Review

Effects of Biochar on the C Use Efficiency of Soil Microbial Communities: Components and Mechanisms

Laura Giagnoni [1,*] and **Giancarlo Renella** [2]

[1] Department of Civil Engineering, Architecture, Environmental and Mathematics (DICATAM), University of Brescia, Via Branze 43, 25123 Brescia, Italy
[2] Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padua, Viale dell'Università 16, 35020 Legnaro, Italy
* Correspondence: laura.giagnoni@unibs.it

Abstract: Biochar production and incorporation into soil is gaining momentum as a sustainable strategy for climate change mitigation, supported by ever increasing reports of significant carbon (C) sequestration in soil and reduction in greenhouse gas (GHG) emissions from the amended soils. With the progression in biochar testing and use, there is also emerging evidence that biochar induces C sequestration in soil, and that it may not be solely caused by its inherent chemical stability, but also by the complex microbially driven processes and an increase in C use efficiency (CUE) through soil microbial metabolism. This evidence contradicts the current paradigm that sees the microbial CUE decrease during the degradation of recalcitrant material due to thermodynamic constraints, as observed only in several short-term and pilot-scale trials. As the CUE in soil results from interactions between several abiotic and biotic factors, in this paper we examine the link between the biochar properties, soil physico-chemical properties and microbial physiology to explain the CUE increase reported for biochar-amended soils. Based on the large body of physico-chemical literature, and on the high functional diversity and metabolic flexibility of soil microbial communities, we hypothesize that the long-term stabilization of biochar-borne C in the soil systems is not only controlled by its inherent recalcitrance, but also by the cooperative actions of improved soil status and increased microbial CUE. Given that the current knowledge on this specific aspect is still poor, in this feature paper we summarize the state of knowledge and examine the potential impact of biochar on some factors contributing to the whole-soil CUE. We conclude that, beside its inherent recalcitrance, biochar weathering and oxidation in soil create physical and chemical conditions that can potentially increase the microbial CUE. While these processes stabilize the microbial processed C in soil and increase soil fertility, more data from long-term field trials are needed to model the relationship between the CUE and the MRT of biochar-borne C. Based on our hypotheses and relying upon analysis of the available literature, we also suggest possible research approaches that may contribute to filling the gaps in the current knowledge on the topic.

Keywords: biochar; soil; soil microorganisms; carbon use efficiency

1. Biochar: Chemical Stability Influences the Carbon Use Efficiency (CUE) in Soil

Biochar is the product of thermochemical decomposition of biomass at temperatures ranging from 300 to 1000 °C in the absence of oxygen, either by pyrolysis or gasification, and can be used for extracting energy from biomass [1]. These processes decompose the organic biopolymers into small gaseous molecules such as methane (CH_4), hydrogen (H_2), carbon monoxide (CO), and carbon dioxide (CO_2), condensable vapours (tars and oils), and a solid phase as a by-product termed biochar. Biochar production is maximized by pyrolysis at low temperatures (e.g., 300–450 °C) and slow heating rates and residence times (hours), whereas gasification is maximized at high temperatures (≥800 °C) and rapid heating rates (seconds), with the biochar production rates of slow and fast pyrolysis being 20–30% and 4–8%, respectively. Moreover, the biochar qualities resulting from pyrolysis and

gasification are also different; for the aims of this paper, it is worthwhile to underline that slow pyrolysis results in less recalcitrant biochar with high porosity as determined by the Brunauer-Emmett-Teller (BET) method and coarse texture. Conversely, gasification results in highly recalcitrant biochar with low BET values and finer texture. In fact, gasification pyrolysis requires finely ground feed of specific biomasses with a narrow range of initial moisture content, a precisely controlled temperature and effective cooling of the vapour phase in order to achieve efficient recovery of the oily product. Therefore, it is a less flexible process mainly aimed at energy production, producing highly inert biochar with a sandy-dusty texture, whereas slow pyrolysis produces manipulable biochar which can be incorporated into soil. For this reason, biochar from fast pyrolysis has been used less than slow pyrolysis for the purposes of achieving agronomic and environmental goals such as climate change mitigation [2].

Based on the potential long mean residence time (MRT) of biochar in soil, the so-called charcoal vision [3] is nowadays considered as a strategy to offset a significant share of anthropogenic C emissions through C sequestration in soil. In addition to its climate mitigation potential, incorporation of biochar into soil also brings significant improvement in soil fertility as it enhances the cation exchange capacity (CEC), neutralizes acidic pH values, changes the soil color and increases the soil thermal capacity, increases water retention, and immobilizes inorganic and organic pollutants [4]. Concurrence of these factors explains the frequently reported increases in crop yields [5–7], especially in highly degraded soils. Owing to its soil-improving and beneficial effects, biochar has been admitted into the new EU Regulation on Fertilizers (EU 1009/2019) under the product of pyrolysis component material category (CMC). The ever-increasing mass of information about the positive effects of biochar parallels to the historical evidence of the transformation of Amazonian dark earth soils (Terra Preta dos Indios), which maintain significantly higher pH values and fertility compared with the surrounding soils even after millennia or centuries from the charcoal burial [8].

Biochar-induced positive effects on soils and related ecosystem services are related to its stability. Initially, potential biochar stability in the environment was estimated through the process parameters such as pyrolysis temperature and feedstock types, but such parameters are currently no longer considered valid. Conversely, chemical properties such as O/C molar ratio [9], the H/Corg ratio value [10], and both the H/Corg and O/Corg ratio values are nowadays considered better descriptors of biochar stability [11,12]. In fact, these parameters reflect the labile C/recalcitrant C ratio value, and are well correlated to the results of thermal/chemical oxidation resistance tests. The molecular structure of biochar is predominantly aromatic, and its inherent stability depends upon this level molecular arrangement. Aromatic substances can form either amorphous phases, in which the aromatic substances are randomly organized, or crystalline phases, in which the aromatic structures form ordered condensed sheets, as observed in the pyrolysis and pyrogasification processes, respectively. Because more aromatic and more condensed molecular structures are supposed to be more resistant to chemical and biological degradation [2], the evaluation of both aromaticity and degree of aromatic condensation, for example by Nuclear Magnetic Resonance (NMR) spectroscopy, is increasingly used as a chemical indicator of biochar stability [13]. Based on the microbial degradative mechanisms we describe in the following paragraphs, in our opinion the quantification of benzene polycarboxylic acids (BPCA) and molar H/C ratio, indicating the degree of molecular unsaturation and H deficiency [14], can be reliable indicators of potential microbial attack on biochar C. The analysis of hydroxylic and carbonylic functional groups, nonaromatic C branches, or N-containing functional groups using Fourier transform infrared spectroscopy (FTIR) [15], or wet chemistry oxidation assays [15,16], provides additional and complementary indications of biochar stability towards potential microbial attack.

Biochar persistence in soil can be also evaluated by batch incubation and C mineralization experiments, in which both biotic and abiotic degradations occur. As compared to the chemical tests, that provide indirect stability indications, the biochar stability, assessed

by soil incubation tests, is increasingly adopted to obtain direct evidence of persistence of different biochar in soil. Significant correlations between biochar C stability, C mineralization rate, the degree of aromaticity and molecular condensation of biochar have been reported [10]. A meta-analysis of biochar incubation experiment reported that the MRT of biochar, added at a rate of 3% and having an initial recalcitrant C proportion of 97%, has an MRT in soil spanning from 100–1000 years [17].

