer SEM–EDS examination, the facilitated biosynthesis, microbial growth, and thus MAOM, were supported by fine particles embedded within the pores of biochar matrix with noticeable Fe and C signals (Figure S9c,d). In summary, BC soils not only exhibited substantial $^\cdot$OH formation potential, but also increased extracellular enzyme activity and carbon utilization, resulting in lower CO$_2$ emission and higher SOC accumulation compared to CK soils.

To further verify the role of microbes in Fenton(-like) reactions and CO$_2$ emissions, we conducted a 10-h slurry experiment with either $\gamma$-sterilized or non-sterilized C-W and B-W soils. Since no significant differences were revealed between soybean or wheat straw-added soils, only wheat straw was used as the FOM input in the slurry experiment. CO$_2$ emissions of $\gamma$-sterilized soil slurries ($\gamma$CW, $\gamma$BW) were ~70% lower than non-sterilized groups (CW, BW) (Figure 5a), indicating microbial respiration as the primary contributor to the loss of SOC. Moreover, after 14-day incubation, $\gamma$CW and $\gamma$BW showed negligible $^\cdot$OH formation potentials (<1.94 μM kg$^{-1}$ soil) (Figure 5b), confirming the critical role of microbial activities in ROS production. Owing to the necessary roles of both Fe(II) species and H$_2$O$_2$ for $^\cdot$OH formation via the Fenton(-like) reactions, in under dark and alkaline conditions, $\gamma$-sterilized soils with low levels of Fe(II)$_{HCl}$ would hardly generate $^\cdot$OH without biogenic H$_2$O$_2$ (Figure S14). Previous studies reported that the addition of ferrous iron stimulated rapid mineralization of organic matter, contributed by formation of $^\cdot$OH via the abiotic Fe(II) oxidation process,\textsuperscript{32, 91} thus FeCl$_2$ solution was used as a positive control in slurry experiments. Here, the addition of 10 mM aqueous Fe(II) to both $\gamma$-sterilized and non-sterilized soil slurries under oxic conditions notably increased the CO$_2$ emissions and $^\cdot$OH production (Figure 5b), validating the significance of abiotic Fenton reaction in carbon mineralization. Interestingly, CW and $\gamma$CW soils exhibited higher CO$_2$ emissions than BW and $\gamma$BW, even with comparable or lower $^\cdot$OH levels. Besides, $\gamma$BW with aqueous Fe(II) addition showed lower $^\cdot$OH formation but higher CO$_2$ emission rates compared to BW. Therefore, these disproportional findings reinforce the crucial role of microbes in BC soils, where a portion of organic carbon released by $^\cdot$OH attack was probably assimilated by microbes. Unlike bulk soil incubation, the soil slurry experiment minimized the physical protection of MAOM on organic carbon, spotlighting microbial activities that curtailed the CO$_2$ emissions of abiotic SOC mineralization.

**Environmental Implications.** Biochar, whether naturally deposited in grass and forest soils following wildfires or intentionally applied as a soil-enhancing agent in agricultural lands, holds promise in enhancing soil carbon pools.\textsuperscript{13} Our study presents compelling evidence that a decade-long biochar amendment can effectively reduce soil CO$_2$
emissions by approximately 11% when supplemented with fresh organic matter, such as plant debris. Such a comparison indicates a substantial carbon accrual in long-term biochar-amended soils, reinforcing the value of biochar as a carbon sequestration tactic, especially in prime agricultural regions like North China Plain, Central Plains of the United States, and central-western Europe, where wheat and maize cultivation predominates. In particular, we revealed that microbes initiated the formation of Fe(II) species and ‘OH during the incubation of upland soils with plant debris and biochar. The pronounced iron redox cycling and accumulation of ‘OH emphasize the need to also consider soil Fenton(-like) reactions in uplands, in addition to lowlands like paddy fields and wetlands. While the formation of Fe oxides is generally considered one of the major processes influencing SOC conservation in upland soils, our findings suggest that the cryptic Fe(II/III) species cycling along with microbial activity would be pivotal to modulate SOC turnover in the biochar-amended soil. Furthermore, the correlation analysis and slurry experiment underscore the importance of reassessing microbially extracellular ROS formation pathways in soils. However, the contribution of abiotic generation of ROS to SOC mineralization might have been overestimated if the coupling of microbial processes with abiotic processes is overlooked. Thus, we spotlight the critical role of microbial activities in the biotic–abiotic ROS production within soil aerobic–anaerobic microsites and in the accumulation of microbial-derived SOC in biochar-amended soils.

