***EFFECTS OF AGING UNDER FIELD CONDITIONS ON BIOCHAR STRUCTURE
AND COMPOSITION: IMPLICATIONS FOR BIOCHAR STABILITY IN SOILS***

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Abstract

The effects of aging on biochar (BC) properties, composition and carbon sequestration are still under debate. This study aimed at illustrating the qualitative alterations of five different BCs aged during a 24-month field experiment located in Southwest Spain. To determine the recalcitrance of each BC, physical fragmentation test, scanning electron microscopy, ^{13}C NMR spectroscopy and CO_2 -respiration experiments were performed.

The physical fragmentation values of all types of BC increased significantly over time at field conditions. FESEM examinations of aged BCs showed collapsed structures and the presence of entrapped soil material and microbial mats into the BC pores. The ^{13}C NMR spectroscopy demonstrated an increase of the relative abundance of O-alkyl C and alkyl C at expenses of aromatic-C in aged BCs. The C losses of all BCs ranged from 27 % to 11 % of the initial C. In contrast, the nitrogen (N) content of wood-derived BCs significantly increased probably due to the sorption of nitrogen containing compounds into these highly-porous weathered chars. With the exception

of that for the sewage sludge-BC, the pH of all aged BCs decreased from > 9 to the soil pH, indicating a short lasting of the liming effect caused by BC addition.

The respiration experiment revealed that BC recalcitrance was much lower than expected and, within the range of decades. Only wood-derived BCs significantly increased the mean residence time of the slow C pool of the Cambisol by factors between 3.4 and 7.7. Mediterranean climate conditions and the characteristics of the Cambisol used probably accelerated the microbial degradation of BCs.

Keywords: organic amendment; Mediterranean climate; physical and chemical parameters; soil properties; carbon sequestration, pyrogenic carbon.

36 **Abbreviations**

37 BC: Biochar

38 CPMAS: Cross-polarization magic angle spinning

39 EC: Electrical conductivity

40 EDS: Energy dispersive spectroscopy.

41 FESEM: Field emission scanning electron microscopy

42 KWB: Vineyard wood biochar pyrolyzed in kilns

43 MRT₁: Mean residence time of labile organic carbon

44 MRT₂: Mean residence time of slow turning organic carbon

45 ms: Millisecond

46 MWB: Mix of wood chips biochar

47 NMR: Nuclear Magnetic Resonance

48 OC: Organic carbon

49 PSB: Paper sludge biochar

50 PWB: Pine wood biochar

51 PyC: Pyrogenic carbon

52 SE: Secondary electron

53 SOM: Soil organic matter

54 SSB: Sewage sludge biochar

55

Highlights

- Biochar aging grade is influenced by feedstock nature•
- Physical fragmentation of biochars increased during aging
- Increase of O-alkyl C and alkyl C in aged biochars at expenses of aromatic-C (^{13}C NMR)
- Mean residence times of C from biochars were much lower than expected, in the range of decades.

BIOCHAR AGING UNDER FIELD CONDITIONS

✓ Physical fragmentation degree

✓ Scanning electron microscopy

✓ ¹³C NMR Spectroscopy

✓ Monitoring of elemental (C,N) composition and basic features (pH, conductivity)

1. Introduction

Biochar (BC) is the carbonaceous solid residue produced through the pyrolysis of organic residues and used as soil ameliorant. It offers a sustainable tool for agriculture management (De la Rosa et al., 2014) and soil remediation (Lehmann and Joseph, 2015). Numerous research studies pointed out that BC can act as a soil conditioner enhancing plant growth by retaining nutrients and improving soil physical and biological properties (Lehmann and Joseph, 2015; Lehmann et al., 2009). In addition, BC can act as an agent for carbon sequestration (Goldberg, 1985; Lehmann, 2007), due to its highly aromatic nature which turns it less available to microbial degradation (Sohi et al., 2010). Thus, BC is often referred to as a material of high chemical and biochemical stability, which may persist over long periods of time (Kuzakov et al., 2009). Nevertheless, there are evidences that the paradigm of recalcitrant pyrogenic carbon (PyC), including BC, has to be revised, since newer results (Knicker et al., 2013a; Watzinger et al., 2014) indicate a much lower biochemical stability of PyC as formerly assumed. This could explain why, at a global scale, the content of PyC stored in soil is low with respect to the annual production rate by wildfires. Masiello and Druffel (2003) demonstrated that a substantial fraction of the annually produced PyC from biomass burning must be mineralized or the accumulation of PyC in the environment over time would be sufficient to perturb global atmospheric oxygen levels. However, regardless microbial decomposition (Wengel et al., 2006), a considerable loss of PyC from soil (Santin et al., 2016) is caused by erosion (Rumpel et al., 2006) or dissolution and transport by water fluxes (Jaffé et al., 2013). Evidences of PyC degradation were obtained by solid-state ^{13}C and ^{15}N NMR spectroscopy (De la Rosa and Knicker, 2011). This study revealed that already after two months of incubation the chemical structure of grass char was

significantly altered, including partial oxidation of aryl structures and degradation of N-heterocyclic constituents. Once deposited in the environment, a range of reactions occur, which also induce changes to physical and chemical properties of BC. Aging of BC was suggested to consist mainly of oxidation of exposed C rings with a high density of π electrons and free radicals (Joseph et al., 2010), which creates a high density of O-rich functional groups at the BC surface. It was further proposed that oxidation starts at the surface and propagates to the core of particles over time (Lehmann et al., 2005, Sorrenti et al., 2016), promoting further physical, chemical and microbial degradation (Hammes and Schmidt, 2009).