Despite the large number of reports on the topic, in our opinion, the following question is still open: is biochar chemical stability sufficient to forecast its persistence in microbiologically soils? This question led us to a twofold reflection on the microbial C use efficiency (CUE) in biochar-amended soils: a chemical and a microbiological one. From the chemical point of view, microorganisms have a nominal cellular C oxidation state close to the average sugar's C oxidation state. For this reason, they mainly derive energy and produce cell components from anabolic pathways fueled by more reduced C substrates. Classic microbiological experiments have shown lower CUE of single isolates grown on substrates with higher C oxidization degrees (e.g., mono- or bicarboxylic acids), and peak CUE values for microorganisms grown on C substrates with an oxidation state (e.g., sugars) close to that of microbial cells [18]. However, for the highly diverse and metabolically flexible soil microbial communities, the relationship between the oxidation state of C substrates and CUE may depend on more factors that the relative C oxidation states of substrates [19]. In terms of microbial physiology, soil microbial communities have been hypothesized to be characterized by the 'metabolic infallibility', i.e., soil microorganisms have the potential capability to use any known substance as an energy source, even one of high molecular complexity and recalcitrance, especially if limiting environmental conditions are alleviated. Though this concept has been debated for decades [20], the microbial metabolic infallibility principle has received confirmation from an increasing body of evidence [21]. This concept is highly relevant for biochar-amended soils, as it implies that under suitable conditions microorganisms can oxidize any substance which is theoretically capable of being oxidized [22]. Since its earlier formulation of the metabolic infallibility concept, environmental microbiologists have demonstrated that microbial degradation processes have two main components, the genetic-based metabolic potential of microorganisms and the chemical recalcitrance of the substrate molecules [20]. The aim of this review is to answer to this question by critically binding the knowledge on the biochar properties to the main soil properties, the fundamental microbial physiology and microbial ecology concepts.

Thermodynamically, soils are open systems that exchange energy and matter with other natural systems, constituted by reactive solid phases and host to large and highly diverse microbial communities characterized by the highest known diversity and metabolic flexibility among the terrestrial ecosystems [23]. The result of soil microbial oxidative activity is that biochar aging in soil turns its properties from inert hydrophobic and neutrally charged matter to increasingly hydrophilic negatively charged solid phases [24], thus increasing its potential interactions with soil microorganisms. We hypothesize that the reported capacity of biochar in stimulating soil microbial activity could be ascribed to the fact that microorganisms possess metabolic degradative potential because black C is ubiquitous to terrestrial and aquatic environments due to natural or human-induced fire and anthropogenic environmental enrichment with organic xenobiotics [25]. Nevertheless, better understanding of the chemical and biochemical mechanisms requires the evaluation of the changes in main soil properties induced by the biochar, treated in Section 3, the alteration of the C:N:P stoichiometry, treated in details in Section 4, and the composition of the soil microbial community, treated in Section 5.

2. Microbial CUE: Definition for Soil Systems and Changes in Biochar-Amended Soils

The simplest definition of carbon use efficiency (CUE) of microorganisms is the ratio between microbial biomass C production and C taken up by microorganisms (1)

$$\text{microbial CUE} = \mu/C_{\text{uptake}} \tag{1}$$

where μ is the microbial biomass growth and C uptake is the sum of C immobilized by microbial growth, plus respired C. The CUE of soil microorganisms represents the efficiency of microbial biomass production resulting from the mineralization of the soil organic matter (SOM). Determination of the microbial CUE can be used to predict the energy flow among trophic levels in different ecosystems, across different soil management and different soil types [26] and represents an integrated parameter that can predict the microbial C turnover in the biogeochemical cycle of the SOM [27]. This highly meaningful soil ecological parameter can be determined with different methods, based on the measurement of soil respiration and variations in the soil microbial biomass C pool. Early CUE estimations were based on in vitro analysis of microbial biomass yields upon exposure to selected C sources. This 'black box' approach mainly takes into account the specific C respiration (qCO_2) during the microbial growth (μ) phase (i.e., qCO_2/μ), which is an expression of the maintenance energy of microbial cells exposed to different C sources [28], not an expression of the microbial CUE.

The CUE values, calculated with by the mass-based approach as in Equation (1), only results in CUE values > 0, with values typically in the order of 0.60 [29,30]. Conversely, theoretically the CUE can also have negative values if C assimilation is lower than the respired C, or when microbial biomass declines due to mortality [31].

As mentioned above, incorporation of biochar also leads to changes in soil properties that affect the CUE. However, from a literature search using SCOPUS as a database in spite of the ca. 11,500 papers retrieved using 'soil' and 'biochar' as search criteria and ca. 1300 paper matching 'soil' and 'CUE' as search criteria, only 12 papers matched the keywords 'soil' and 'biochar' and 'CUE' as search criteria. This literature search highlights the current poor knowledge on the CUE as a key process that controls the biochar C stability in soil.

In structured, open, and highly biodiverse systems like soils, the CUE is not only a function of C availability, but also of the microbial community composition [32]. Moreover, CUE measurements integrate several biotic and abiotic factors such as SOM quality and solubility in the soil solution, availability of other macronutrients than C, soil temperature and moisture levels [33,34]. This only to make mention the major factors, the effects of which will be discussed in detail below. For soil microorganisms, this means that the SOM quality is not the only factor that determines the maximal CUE. Rather, other environmental properties altered by biochar amendment also influence microbial activity and proliferation. In a greenhouse trial, the soil organic C (SOC) mineralization and the microbial community of a soil amended with 20 and 40 t ha^{-1} of biochar Zhang et al. [35] showed the increase in SOC and recalcitrant C, the reduction in SOC mineralization and the temperature sensitivity (Q_{10}) value [35], confirming previous reports on this topic [36]. Concerning the importance of organic substrates quality, microbial CUE is higher when the C availability is limited but, thermodynamically, decomposition of recalcitrant C should reduce the microbial CUE due to the high metabolic costs of the oxidative catabolic steps [37].

Changes in quality and rates of organic substrates upon undergoing biochar aging could induce changes in soil microbial communities, either as individuals or microbial physiological groups, potentially altering the kinetics of C assimilation pathways [38,39]. In enzyme-mediated uptake pathways, C uptake rates are expressed as a saturating function of substrate concentration, well represented by a Michaelis–Menten model [40]. Theoretically, C uptake for soil microorganisms energetically covers the metabolic costs of the substrate uptake mechanisms [41] and the optimal substrate saturation should not vary. Under these conditions, low C availability should decouple catabolic and anabolic pathways and globally lead to low CUE of soil microbial communities [26]. At a microbial community level, the CUE varies depending on major ecological constraints, namely C sources type and bioavailability, relative abundance of different microbial physiological groups, soil moisture level and temperature, and generally decreases with soil depth [42,43]. At an individual level, the CUE values vary in different physiological states of microorganisms, being lower during the C assimilation stage and higher during the exponential growth

phase [44]. This is because, a short time after exposure to energy substrates, microorganisms respire C and thus synthesize extracellular enzymes for SOM decomposition and cell membrane transport proteins. Whereas, in the logarithmic growth phase, the assimilated C is maximally allocated into a new biomass [40,45].