In summary, our findings illuminate the mechanisms underlying mitigated CO₂ emissions in biochar-amended soils and advance our understanding of the biotic–abiotic processes in upland soil carbon dynamics. While facing global climate change accompanied by extreme weather events (e.g., drought, floods, warming, etc.), soils will undergo extensive and intense redox fluctuation, potentially driving CO₂ emissions for elusive mechanisms. While the current results of 56-day incubation may shed light on the mechanisms underlying the FOM-induced priming effect in biochar amended soil, more prolonged laboratory experiments and field observations under complex conditions deserve further investigation to recognize the temporal effect of BC amendment along the long-term application management.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at XXXXXXXXXX. Experimental details and calculation for the cumulative soil CO₂ emissions, microbial biomass carbon, reactive iron, hydroxyl oxygen, and microbial analysis; selected properties of soils and straws; temperature and pH variation; more experiment results of SOC, qCO₂, reactive Fe and ‘OH, SEM–EDS characterization images,
correlation analysis, and microbial analyses are provided (PDF).

**Data availability**

The data used in this article would be available in Figshare (Number: XXX) after the acceptance of the article.

All sequencing data were available in the China National Center for Bioinformation (Number: PRJCA024115).

**AUTHOR INFORMATION**

**Notes**

The authors declare no competing financial interest.

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Figure 1. Soil carbon dynamics during 56-day microcosms incubation. (a) Soil carbon loss represented by cumulative CO$_2$ emissions and (b) the corresponding priming effects; (c) DOC contents, and (d) MBC contents. C, empty green circle, control soil without FOM input; B, filled green circle, biochar-amended soil without FOM addition; C-W and B-W, empty and filled triangles, soils incubated with 2% wheat straw input; C-S and B-S, empty and filled squares, soils incubated with 2% soybean straw input. The error bars represent the standard deviation from triplicate experiments. Notes: The cumulative CO$_2$ emissions in (a) were calculated by integrating the CO$_2$ emission rate at each examination time interval. The cumulative priming effects in (b) were calculated by the ratio of the difference between the treatment groups and the control groups to the control groups, detailed in Text S1.
Figure 2. Microbial responses of bacteria at (a) phyla and (b) genus level, and (c) identified Fe-reducing bacteria at genus level. Only the top 10 identified bacteria or fungi are presented in (a), and those bacteria genera with relative abundance less than 0.025 were cleared in (b). C and B, control soils and biochar-amended soils without FOM input; C-W and B-W, soils incubated with 2% wheat straw addition; -0d, soil samples that after pre-incubation but before incubation; -28d, soil samples on day 28. Different lowercase letters above the columns in (b) indicate significant differences (P<0.05) among soil samples.
Figure 3. Highly reactive iron species and hydroxyl radical indexes. (a) Fe\[^{III}\]HCl contents, (b) Fe(II)\[^{III}\]HCl to Fe\[^{III}\]HCl ratios, and (c) 'OH formation potentials. C, soil without any treatment as control; B, soil with 10-year continuous biochar amendment; -W, treatment with 2% wheat straw addition; -S, treatment with 2% soybean straw addition. Solid lines inside the boxes represent the median values. Squares represent the mean values, while circles within each treatment depict data derived from triplicate experiments at 6 time-points. The bottom and top edges of the boxes represent the 25th and 75th percentiles, respectively. Whiskers demarcate minimum and maximum data points within 1.5× of the interquartile range. Different lowercase letters above the boxes indicate significant differences (P<0.05) among separate treatments.
Figure 4. Pearson correlation analyses between (a) CO$_2$ emission rate and MBC content, (b) CO$_2$ emission rate and •OH formation potential, (c) •OH formation potential and MBC content, and (d) •OH potential and Fe(II)$_{HCl}$ content measured from control soils (CK, blue symbols) and biochar-amended soils (BC, pink symbols) soils. Blue and pink lines indicate linear fitting to the data from CK and BC soils, respectively. $r$, Pearson correlation coefficient; $P$, significant level; $R^2$, the coefficients of determination.
Figure 5. Carbon dioxide emissions and 'OH formation potentials in 10-hour soil slurry experiments.

CW, no sterilized control soils with wheat straw incubation after 14 d; BW, no sterilized biochar-amended soils with wheat straw incubation after 14 d. γCW and γBW, gamma radiation sterilized control and biochar-amended soils, incubated with sterilized wheat straw for 14 days. DI, water control treatment; Fe(II), 10 mg/L FeCl₂ treatment. Numbers above the columns represent the increase (+) or decrease (–) of γ-sterilized samples compared to no-sterilized samples.
Supplementary Files

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