Despite BC is increasingly used as soil amendment and the properties of freshly produced BCs are well documented (De la Rosa et al., 2014; Kim et al., 2013), its biochemical stability and alteration during aging in the soil environment are still unclear (Ameloot et al., 2013). Amazonian dark earths, which triggered interest in BC, probably comprise the best-documented long-term case study of BC in soil. Nevertheless, results from *terra preta* are difficult to extrapolate to other soil, climatic and agronomic contexts (Hardy et al., 2017). Alternatively, studies dedicated to BC aging in agricultural soils have been conducted. Yet, they have been frequently performed using artificially accelerating aging methods, including drying or wetting and chemical oxidants (Sultana et al., 2011; Ascough et al., 2011; Cross and Sohi, 2013; Wang et al., 2015). Others promoted the aging of BC with the aid of microorganisms whose activity was enhanced through supplementation of labile organic compounds such as glucose or fresh biomass (Hamer et al., 2004; Keith et al., 2011). In general, they agreed that with progressive aging, BC is reduced in size, and functional groups such as carboxyl and carbonyl or hydroxyl are formed on the BC surface. Functionalized (aged) BCs can potentially increase interactions between

106 BC, SOM, soil minerals, nutrients, and contaminants (Mia et al., 2017). Spokas
107 (2013) reported that aging greatly alters the greenhouse gas response of the soil
108 systems to BC amendments. Unfortunately, taking into account the complex
109 interactions within the soil matrix, the extrapolation of these impacts to BCs added as
110 agricultural soil ameliorant is very uncertain. As soil amendment with BC targets
111 mainly agricultural soils, a better assessment of the effect of climate and soil
112 characteristics on the fate of BC is crucial for predicting the long-term dynamics of
113 BC in soil. It is well known that intensive tillage disrupts soil aggregates and exposes
114 SOM to physical and microbial degradation (Panettieri et al., 2015). Such soil
115 treatments may also accelerate BC aging. The environmentally more sustainable
116 conservation agriculture enhances not only soil quality but also favours soil
117 aggregation and prevents increasing SOM loss due to enhanced microbial activity.
118 We hypothesize that in spite of the conservation agriculture and the hot
119 Mediterranean climate, BC ages, once it has been applied to soils. Thus, for this study
120 a no-tillage field experiment was conducted during 24 months in an agriculturally
121 used Calcic Cambisol (SW Spain) to determine the alterations induced by the
122 environmental conditions, soil properties and native microbiota to physical and
123 chemical characteristics of five different types of BC. For that purpose, weathered BC
124 particles were collected 6, 12 and 24 months after soil amendment and sunflower
125 sowing. Subsequently, they were analyzed by a multidisciplinary approach which
126 combined the analysis of basic properties (pH, electrical conductivity and physical
127 fragmentation degree), elemental composition (C and N), field emission scanning
128 electron microscopy (FESEM) and ^{13}C nuclear magnetic resonance (NMR)
129 spectroscopy. In addition, the biochemical recalcitrance of the field-aged BCs was
130 assessed through a laboratory-based respiration experiment in which the collected

BCs were mixed with the same soil and incubated for 110 days. To the best of our knowledge, this is the first study on the effects of aging at field conditions on BC properties involving qualitative analyses (composition, physical and chemical characteristics) and quantitating the microbial degradation potential of field-weathered BCs, without applying additional chemical, physical or microbiological procedures for aging acceleration.

2. Materials and Methods

2.1. Biochars aging experiment at field

The field experiment was carry out at the experimental farm “La Hampa” of the Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS), located 13 km southwest of the city of Seville (Spain) in the Guadalquivir river valley (SW Spain; 37° 21.32 N, 6° 4.07' W) from February of 2014 to March of 2016. The soil of the plots used for this experiment is a calcic Cambisol (IUSS Working Group WRB, 2007). This is a calcareous soil (Mudarra-Gómez, 1988), with a sandy clay loam texture (240 g kg⁻¹ clay, 180 g kg⁻¹ silt and 580 g kg⁻¹ sand) which contained 20 g C kg⁻¹ soil (7 g organic C kg⁻¹ soil) and 1 g N·kg⁻¹ soil., Its pH_(H2O) was 7.8 and the electrical conductivity was 68 µS cm⁻² (Panettieri et al., 2015). The climatic conditions are typical Mediterranean with mild rainy winters (496 mm mean annual rainfall) and very hot and dry summers. The mean annual daily temperature at the experimental site is around 21 °C, with maximum and minimum mean monthly temperatures of 33.5 °C and 5.2 °C registered in July and January, respectively (Panettieri et al., 2015). Five types of BCs, produced from pine wood (PWB), paper sludge (PSB), sewage sludge (SSB), old vineyard wood pyrolyzed in traditional kilns (KWB) and a mix of wood chips (MWB) were homogenized, moistured (biochar:water, 1:1) and subsequently