Moreover, in terms of metabolic flexibility, under nutrient-limiting conditions and/or other environmental constraints, soil microorganisms may activate degradation but not growth metabolic pathways. These cometabolic pathways involved in biochar modification due to the release of nonspecific enzymes are capable of chemically modify recalcitrant organics that, though not directly capable of supporting microbial proliferation, can be oxidized or reduced in the presence of SOM-derived substrates providing metabolic energy [46]. For example, cometabolism has been demonstrated as a mechanism of transformation of polycyclic aromatic hydrocarbons (PAHs) and other organic xenobiotics [47]. Based on the above considerations, we hypothesize that in the presence of biochar the microbial physiological groups capable of utilizing biochar-borne recalcitrant substances are positively selected, and their capability to use the biochar-derived substances and native similar organic substances may explain the higher microbial CUE reported for biochar-amended than nonamended soils.

3. Biochar-Induced Temperature and Moisture Effects on Soil CUE

Temperature and moisture are environmental factors that influence microbial CUE [48]. Incorporation of biochar into soil significantly changes soil color and water retention properties. Reduced reflectance of biochar-amended soils increases soil temperature due to changes of soil albedo [49]. By definition, the albedo values range from 0 to 1, and the range is between 0.1–0.2 for dark soils and between 0.4–0.5 for light-colored soils [50], with either geographical, daily and seasonal variations. Beside the incident radiation, soil thermal capacity is also increased by soil moisture content, SOM content, and particle size distribution [50]. The biochar-related increase in water retention has a cooperative effect with albedo, especially in sandy soils that drain and dry out faster than clayey soils, as the specific heat of water in moist soil is ca. 5 times higher than in dry soil [51]. With few exceptions, long-term field trials show that biochar increases the water retention, and higher water retention in dry periods may reduce the accumulation of osmolytes [52] that generally increase the C:N ratio values of the microbial biomass and the apparent CUE values [53,54]. Microbial CUE is generally reduced upon an increase in soil temperature [26], mainly due to the faster acceleration of microbial respiration processes than microbial growth responses [48]. Although at the community level, microorganisms adapt to increased temperatures in terms of species composition, the link between biochar-induced changes in microbial community composition and thermal adaptation of microbial communities still needs to be assessed, and reliable information can be obtained only from the analysis of soils from long-term field trials.

Higher soil temperature can reduce the activation energy of SOM decomposition [34,55], though SOM activation energy depends on its molecular complexity [56] and increases upon the number of enzymatic steps required for substrate modification and decomposition [57]. Under different temperatures, changes of CUE values in the presence of molecular complex substrates are generally less pronounced than those recorded during the decomposition of low molecular weight organic compounds (LMWOCs) [58], and biochar generally has lower temperature sensitivity than native SOM [57]. In this regard, higher mean temperature and more constant moisture levels may facilitate microbial oxidative enzymes synthesis and release, and in cooperation with the nonspecific enzymatic mechanisms, they may reduce the activation energy for microbial respiration [59]. Overall, these mechanisms can make microbial oxidation of biochar in soil less dependent on its inherent thermal stability and more dependent on microbial enzymatic activity. However, to our knowledge, no experiments aiming at determining the changes in the activation energy of SOM of biochar-amended soils have been conducted.

Biochar stability also depends on soil texture and its eventual association with soil minerals, or minerals deliberately associated with biomass feedstocks for producing different biochar types [60]. Because microbial activity occurs in hot spots mainly present in soil aggregates, the diffusion of biochar-borne substrates into the aggregates can possibly determine an 'abiotic gate' limiting their decomposition, at least shortly after soil amendment [61]. Limitations in the use of insoluble pools of biochar-borne C by soil microorganisms, due to physical and chemical protection in soil aggregates, can be alleviated when the decomposition process initiated by the synthesis of enzymes and sustained by the subsequent formation of more hydrophilic and soluble C pools, increase the microbial accessibility to organic substrates. Physico-chemical mechanisms occurring in soil such as sorption, diffusion, and occlusion into aggregates exert additional control on biochar stability in soil, because they increase the microbial energy investment in enzyme synthesis for C acquisition. These mechanisms, that depend on the properties of the soil solid phases and the soil structure complexity, along with surface hydrophobicity and molecular recalcitrance of biochar, control the biochar C transfer from stable to more labile pools. In our opinion, the biochar decomposition rate in soil is co-controlled by the diffusion of LMWOCs from the biochar particles surface towards soil aggregates driven by moisture (Figure 1), and from their sorption onto organic and inorganic soil solid phases [62].
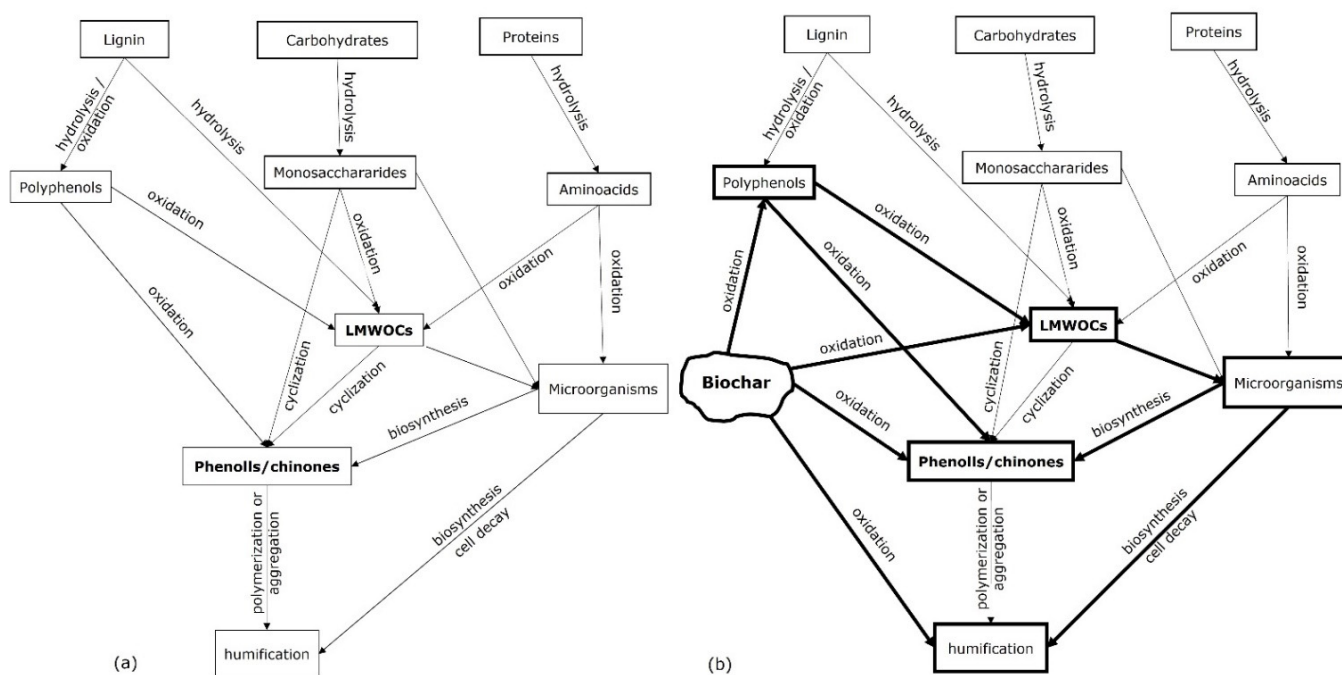

Figure 1. Organic matter decomposition in soil (**a**) and biochar decomposition in soil (**b**) processes mainly controlled by hydrolase and oxo-reductase enzymatic activities. The bold lines indicate the decomposition processes stimulated by the biochar amendment in soil. LMWCOs are the low molecular weight organic compounds.