applied to the first 5 cm of the topsoil at a dose equivalent to 15 t ha⁻¹. Additionally, KWB was crushed and sieved (< 2 cm) before application. Table 1 comprises a complete description of the production conditions of the used BCs.

Certified seeds of *Helianthus annuus* L. (sunflower) were planted on the 20th of February 2014. A detailed description of the experimental conditions and of the effects of BCs addition on sunflower productivity are described in Paneque et al. (2016). No tillage operations were performed, except for the crumbling of the sunflower stalks, and the sowing was performed by direct drilling. Sunflower plants were harvested 6 months after sowing. During that initial period weeds were removed manually. Subsequently, the plots were kept fallow at the field from month 7 until the end of the experiment (month 24). Soil samples were taken with a manual gouge auger drill (0-15 cm depth; 5 replicates per treatment) at months 6, 12 and 24. Subsequently, dry soils (40 °C) were homogenized, hand crushed and passed through 2 mm sieves. All BC particles were removed manually from the sieves. For the fraction of BC ≤ 2 mm, the remaining material was placed in Petri dishes and BC was removed manually using tweezers. Biochar particles were stored in glass flasks at 4 °C until being analyzed.

2.2 pH, electrical conductivity and elemental analysis (C, N content)

The pH and electrical conductivity were measured using a Crison 40 pH-meter and a conductimeter Crison Basic 20 (Crison, Spain) respectively in a mix of hand-picked BC and distilled water (1:10) according to De la Rosa et al. (2014).

Total carbon (C) and total nitrogen (N) were determined in triplicates by dry combustion (1000 °C) using a LECO Truspec micro CHNS (LECO, United States) detecting N as N₂ and C as CO₂.

2.3. Physical fragmentation of biochars

The physical fragmentation stage of the BC particles was determined by counting the number of fragments comprising 0.50 g of BC yielded at 0, 6, 12 and 24 months after starting the experiment. The respective standard deviations were calculated after the analysis of three replicates.

2.4. ^{13}C Solid-State Nuclear Magnetic Resonance Spectroscopy

Cross-polarization magic angle spinning (CPMAS) ^{13}C NMR experiments were carried out with a Bruker Avance III HD 400 MHz spectrometer (Bremen, Germany). Therefore, BC samples were homogenized and crushed in a mortar before being placed into zirconium rotors of 4 mm OD with KEL-F-caps. The rotors were spun at 14 kHz. A ramped ^1H -pulse was used during a contact time of 1 ms to circumvent Hartmann-Hahn mismatches. A 90° ^1H -pulse width of 2.4 μs was used for all spectra. The ^{13}C chemical shifts were calibrated relative to tetramethylsilane (0 ppm) with glycine (COOH at 176.08 ppm). Over 10,000 scans were accumulated for each sample, applying a pulse delay between each single scans of 300 ms. The spectra were quantified by integration of the following chemical shift regions (Knicker, 2011) using MestreNova 10 (Mestrelab Research S.L., Santiago de Compostela, Spain): Alkyl C (0–45 ppm); N-alkyl/methoxyl C (45–60 ppm); O-alkyl C (60–110 ppm); aromatic C (110–160 ppm); carbonyl/amide C (160–250 ppm).

During the NMR experiment of the fresh PWB, the presence of graphitic C, which was previously confirmed by FT-IR spectroscopy (De la Rosa et al., 2014), hindered the entrance of the excitation energy into sample. Since the reflection of the energy can damage the NMR probe, the ^{13}C NMR spectrum of that sample was not acquired.

2.5. Field Emission Scanning Electron Microscopy (FESEM)

The alterations on surface topography and chemical composition on BCs after staying 24 months in the field were determined by FESEM. Therefore, hand-picked BC fragments were directly mounted on a sample stub and sputter coated with gold/palladium film. Subsequently, samples were examined on a Jeol JSM-7001F microscope equipped with an Oxford X-ray energy dispersive spectroscopy (EDS) detector using standard ZAF corrections that allow semi-quantitative microanalysis. FESEM examinations were operated in secondary electron (SE) detection mode with an acceleration potential of 15 kV.

2.6. Respiration experiment under controlled conditions

The microbial degradation of the five BCs under controlled conditions was performed using a Respicond Apparatus IV (Nordgren Innovations, Sweden) as previously described (Knicker et al., 2013b). Therefore, for each field plot three replicates (containing both BC and soil) were taken after 6 months field experiment time. They were placed into closed 250 ml vessels, inoculated with 1 ml of a microbial suspension, obtained from the extraction of fresh soil from the field trial, and subsequent filtering (5 µm pore size). The water content was adjusted to ca. 60 % of the maximum water holding capacity, and finally all the samples were incubated for 110 days at 25 °C. The CO₂ released was monitored every 3 hours by determining the changes in the electrical conductivity induced by the absorption of CO₂ in a KOH solution (10 mL; 0.6 M) placed inside the vessel (Nordgren, 1988). The C content of each vessel was measured in triplicates at the beginning and at the end of the incubation. The determined CO₂ production was plotted as a function of incubation time. Subsequently, the curves were optimized and fitted with Sigmaplot 13 (Systat

Software Inc). The mean residence times and decomposition rates were estimated with a double first-order exponential decay model according to Eq. (1) as described by Knicker et al (2013b).

$$(1) A(t) = A_1 \times e^{-k_1 t} + A_2 \times e^{-k_2 t}$$

Where: A (t) is the remaining C (% of total OC); A₁ is the amount of C which is labile against mineralization (% of total OC); A₂ is the amount of C which is more stable (refractory) against degradation (% of total OC); t is incubation time; k₁ and k₂ are degradation constants for the labile and refractory pool (y⁻¹). All obtained curves showed coefficients of determination (r²) > 0.998.

3. Results and discussion

3.1. Alterations on the elemental composition (C, N) and basic features of biochars with aging at field

At time 0, the BCs produced from wood (PWB, KWB and MWB) showed comparable C (764-847 g kg⁻¹) and N contents (1.7-1.9 g kg⁻¹). The PSB, derived from paper-sludge, comprised 475.4 g kg⁻¹ of C and 12.3 g kg⁻¹ of N, whereas SSB (from sewage-sludge) showed the lowest C (less than 200 g kg⁻¹) and the highest N values (20.0 g kg⁻¹). These data show that, as expected, the nature of the feedstock drives the elemental composition of BC (Zhao et al., 2013). Wood BC typically contain around 1-2 g kg⁻¹ of N (Enders et al., 2012) and above 500 g kg⁻¹ of C (Brewer et al., 2012). On the other extreme, sludge contains considerable amounts of peptidic materials which are transferred to heterocyclic aromatic N compounds during heating (Knicker, 2010).

As indicated by Table 2 in general, the C contents of all BCs were reduced after 24 months at field. This trend was more pronounced in absolute terms for PSB, PWB and MWB. The relative losses of C ranged from 27 % (PSB) to 11 % (KWB) of the initial C. Sorrenti et al (2016) reported losses of total C by 14.5 % for aged BCs, which was partly attributed to mineralization. Beside this, Mukherjee and Zimmerman (2013) measured a significantly high amount of dissolved organic carbon in the soil leachate of BC amended soils. Most likely, such losses were also contributing to the initial fast C loss observed in our study. For SSB, MWB and KWB the C content drastically decreased during the first year and remained statistically unaltered during the second year at field. In contrast to them, the C content of PWB and PSB was gradually reduced throughout the experiment.

Concerning the N content, the effects of weathering were not universal for all BC types. The N content decreased until the 6th month at field for SSB and remained practically constant (around 15-18 g kg⁻¹) afterwards. Similarly, PSB showed no significant alteration of its N contribution. In contrast, wood derived BCs (PWB, KWB and MWB), originally low in N, revealed a significant increase of their N from the harvesting (month 6th) until the end of the experiment. Aside from a relative enrichment caused by preferential C-losses, this could be due to contributions of dead vegetation from abundant weeds, which were incorporated into the pores or adsorbed to the surface of the BC during the 18 months following period. However, alternatively, inorganic N may have been sequestered. Numerous studies have reported increases in organic and inorganic N content of aged BCs (Spokas et al., 2012; Sorrenti et al., 2016; Joseph et al., 2010). The impact of aging has been hypothesized as a critical factor for the interaction of BC with the plant and soil systems, particularly for the sorption of nitrogen containing compounds (Seredych

and Bandosz, 2007; Wang et al., 2012). The pH values of BCs at time 0 depend upon the nature of the feedstock. As expected, pH of wood derived BCs at time 0 was very alkaline (> 9) (Bachmann et al., 2016), whereas the pH of SSB₀ was practically neutral (6.9). In relation with the sludge BC, Agrafioti et al. (2013) reported a pH around 6 for BCs derived from sewage sludge. The pH of all BCs weathered for 24 months except that of SSB, dropped significantly from 9.2-9.4 to 7.7-7.9, which is similar to the pH of the Cambisol. A similar result has been observed in other studies (Joseph et al., 2010; Spokas, 2013; Van Zwieten et al., 2010; Yao et al., 2010). It was suggested that the weathering-induced carboxylic groups lead to a decrease of basic sites on the wood derived BCs (Sorrenti et al., 2016). Alternatively, the leaching of cations from the BC to the soil may have caused the pH-drop. The fast decline of the pH supports that the liming potential of BC is limited to a short time after its application. This was previously observed for the charcoal deposited over the soil after wildfires, but still requires further investigations with respect to BC amendments.