These considerations let us also to propose that physical aspects are important for future formulation of biochar-based fertilizers, which are supposed to be more efficient for crop nutrition [63] but may not contribute to the maintenance of a porous soil structure as compared to organic amendments such as compost [64].

Overall, we support the idea that, by increasing soil thermal capacity and water retention, biochar confers resilience to soils allowing more constant microbial activity, attenuating the seasonal variations or eventual environmental drought stressful conditions, and enhancing the C stabilization through the 'Microbial Carbon Pump' mechanism [65,66]. Such fluctuations are particularly broad in agricultural soils, where biochar can be incorporated, because microbial CUE also decreases with soil depth due to energetic limitations [43], for example

due to unfavourable C:N and C:P ratios. Biochar incorporation in the deeper horizons for an effective C storage may also increase the CUE in the long term owing to the release of LMWOCs (Figure 1) and let microorganisms living deeper soil layers reach CUE values similar to those of microorganisms of the surface horizons. To our knowledge, there is no information on the effects of the biochar on the CUE in deeper soil horizons, and if proven, such a change may become an additional factor stabilizing organic C in the subsoil.

4. Biochar Effects on C Availability, Nutrient Stoichiometry and CUE in Soil

Several studies have demonstrated that biochar amendment improves the soil habitat for microorganisms, and that the positive effects on microbial activity are mainly related to biochar and properties of soil [6,67,68]. These results provide a ground base for a critical evaluation of the reported increase in microbial CUE in biochar-amended soils. In a laboratory incubation experiment, the higher CUE of biochar-amended soils was attributed to the biochar protection of hydrophilic SOM towards microbial mineralization, whereas no such effect was observed for hydrophobic SOM [69]. Soil amendment with aged biochar increased microbial CUE and significantly decreased the biomass turnover time compared to the amendment with fresh biochar. However, such effects were observed in a sandy, as opposed to in a clayey, soil [70,71]. This result could be explained by the fact that clay soils better stabilize SOM than sandy soils [72]. A support to this hypothesis is a study of the short-term effects of biochar by pyrolyzed maize straw pyrolysis at different temperatures showed that biochar changed the microbial CUE, inducing the release of more biochar-borne LMWOCs [73]. These results indicated that, while biochar stimulates the mineralization of LMWOCs, it may reduce the decomposition of native SOM and thus increase the potential C storage in soil (Figure 1). The two above-mentioned studies confirmed that changes in pH value, availability of primary nutrients and SOM quality, induced by fresh or aged biochar, were all covariates that influenced microbial CUE in biochar-amended soils [74].

However, though higher CUE is increasingly reported for biochar-amended than unamended soils, considering only the C availability, higher microbial biomass or activity in biochar-amended soils may not necessarily result in higher CUE.

4.1. Biochar Changes the Soil N and P Contents and the C:N:P Stoichiometry

A large number of studies have demonstrated that, though microbial metabolism in soil is limited by C availability, microbial CUE can be also limited by the availability of other nutrients, primarily N and P [75,76]. The soil C:N:P stoichiometric ratio only significantly varies in different soil types or after major changes in soil use and management. Additionally, the physiological effects of nutrient stoichiometry, as the main forces that drive the biochar transformation and regulate SOM stability in soil, have received experimental confirmation. In highly degraded soils, P availability is considered the primary limitation on microbial growth [77]. In severely degraded soils, colimitation of microbial activity by N and P have been also reported [78], and this may explain why in degraded soils the potential of biochar to increase soil microbial biomass and stimulate microbial activity is stronger than in more healthy soils.

Soil C:N:P stoichiometry affects microbial CUE in soil because, while C substrates are oxidized for energy production in the catabolic pathways, N is needed for protein synthesis and P is required for nucleotide synthesis and ribosome activity [77,79]. For these reasons, nutrient stoichiometry is also considered in SOM turnover models e.g., [26,80]. Due to the high condensation degree of C structures, biochar incorporation into soil increases the C:N:P ratio of amended soils. However, the literature data show that the biochar amendment of soils increases total concentration and availability of N, P and K in the long-term [5,81]. Biochar enriches in phosphate P during carbonization [82] or retains P on its surface due to the presence of sorption sites [83,84]. Higher P availability in biochar-amended soils may be a major factor in improving microbial CUE because the microbial biomass C:P ratio varies more than the biomass C:N ratio. The C:N:P stoichiometry in