Electrical conductivity (EC) of BC is responsible for exchange of ions with the soil matrix. This is a significant parameter when considering BC as soil ameliorant. All BCs exhibited EC values over $1000 \mu\text{S cm}^{-1}$, excepting for BC SSB that had a lower conductivity ($814.0 \mu\text{S cm}^{-1}$), they are comparable to those obtained by Lehmann (2007) and Liu and Zhang (2012) for analogous samples. The evolution of EC with time in the field showed a sharp decline (final ECs ranged between 74.5 and $150.4 \mu\text{S cm}^{-1}$) which is best explained by leaching of salts and ions (Joseph et al., 2010; Wu et al., 2014). The dissolution and leaching of soluble salts present in the BCs will be among the first reactions, especially after rain events. The initial dissolution of soluble salts (e.g. K and Na carbonates and oxides) may produce a pH increase in

the water-film around BC particles. However, in leaching environments, as occurred in this case, the pH of alkaline chars will decrease as these salts are lost from the system. The magnitude of this decrease is determined by the intensity of the leaching and the acid-buffering capacity of the system.

3.2. Physical fragmentation

As expected, the number of fragments per g of BC increased for all BCs after 24 months at field (Table 2). Nevertheless, the physical fragmentation was not increasing homogeneously and the largest variances were noticed for the last 12 months. In addition, in line with the heterogeneity of the BCs, diverse fragmentation degrees were observed for the different BC types. The KWB showed a better physical preservation (39 ± 5 fragments at month 24), whereas PWB and PSB indicated considerable fragmentation (125 ± 11 and 140 ± 14 fragments g^{-1} BC, respectively). If the number of initial fragments (at time 0) is considered, the relative physical disintegration grade at the end of the experiment can be estimated. In this case, it followed the order $\text{MWB} \geq \text{PSB} > \text{KWB} > \text{PWB} > \text{SSB}$. Nocentini et al. (2010) reported that the initial structure of feedstock is the key factor responsible for the physical fragmentation of BC during pyrolysis. Nevertheless, the role of the properties of the raw material in its subsequent fragmentation in soil is not well-defined yet. The resulting fragmentation is probably a combination of the effects of physical erosion (by wind, temperature, pressure, roots and water-infiltration/precipitation events in the field), microbial degradation and interactions between BC particles and soil components (including for instance SOM, soil minerals and nutrients).

3.3. ^{13}C Solid-State NMR spectroscopy

The ^{13}C NMR spectra and their intensity distribution (in percentages) among the different chemical shift regions for all BCs with time (0, 12 and 24 months at field) are shown in Figure 1 and Table 3 respectively. The ^{13}C NMR spectra of all BCs at t_0 are very similar. They are dominated by an intense and broad signal at 125 ppm corresponding to aryl C (160-90 ppm), which represents over 82% of the total intensity for PSB and KWB. Since tuning of the original PWB and MWB was not possible due to high reflection of the excitation energy, we can assume high aromatic C contents, occurring in strongly condensed aromatic networks. At t_1 , aryl C of PWW contributed with 88% to the total organic C. In contrast, SSB showed the lowest concentration of aryl C (75 %). The small shoulders at 151-153 ppm within the aryl C region for PSB and SSB may be caused by the presence of furfurals or N-heterocyclic aromatic structures (Knicker et al., 2007). The ^{13}C NMR spectra of BCs at t_0 also showed small signals in the regions corresponding to carbonyl-C (245-185 ppm) and alkyl-C (45-0 ppm), which accounted for 1 to 6 % and 6 to 10 %, respectively.

The aging of BCs in the field caused some alterations in the ^{13}C NMR spectra. In general, the relative contribution of aryl C decreased (up to 11%) whereas the alkyl C and O-alkyl C increased. In summary, aged BCs were less aromatic and more functionalized than initial ones. For instance, for SSB at t_2 , the O-alkyl C reached 10 % whereas the alkyl C fraction accounted for 14 %. Similar results were obtained by Zhao et al (2013), who suggested that this is caused by the degradation of aromatic rings in BCs and its partial transformation into linear C alkyls and O alkyls. The increased functionality of aged BCs could be also connected to an alteration of its retention/adsorption capacity with aging (Laird, 2008). Nevertheless, the relative contribution of carboxyl C did not change considerably over time. The fact that the

chemical composition of all BCs is noticeably altered already after a short period of time (only 24 months) in a field under conservation agriculture practise, indicates that BCs degradation and modification occurs on timescales much shorter than hundreds of years.