soils also influences the nutrient availability in soil as soil microorganisms tend to regulate the SOM stoichiometry by releasing nutrients absorbed in excess [85]. Therefore, C:N:P stoichiometry is a main parameter conditioning the activity of soil microorganisms during the SOM decomposition and nutrient mineralization, and significant variations of soil stoichiometry can induce changes in soil microbial diversity and soil microbial activity, because it can make one of the nutrients limiting [86,87]. For these reasons, P availability influences microbial CUE, and the use of decomposition models indicates that the CUE should decrease upon an increase in the C:P ratio of the SOM [88]. However, a decrease in microbial CUE concerns forest litter, which typically has C:P ratios in the order of 1000 on a mass basis [89], whereas in biochar-amended soils the C:P ratio values are in the order of 100, i.e., in the typical stoichiometric range of the microbial biomass C:P ratio [88]. Moreover, soils amended with biochar increase the abundance of P-solubilizing bacteria in a forest soil [90], and only in soils where biochar reduces P availability may it impair microbial activity of amended soils [91]. Accordingly, CUE in biochar-amended soils may have an opposite trend as compared to that of native SOM, as for the latter the C:N ratio moves below the N immobilization–mineralization threshold as C becomes increasingly recalcitrant upon decomposition, thus limiting microbial activity. Differently, biochar aging in soil increases C lability and the C:N ratio may not decrease, leading to a faster microbial growth and increase in the CUE to a plateau level, with timing and increments depending on the biochar quality, soil type and soil use and management (Figure 2).
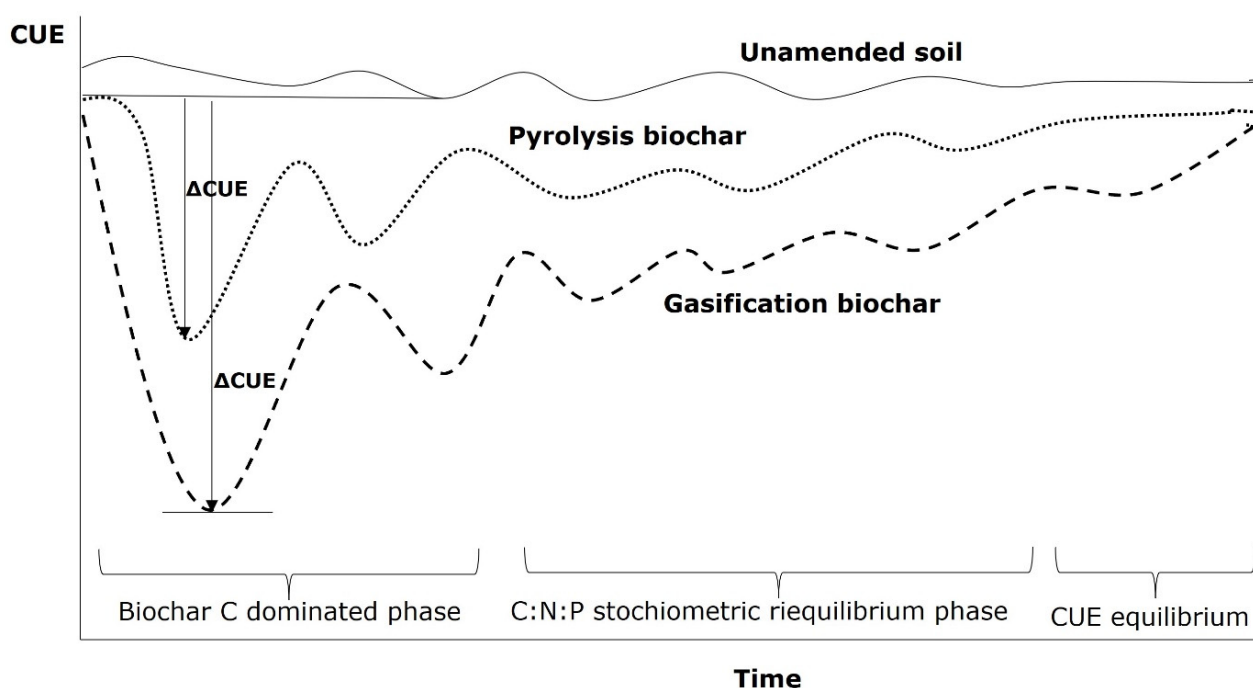

Figure 2. CUE in unamended soil, in soil amended with biochar produced by pyrolysis (pyrolysis biochar) and in soil amended with biochar produced by gasification (gasification biochar).

Overall, we hypothesize that the few reports on high CUE in biochar-amended soils could be due to the reaching of a C:N:P ratio that favors metabolically efficient microbial communities.

4.2. Determination of Soil Microbial Biomass Homeostatic C:N:P Ratios in Biochar-Amended Soils

To date, studies of biochar-amended soils have seldom focused on the release of nutrient limitations to microorganisms, which is crucial to understand the adaptation of microbial metabolism in soil. Relying on the concept that microorganisms have an homeostatic C:N:P composition, altered availabilities of N and P, induced by biochar amendment in soil, have the potential to favour microbial communities that can initially

cope with the stoichiometrically altered substrates [80]. Such changes could be estimated by the measurement of nutrient assimilation efficiency by soil microbial communities [92] or maximum microbial growth efficiency [93]. Such estimations can be complicated in soil because it is a structured environment and biochar movement can cause the creation of hot spots of nutrients, or transfer nutrients along the soil profile and also between distinct microbial populations. Decomposition studies showed that SOM residues with high C:N ratio (e.g., conifers wood) indicate that microbial CUE decreases upon an increasing in the C:N ratio, something which normally leads to N immobilization [94]. Limited use of insoluble pools of biochar-borne C by soil microorganisms due to physical and chemical protection into soil aggregates, as discussed in Section 3, can be overcome when the decomposition process is initiated by the synthesis of enzymes and the subsequent formation of more hydrophilic and soluble C pools that can be used by microorganisms.

5. Microbial Community Composition and CUE in Biochar-Amended Soils

While at a single-species level CUE depends on cellular metabolism and on the production of cell structures and storage compounds, at the community level variation in CUE is related to the soil microbial communities composition, changes of dominant microbial species and relative abundance of microbial groups, exemplified by the fungi:bacteria biomass ratio. Differently from natural organic polymers that are mineralized after depolymerization and solubilization processes [95], biochar-C can be used by soil microorganisms after oxidative and hydrolytic reactions that should be theoretically restricted to a subset microbial physiological group capable of performing the highly demanding reactions (Figure 1). Such decomposer microorganisms, characterized by low CUE values, should be adapted to low substrate concentrations and be slow growing [95]. Based on these assumptions, initial steps of biochar use should be characterized by a drop of CUE related to biochar recalcitrance and to the relative abundance of degradative keystone microbial species (Figure 2).

Biochar incorporation into soil significantly increased both Gram-negative and Gram-positive bacteria and decreased the fungi:bacteria ratio [70]. Fungi, which have higher C molar ratio than bacteria, could be mainly involved in the 'char dominated phase' because theoretically they have a higher C-demand and slower growth rate than bacteria [96]. Moreover, an increase in fungal activity during early biochar decomposition may justify a high CUE due to their large C:N and C:P ratios, which imply larger C demands [97]. However, different fungal phylogenetic groups have different C:N:P ratio values [98]. In particular, the C:N:P ratio for free living fungi varies broadly with lower C:P and N:P ratios found in *Ascomycota* as compared to *Basidiomycota* fungi, whereas lower C:N ratios and higher N:P ratios were found in mycorrhizae than in saprotrophic fungi [98]. Interestingly, fungal C:N:P: ratios were correlated with environmental parameters such as moisture and temperature, and all parameters varied by biochar amendment of soil (cfr. Section 2). A field experiment showed that after 3 years biochar amendment in an alkaline soil cultivated with soybean, *Ascomycota* significantly decreased and *Mortierellomycota* significantly increased, whereas no significant changes in the bacterial community was observed [99]. Overall, changes in bacterial and fungal communities were associated with soil properties such as SOC and TN [99].

Bacteria also exhibit highly flexible metabolism under various environmental conditions e.g., [100], and may contribute to biochar oxidation.

Later, when a significant share of biochar has been weathered, the CUE values of the soil microbial community should approach a plateau level (Figure 2). While the length of the char-dominated phase mainly depends on the biochar type, soil properties and environmental conditions, the new CUE level should mainly depend on the microbial community composition. Changes in soil microbial community structure have been reported [101,102], but the effect of biochar on total microbial diversity and functional gene abundance and expression are still controversial. Changes in the soil microbial community after biochar amendment were observed, and an increase in the abundance of soil microbial communities

in a dose-related relationship has been reported [103]. An increase in fungal abundance after biochar application in an alkaline soil was observed, highlighting that the effect of biochar is related to different types of biochar and soil properties [99]. The addition of biochar (10–15% w:w) induced modification of the microbial community structure, and a significant increase in the richness and diversity index of total microbes [64]. Differently, no significant effect on microbial community structures and extracellular enzyme activities was observed in a short-term experiment, in which the biochar application (22 t ha^{-1}) was compared with manure amendment [104]. This result could be attributed to the relatively low application rate of biochar or to site-specific environmental conditions.

Current information on microbial groups potentially active in biochar-amended soils is inconclusive. Future studies based on stable isotope probes may clarify the main biosynthetic pathways and improve the models applicable to microbial communities of biochar-amended soils, especially considering long-term trials. For example, laboratory incubation studies, based on pulsed additions of large amounts of LMWOCs, may allow us to quantify the maintenance energy and growth respiration of specific substrates released during biochar aging. These substrates could be representative of in situ microbial metabolism activated by LMWOCs release, and also analyze the influence of environmental factors such as soil temperature and moisture level.

5.1. Polycyclic Aromatic Hydrocarbon Degraders: The Chemical Gate Operators

Specialized polycyclic aromatic hydrocarbon (PAH) degraders may be among the first microbial groups attacking the biochar C. In a pot experiment, the biochar amendment increased the PAH-ring hydroxylating dioxygenase (PAH-RHD) genes coding for the enzyme involved in the initial step of the microbial degradation of PAHs [105]. In the same soils, the increase in K, P and N availability in biochar-amended soil was also highlighted [105]. These results from short-term laboratory or greenhouse scale experiments are in agreement with evidence from the Amazonian dark earth (ADE), anthropogenic dark soil horizons formed by the deposit of organic materials that date back from ca 8000 years before the Columbus age [106]. Such deposits turned infertile Oxisols into highly fertile hotspots, mainly due to the stabilization of organic matter as a result of incompletely combusted biomass, similar to the biochar [107]. An ADE soil had similar bacterial community compositions, as well as a significantly greater species richness than a pristine forest soil [108]. The relative abundance and diversity of the biphenyl dioxygenase (*bph*) gene involved in aromatic hydrocarbon degradation in an ADE and a nonanthropogenic-adjacent soil, and of the bacterial genera harbouring the *bph* genessuch as *Streptomyces*, *Sphingomonas*, *Rhodococcus*, *Mycobacterium*, *Conexibacter* and *Burkholderia* with known aromatic hydrocarbon degradation capacity, were more abundant and diverse in the ADE than non-ADE soil [109]. The *bph* and *thp genes*-coding dioxygenase and aromatic ring-hydroxylating dioxygenases have been identified in genomic and plasmidic DNA of aromatic hydrocarbon-degrading bacteria also detected ADE soils [110]. Overall, evidence from short- and long-term studies demonstrates that biochar oxidation is energetically permissible, but that it likely occurs in microbial communities hosting physiological groups of microorganisms that use catabolic pathways to obtain energy. The PAH-degrading microorganisms could constitute one of the operational groups responsible for the metabolic infallibility of soil microbial communities.

5.2. Enzyme Activity: The Toolbox

Changes in enzyme activity profile from biopolymer hydrolase to condensed C oxidase allow us to hypothesize that, in biochar-amended soils, the respiration rate should be lower than thermal oxidation. Activation energy for the mineralization of recalcitrant biochar matter by microbial communities, which is greater than the electromotive force of the cell oxidative metabolism, is lowered due to the multiple enzymatic steps needed to transform the organic C into CO_2 [111]. In fact, the relation between temperature dependency for chemical and microbial oxidation of SOM was studied, and the release of specific oxidative enzymes was considered as a factor that makes the microbial decomposition

capacity relatively independent of the SOM recalcitrance [112]. This hypothesis should be tested in different soils, especially from long-term trials, because under natural conditions the temperature sensitivity of microbial respiration is far higher than chemical oxidization, and other main soil factors (e.g., C:N ratio, pH value, salinity) influence microbial activity, not only SOM chemical oxidability. If proven operating, this mechanism could explain the higher CO_2 release generally reported for biochar-amended soils compared to nonamended soils.

An alternative hypothesis that may explain higher abundance of dioxygenase, phenol oxidase enzyme activities and relevant encoding genes could be the need to mitigate the impact of toxic compounds. In fact, increased expression of genes and enzyme activities involved in the degradation of PAH has been reported for contaminated soils [113]. In this light, other well-characterized genes involved in the oxidation and detoxification of recalcitrant C compounds, such as the naphthalene dioxygenase genes (*nah*A), may be involved in the biochar modification, as such genes are ubiquitous in soil. In particular, the larger abundance of *nah*A genes in the rhizosphere, i.e., in the soil portion modified by the plant roots, may explain the faster biochar C turnover in soils under permanent grass, which can be considered as an entirely rhizospheric soil. A rapid increase in the relative abundance of dioxygenase genes in PAH polluted soils amended with biochar has been frequently reported e.g., [101,114,115], although no effects have been also reported e.g., [116,117]. While biochar used in agriculture may contain low levels PAHs, amendment generally does not induce soil toxicity to microorganisms [118]. In addition to oxidase, hydrolytic enzymes also play fundamental role in CUE because catalyze reactions necessary for soil organic C decomposition and mineralization of N, P and S [119]. Recent research has shown that also the profile of the hydrolytic enzyme activity changes in long-term biochar-amended soils [81]. Soil enzymatic activity is an integral component of the whole CUE as it regulates the nutrient availability to soil microorganisms [120], and such changes in substrate utilization can potentially affect the enzymatic stoichiometry, and in turn the biogeochemical cycle of nutrients in soil [80,121]. According to the economic theory of soil enzymatic activity [40,122], microorganisms synthesize and release extracellular enzymes to decompose SOM into LMWOCs [123]. The release of LMWOCs in biochar-amended soils can also be enhanced by the increase in arylesterase enzyme activity that cleaves carboxylic branches from aromatic compounds. This change is important because it was estimated that LMWOCs microbial uptake and mineralization accounts for a significant share (up to 30%) of soil CO_2 emission from soil [124]. Thus, the LMWOCs pool should be included in models of SOC dynamics. Shifts in soil enzyme activities have been reported from both short-term laboratory/greenhouse experiments [125] and long-term field trials [126]. Because it can be considered as the last enzymic step prior to utilization of LMWOCs as energy sources by microorganisms actively degrading biochar. The ratios of oxidative and carboxyl esterase to hydrolytic enzyme activity as proxies for SOM were proposed [127]. Here, we propose the following enzyme activity ratio as a proxy for describing the potential biochar degradability in soil, as a potential C source to soil microorganisms (Equation (2)), with the highest value indicating faster potential microbial C turnover and microbial CUE in biochar-amended soils.

$$\text{Biochar C use} = f\ [(\text{oxidase} + \text{carboxyl-esterase})/\text{hydrolase}] \qquad (2)$$

As mentioned above, microbial CUE is assumed to be lower during the use of recalcitrant substrates due to the high metabolic energy investment in enzymes and cell uptake systems, but in the soil environment the CUE may non increase owing to the accumulation of extracellular oxidase and hydrolase enzymes. This hypothesis converges with those deriving from the degradation of fresh biomass polymers, as the available C molecular can be used to offset a significant part of the energy dissipation due to the initial substrate recalcitrance (Figure 2). This change in CUE can be realized by adaptation of microbial groups capable of using biochar-derived C substrates after the first oxidative reactions that enrich the condensed C in carbonyl, carboxyl- and phenolic functional groups, open the

aromatic rings and cleave the lateral branches, releasing LMWOCs (Figure 2). Release of oxidase and hydrolase enzymes coupled with higher mean temperature, moisture, and N and P availability, can improve C acquisition and maintain the C:N:P stoichiometric balance of adapted microbial consortia. In these conditions, microbial taxa with high CUE reduce energy investments for enzyme synthesis, especially for catalysing the first steps of biochar degradation. Possibly, the greater CUE in biochar-amended soils is reached after the biochar-borne C structure modification releases sufficient LMWOCs, and less degradative enzymes need to be produced per unit of biomass formed [128]. This CUE adaptation stage at microbial community level may be responsible for the C storage in biochar-amended soils due to sorption of SOM of microbial origin [129], and microbial colonization of biochar habitats.