3.4. Field Emission Scanning Electronic Microscopy (FESEM)

Figure 2 shows FESEM images of the BC samples before and after 24 months of exposure to field conditions. The characteristic morphology and structure of the BC feedstock are clearly observed in the original BCs, in particular their porous structure. The shape and size of the pores are related to the feedstock type and pyrolysis conditions (Downie et al., 2009).

In contrast to the fresh PWB, where macropores ($>13\ \mu\text{m}$) corresponding to hollow fibers are observed (Figure 2A), the aged sample after 24 months (Figure 2B and 2C), depicts great amounts of SOM and microbial materials on its surface. In addition, FESEM images show the presence of entrapped mineral grains into the BC pores and microbial mats (Figure 2B and 2C), probably from soil minerals, as also noticed by Spokas (2013). The FESEM images of PSB (Figure 2D-F) revealed a high amount of collapsed structures, especially from cellulose and lignin, proceeding from the feedstock (paper sludge). Collapsed treachery elements with small pits are particularly evident (Figure 3D). After 24 months, the same collapsed structures are clearly observed (Figure 2E and 2F), usually filled with mineral particles (Figure 2F). This is consistent with soil-exposed BCs as demonstrated by several authors (Spokas, 2013; 2014; Sorrenti et al., 2016), as well as with precipitation reactions within the BC pores. According to Joseph et al. (2010), the precipitation of inorganic compounds in BC pores may occur in soils with minimal leaching during dry-wet

cycles. These processes are enhanced within small pores due to their slow water percolation.

The structure of the SSB sample is different from the rest of the BCs studied. The great variety of constituents from sewage sludge is responsible for the high heterogeneity in morphology and composition observed for this BC (Figure G-I). Both organic and inorganic structures are evident, which can be assigned to mineral grains including clay minerals, microbial mats and filaments (Figure 2G). After 24 months, the same materials are identified together with OM-SOM and microorganisms, such as fungal hyphae (Figure 2H and 2I).

The FESEM images of KWB exhibited a good anatomical preservation of the vineyard wood feedstock, being mainly formed by macropores (Figure 2J). After 24 months of aging, the vineyard wood BC was greatly coated by microbial mats and SOM (Figure 2K and 2L). An organic matrix including microbial structures such as filaments and extracellular polymeric substances fill the pores and coat the surface of this BC. Considering that this BC has the highest water holding capacity and total organic matter content (De la Rosa et al. 2014), a high susceptibility to microbial colonization is consistent for this BC. Hence, KWB seems to offer a suitable habitat for the development of microorganisms (Figure 2K and 2L).

3.5. Respiration experiment under laboratory conditions

Soil organic carbon (SOC) is usually divided into labile C with a small size and rapid turnover and recalcitrant C with slow turnover (Schmidt et al., 2011). The analysis of the biochemical degradability via respiration experiments revealed that the soils amended with wood-BCs contained comparable proportions of labile organic carbon (OC); A1 ranged between 2.7 % (KWB) and 2.9 % (MWB) (Table 4). These data are

397 in agreement with the calculations performed by Wang et al. (2016), which estimated
398 in a meta-analysis that only about 3 % of the BC carbon amounts the labile pool. In
399 contrast, the quickly mineralized C pool was considerably higher for SSB. With 12.5
400 % of the total OC, it is comparable to that measured for the bulk soil without
401 amendment (15.9 %). The mean residence times of the labile fraction (MRT_1) for the
402 BC-soil mixtures were very short (0.020-0.061 years). Knicker et al (2013a) reported
403 similar MRT_1 values in fire-affected soils from SW Spain. However, here one has to
404 bear in mind that not only the degradation of labile organic compounds contributes to
405 the fast cycling pool, but also the release of CO_2 captured in the soil and in the BC
406 (Potter, 1908). Biochemically labile residues formed during the thermal break down of
407 macromolecules during pyrolysis are unlikely to contribute since they should have
408 been already metabolized during the field study. Alternatively, organic matter
409 accumulated within the BC and residues of soil microorganisms that died during
410 drying of the soil samples are more likely candidates to explain the quickly
411 mineralizable C pool. However, for our objective, the relative content of the slowly-
412 degrading OC and its mean residence time (A_2 and MRT_2 respectively) are more
413 informative for the long-term stability of BCs. All the BCs show elevated A_2 , ranging
414 from 87.5 (SSB) to 98.6 % (PSB). The wood derived BCs display MRT_2 in the range
415 from 19.3 (MWB) to 43.9 years (PWB), which is only 3.4 to 7.7 times longer than the
416 MRT_2 of the unamended Cambisol. Major differences were not observed between the
417 latter and the MRT_2 of the SSB amended soil. Thus, our results indicate that BC
418 addition did not or did only slightly increase the MRT_2 of the SOM of the studied soil.
419 As a consequence one has to assume that MRT_2 of aged BCs are in the range of
420 humified SOM, as it was recently also observed for aged charcoal residues in fire-
421 affected soils (Knicker et al. 2013b). This may be shorter than those reported from

other studies (Kwzyakov et al., 2009; Singh et al., 2012; Wang et al., 2016), claiming MRTs of BC in the range of hundreds of years, but it is still slower than MRTs expected during degradation of fresh plant litter. Since the organic matter of the latter is mostly part of the fast turning pool, that of the BC is almost completely composed of biochemically more resistant components. Thus, with its application to soil, its slow C pool is directly refilled without prior CO₂-losses due to fast mineralization.