　　　In amended soils the chemical modification of biochar induces modification of the community metabolism and the stimulation of a potential metabolic mechanisms controlling microbial nutrition and microbial energy (Figure 3).
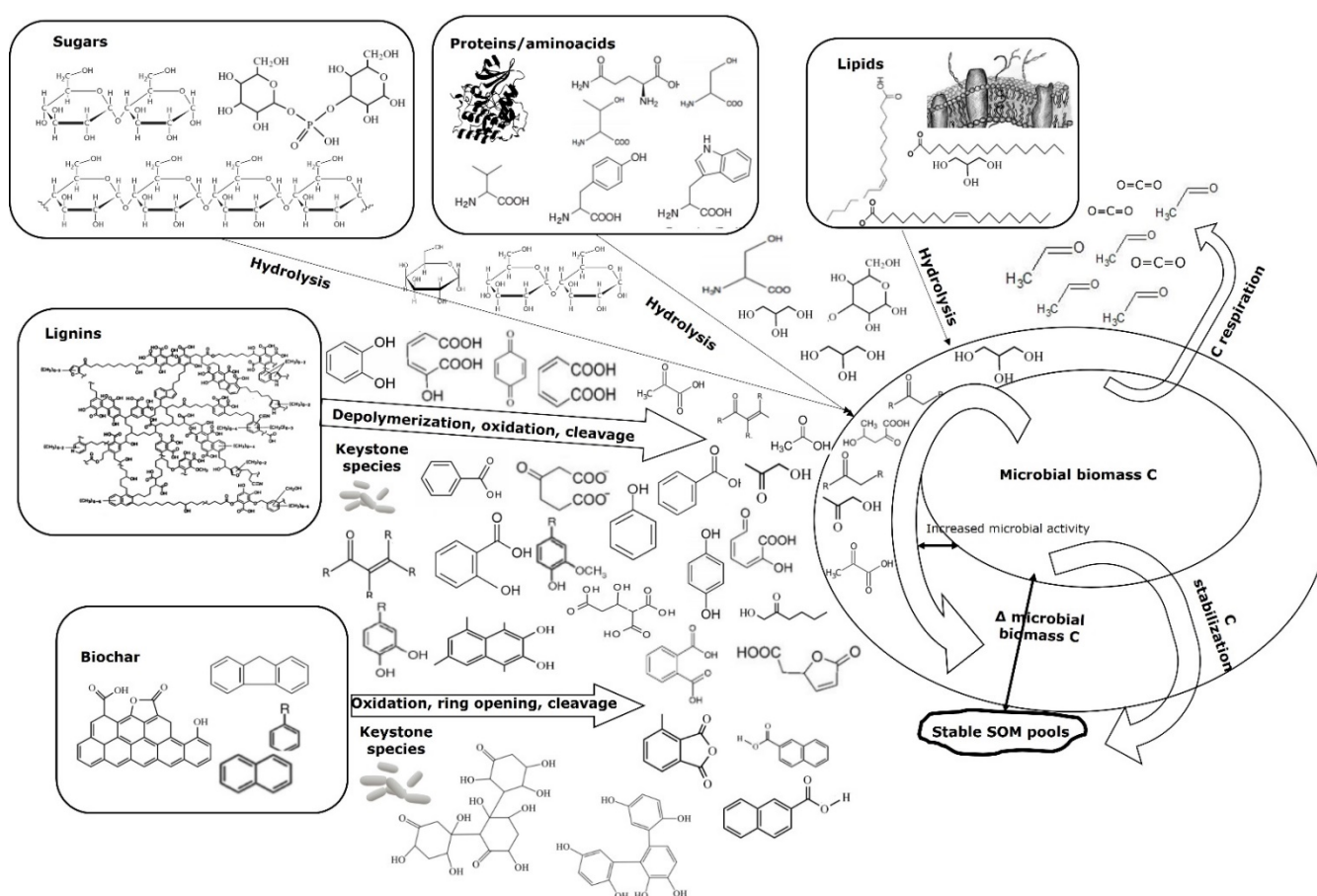

Figure 3. The chemical modification of biochar and organic substances in biochar amended soils and the potential metabolic mechanisms.

　　　The above hypothesized CUE adaptation based on the utilization of low molecular weight carbonyl- and acyl- compounds requires the oxidation and modification of the poly- and branched aromatic molecules, formed during the biomass pyrolysis. This preliminary step can be conducted by various bacterial and fungal groups peroxidases releasing extracellular peroxidases, phenol oxidases and laccases enzymes [130,131]. It is important to underline that, independently on the involved microbial species, the mentioned oxidative enzymes are those involved in the degradation of lignin and other biopolymers. The convergence of molecular structure during the biochar oxidation and SOM decomposition

will result in an optimization of the enzymatic efforts of soil microorganisms which will benefit from additional C sources from the same enzyme activities (Figure 2).

5.3. P and N Mineralizing Microorganisms: The Helpers

The above discussed importance of nutrient stoichiometry should be reflected in changes of microbial physiological groups involved in N and P turnover. In particular, the release of abundant oxidized LMWOCs from biochar decomposition should influence the diversity and relative abundance of functional genes coding for the enzymes involved in the N and P cycles, but evidence on the relevant microbial groups is recent and still conflicting. In a short-term field trial an increased total soil organic C, the decrease in extractable nitrate pool and the reduction in gross rates of organic N transformation were reported, whereas the increase in ammonia-oxidizer bacterial and archaeal populations that paralleled a two-fold increase in the gross nitrification rate were observed [132]. Soil urease activity paralleled to the increased abundance of the PAH-degrading bacteria in biochar-amended soils was reported [101]. No or variable effects of biochar addition on ammonia-oxidizing Archaea and ammonia oxidizer and nitrite-reducing bacteria were reported [133,134]. The bacterial community involved in the Dissimilatory Nitrate Reduction to Ammonium (DNRA) was found not significantly affected by amendment with biochar of neutral and alkaline soils [135], whereas biochar amendment was reported that it could increase both potential nitrification and denitrification activity in acidic soils as the biochar alkalinity could create a favorable environment for the activity of nitrifying and denitrifying communities [136]. In a pot experiment biochar amendment of an acidic soil stimulated both nitrification and denitrification processes, reduced N_2O emissions which paralleled the increased *nos*Z gene expression, indicating an increased tendency of reduction in N_2O to N_2 [121]. Differently, biochar addition reduced the relative abundance of ammonia oxidizers and the nitrification potential of soil [137]. The addition of leaf and woodchip biochar showed the increase in the abundance of P-solubilizing bacteria and the diversity of soil bacterial community in the forest soil [90]. These inconsistent results could be due to differences in plant species, biochar characteristics, soil types and biochar application periods [138,139]. Biochar plus chemical fertilizers increased the abundance of bacteria in the rhizosphere [140]. It is important to underline that most of the studies on changes in diversity and abundance N cycling microbial populations in biochar-amended soils have been conducted in short-term microcosm experiments e.g., [141–146].

5.4. Sulfur Reducing Bacteria: The Stone Guest?

When compared to N, P and K, little information on total S and sulphate in biochar-amended soils is currently available. Sulfur is essential for plant growth in lower amounts as compared to N, P and K, and an increase due to biochar dose may release large amounts of sulfates, triggering sulfate-reducing bacteria. Such bacteria are known to degrade aromatic hydrocarbons with various degree of condensation and molecular complexity, and under optimal conditions they can mineralize completely aromatic C up to CO_2. Sulfate-reducing bacteria have been detected in hydrocarbon-contaminated environments, and their degradative activities result in release LMWOCs (e.g., acetate, propionate) which are mineralized by the same bacteria or other bacterial groups [147]. Microbial acetogenic activity of sulfate-reducing bacteria was enhanced by biochar added to a wastewater treatment sludge [148]. Higher emissions of acetylene, propene, and C_4 aldehydes from long-term biochar-amended soils as compared to nonamended soils were observed, but no information on the source of such LMWOCs was investigated [126]. Therefore, the potential role of sulfate reducing bacteria in biochar-amended soils remains obscure. Sulfate-reducing microorganisms can occur in association with fermentative, syntrophic microorganisms, such as methanogenic bacteria with other metabolic pathways involving Fe (III)$^-$, Mn (IV)$^-$, NO_3^--N reduction [149]. Archaea can also degrade aromatic hydrocarbons into acetate followed by acetate syntrophic conversion into CH_4 and CO_2 [150]. Soil amended with biochar could increase the number of sulfur-mobilizing bacteria, which may explain

the enhancement of sulfur cycle functions [151]. The combined use of ferrous sulfate and biochar significantly reduced emissions of NH_3, N_2O, CO_2, and CH_4 and increased of the richness of bacterial communities during composting of pig manure and straw mixture more than single ferrous sulfate and biochar [152]. Biochar acting as an electron shuttle in the microbial reduction in Fe (III) in ferrihydrite has been reported [153]. In our opinion, the biochar-mediated S and Fe transformations and the involved microbial groups, deserve higher attention in future studies to better understand the biochar influence on nutrients biogeochemistry with the complex mechanisms illustrated in Figure 4, not only on microbial CUE.
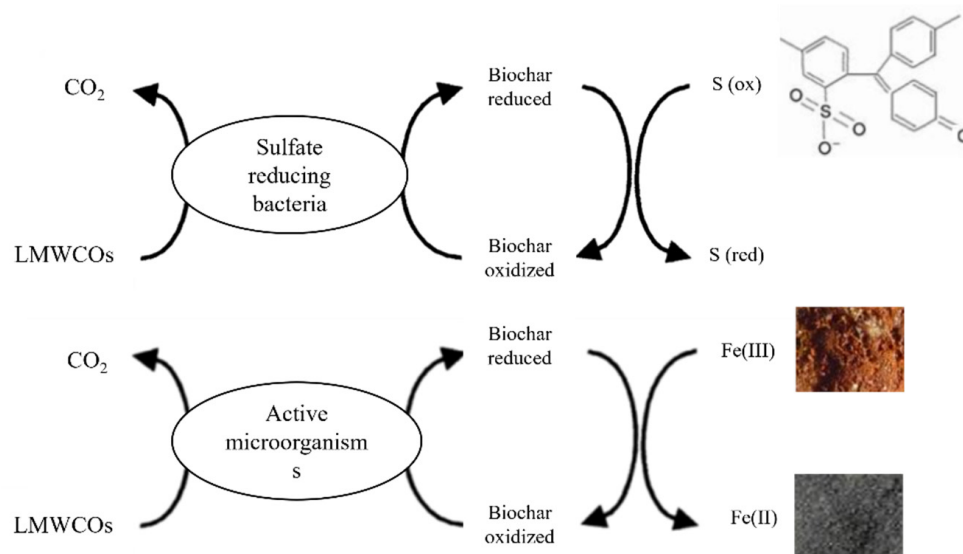

Figure 4. Hypotetical pathway of S and Fe reductions by electron shuttling via biochar.

Summarizing, in biochar-amended soils after the char-dominated phase initiated by specialized microbial physiological groups, two major and related features can be anticipated: (i) an abundance of microbial species can process the biochar-borne LMWOCs and (ii) microbial CUE of the soil tends to a new stable value, typical for each specific soil (Figure 2). Duration of the different processes, depicted in Figure 2, will depend on the biochar type, pedoclimatic conditions, and soil management, but in our opinion reaching new equilibria may require decades. The increased CUE values reported shortly after fresh additions of biochar to soil may result from the use of labile C associated with biochar, formed onto the biochar surface during the condensation of syngas during the cooling phase of the biochar preparation.

6. Conclusions and Research Needs

We support the idea that biochar amendment can improve the fertility of degraded and intensively cultivated soils and can lead to significant C sequestration. Nevertheless, we hypothesize that, in the long term, stabilization of biochar C in soil is not solely due to biochar's inherent chemical stability, but also to the stabilization of microbial processed C released by the oxidation and cleavage of biochar C. This process should be initiated by soil microbial physiological groups possessing the suite of functional genes for biochar oxidation. We hypothesize that microbial activation is facilitated by higher thermal capacity, greater water retention, and larger C N and P availability induced by biochar addition. Biochar porosity also increase the biological space and create favorable conditions for microbial activity, stabilization of extracellular oxidase and hydrolase enzymes and adaption of microbial groups with relatively high CUE values. To test these hypotheses, we suggest the following research objectives:

i. Determine the theoretical CUE of biochar-amended soils and under different environmental conditions, soil types and management. Determination of the thermodynamic

maxima of different biochar types can also allow the improved determination the limiting or unlocking effects of increased nutrient availability;

ii.   Models operating on finer time scales (days to seasons) should consider the effects of changing SOM molecular composition, multi-element stoichiometric constraints, and microbial community physiology. In addition, environmental drivers should be implemented on data from long-term field trials to predict the CUE of biochar-amended soils under different management. The effects of larger N and P availability as driving forces, and changes in enzymatic activity, should be tested, particularly in arable soils amended with biochar and under chemical fertilization, in order to determine the stability and MRT of the biochar-borne C. An interesting technique that could be tested to assess the extent of biochar stability in soil could be the reverse stable isotope labelling [154];

iii. Perform hypothesis-driven metagenomic research to reveal presence, activity, and evolution of microbial metabolic pathways in long-term biochar-amended soils. Complementary proteomic and metabolomic studies may help to elucidate the hypothesized pathway of release of LMWOCs from biochar, and better estimate the C partition in CO_2, microbial and SOM pools, also analyzing the changes of the ^{13}C signature of biochar and SOM;

iv.   Adopt imaginative direct observation approaches to observe the surface of weathered biochar, also extracted from amended soils, to describe the formation of the 'charrosphere' and its relations with biochar biodegradation and the release of LMWOCs into the soil solution.

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