Several laboratory studies conducted at temperatures above 30 °C concluded that BC degradation is accelerated with increasing incubation temperature (Cheng and Lehmann 2009; Zimmermann et al., 2012). Bearing in mind that the temperature of 25 °C applied during the incubation experiment is very close to the average temperature of Seville province, which exceeds 32 °C over 70 days per year (www.aemet.es), deceleration of BCs degradation by lower temperatures should not be relevant in the field. Another factor which may have contributed to the relatively short MRT₂ of BC in our study compared to those reported in the literature, could be the low fertility of the used soils. It is well-known that BC addition stimulates microbial activities and consequently organic matter mineralization in low-fertility sandy soils (Wang et al., 2016). One can hypothesize that the addition of nutrients with the BC did not only increase the growth of the plants but also that of the microbial community (De la Rosa et al., 2014). Their enhanced activity, on the other hand may have also accelerated BC degradation.

4. Conclusions

Our results proved that even under the conservative management applied to Calcic Cambisols with low C contents in the Mediterranean region, BC amended to the soils, suffers physical and chemical degradation which is notable within two years of

experiment time. The fact that BC addition did only slightly increase the MRT of the slow-turning C pool in the studied soil may question their longevity in the studied environment. However, BC addition increases the slow C pool, composed of humified organic matter, without prior elevated CO₂-production as it would be the case for fresh plant residues. Therefore BC amendment still can be a valuable tool for an efficient long-term elevation of organic C contents in C-poor soils. A second observation of our study revealed that in contrast to artificial chemical weathering, carboxylation of BC did not represent a major process during aging at field. Thus, we have to assume that either nutrient adsorption will not increase with aging at field or functional groups other than carboxyl C are responsible for the process.

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661

662 **FIGURE CAPTIONS:**

663

664 Figure 1. Solid-state ^{13}C NMR spectra of the studied BCs taken from the Calcic
665 Cambisol after 12-months aging.

666

667 Figure 2. FESEM images of the studied BCs before and after 24-month aging: A-C)
668 PWB; D-F) PSB; G-I) SSB, and J-L) KWB.

669

Table 1. Details of biochar samples

Sample (label)	PWB	PSB	SSB	KWB	MWB
Feedstock	Pine wood sievings	Paper sludge & wheat husks	Sewage sludge	Vineyard wood (>1 year old)	Mixed wood chips
Maximum Pyrolysis Temperature (°C)	620	500	600	Unknown	450-600
Duration of pyrolysis (min)	20	20	20	Unknown (>120 min)	20-30
Producer and pyrolysis system	Swiss biochar (Switzerland); PYREG® 500	Sonnenerde GmbH (Austria); PYREG® 500	Pyreg (Germany); PYREG® 500	Bodegas Torres (Spain); Traditional kiln-method	Swiss biochar (Switzerland); PYREG® 500
Conditioning and storage	Quenching with water to 30%; dried 40 °C; stored at 5 °C	Quenching with water to 30%; dried 40 °C; stored at 5 °C	Quenching with water to 30%; dried 40 °C; stored at 5 °C	Dried 40 °C; stored at 5 °C	Quenching with water to 30%; dried 40 °C; stored at 5 °C
Average particle size and shape	8 mm; elongate-not homogeneous	12 mm; rounded	5 mm; granulated (spherical)	5-20 mm; very irregular- not homogeneous	10 mm; elongate-not homogeneous

Table 2
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Table 2. Evolution of elemental composition (C, N) and basic properties of biochars with time at field							
Biochar label (feedstock)	Time at field (months)	TC (g kg ⁻¹)	TN (g Kg ⁻¹)	C/N	pH	Electrical conductivity (μS cm ⁻¹)	Physical fragmentation (number of fragments per gram of biochar)
PWB (Pine wood sievings)	0	847.7 ± 2.0	1.8 ± 0.3	471	9.4 ± 0.2	1187.7 ± 56.6	75 ± 4
	6	751.3 ± 9.2	1.5 ± 0.4	501	8.7 ± 0.2	1181.5 ± 50.1	81 ± 9
	12	723.7 ± 3.4	1.4 ± 0.7	517	7.9 ± 0.1	1179.7 ± 57.7	77 ± 8
	24	679.4 ± 2.6	2.3 ± 0.4	295	7.9 ± 0.1	101.3 ± 4.2	125 ± 11
PSB (Paper sludge & wheat husks)	0	475.4 ± 4.3	12.3 ± 0.1	39	9.2 ± 0.3	1045.7 ± 13.7	46 ± 4
	6	461.3 ± 6.3	13.1 ± 0.7	35	8.6 ± 0.1	989 ± 25.5	49 ± 5
	12	419.8 ± 10.5	14.2 ± 0.6	30	8.0 ± 0.1	190.5 ± 5.1	90 ± 7
	24	349.1 ± 4.8	13.4 ± 0.5	27	7.8 ± 0.1	139.5 ± 1.7	140 ± 14
SSB (Sewage sludge)	0	182.0 ± 2.1	20.0 ± 1.4	9	6.9 ± 0.1	814.0 ± 34.4	75 ± 6
	6	149.0 ± 14.1	15.1 ± 0.9	10	7.1 ± 0.1	801.2 ± 12.9	83 ± 7
	12	153.3 ± 2.4	18.4 ± 0.8	8	7.7 ± 0.1	150.4 ± 2.5	80 ± 6
	24	154.4 ± 3.1	15.9 ± 0.3	10	7.7 ± 0.1	117.3 ± 0.3	102 ± 6
KWB (old Vineyard wood-in kiln)	0	764.0 ± 10.4	1.9 ± 0.1	402	9.3 ± 0.2	1082.3 ± 2.9	16 ± 4
	6	752.6 ± 13.4	1.7 ± 0.2	443	8.9 ± 0.1	1079.9 ± 25.8	17 ± 3
	12	644.2 ± 15.5	3.0 ± 0.6	215	8.1 ± 0.1	112.0 ± 1.3	20 ± 3
	24	676.8 ± 17.1	3.5 ± 1.0	193	7.8 ± 0.1	74.5 ± 0.9	39 ± 5
MWB (Mixed wood chips)	0	829.4 ± 5.3	1.8 ± 0.1	461	9.3 ± 0.2	1096.7 ± 5.1	24 ± 6
	6	721.6 ± 9.3	1.7 ± 0.2	425	8.7 ± 0.1	1070.1 ± 8.5	29 ± 6
	12	718.0 ± 4.9	2.1 ± 0.3	342	8.1 ± 0.1	222.7 ± 3.8	44 ± 3
	24	707.0 ± 6.5	2.2 ± 0.3	321	7.8 ± 0.1	93.3 ± 4.4	80 ± 12

Table 3. Assessment (%) of the different C types as seen by ¹³C NMR spectroscopy integration regions.

Sample	Aldehyde & ketone	Carboxyl & amide	Aromatic	O-alkyl	N-Alkyl & Methoxyl	Alkyl C
	245-185 ppm	185-160 ppm	160-100 ppm	100-60 ppm	60-45 ppm	45-0 ppm
PWB (t ₀)	-	-	-	-	-	-
PWB (t ₁)	4	3	88	3	1	2
PWB (t ₂)	9	4	77	3	2	5
PSB (t ₀)	6	3	82	2	1	6
PSB (t ₁)	5	4	80	4	2	7
PSB (t ₂)	3	2	77	7	3	8
SSB (t ₀)	5	4	75	5	2	9
SSB (t ₁)	4	4	64	9	4	14
SSB (t ₂)	5	4	64	10	4	14
KWB (t ₀)	1	1	82	2	3	10
KWB (t ₁)	5	3	80	6	2	5
KWB (t ₂)	4	2	75	4	2	13
MWB (t ₀)	-	-	-	-	-	-
MWB (t ₁)	5	3	79	6	2	5
MWB (t ₂)	4	2	76	5	3	10

t₀: 0 months at field; t₁: 12 months at field; t₂: 24 months at field. -: not analyzed

Table 4. Parameters calculated by the incubation-respiration experiment. Percentages of CO₂ released by the fast (A₁) and slow (A₂) organic C pools, degradation rate constants (k₁ and k₂) and their respective mean residence times (MRT₁, MRT₂) for each biochar-soil mixture and for the unamended Cambisol (control).

Sample	Fast (labile) C pool			Slow (recalcitrant) C pool			MRT _{2-BC} / MRT _{2-control}
	A ₁ (% OC)	K ₁ (y ⁻¹)	MRT ₁ (years)	A ₂ (% OC)	K ₂ (y ⁻¹)	MRT ₂ (years)	
Pure Cambisol (control)	15.9	12.3	5.7	84.1	0.12	5.7	--
PWB	2.7	11.4	0.061	97.3	0.016	43.9	7.7
PSB	1.4	22.8	0.044	98.6	0.099	10.1	1.8
SSB	12.5	35.0	0.020	87.5	0.088	7.9	1.4
KWB	2.7	32.4	0.031	97.3	0.016	32.4	5.7
MWB	2.9	19.3	0.052	97.1	0.050	19.3	3.4

Figure 1
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Figure 2
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