Universitat Autònoma de Barcelona

Effects of applying pine and corn cobs biochar on soil organic carbon in a Mediterranean agricultural land

Irene Raya Moreno

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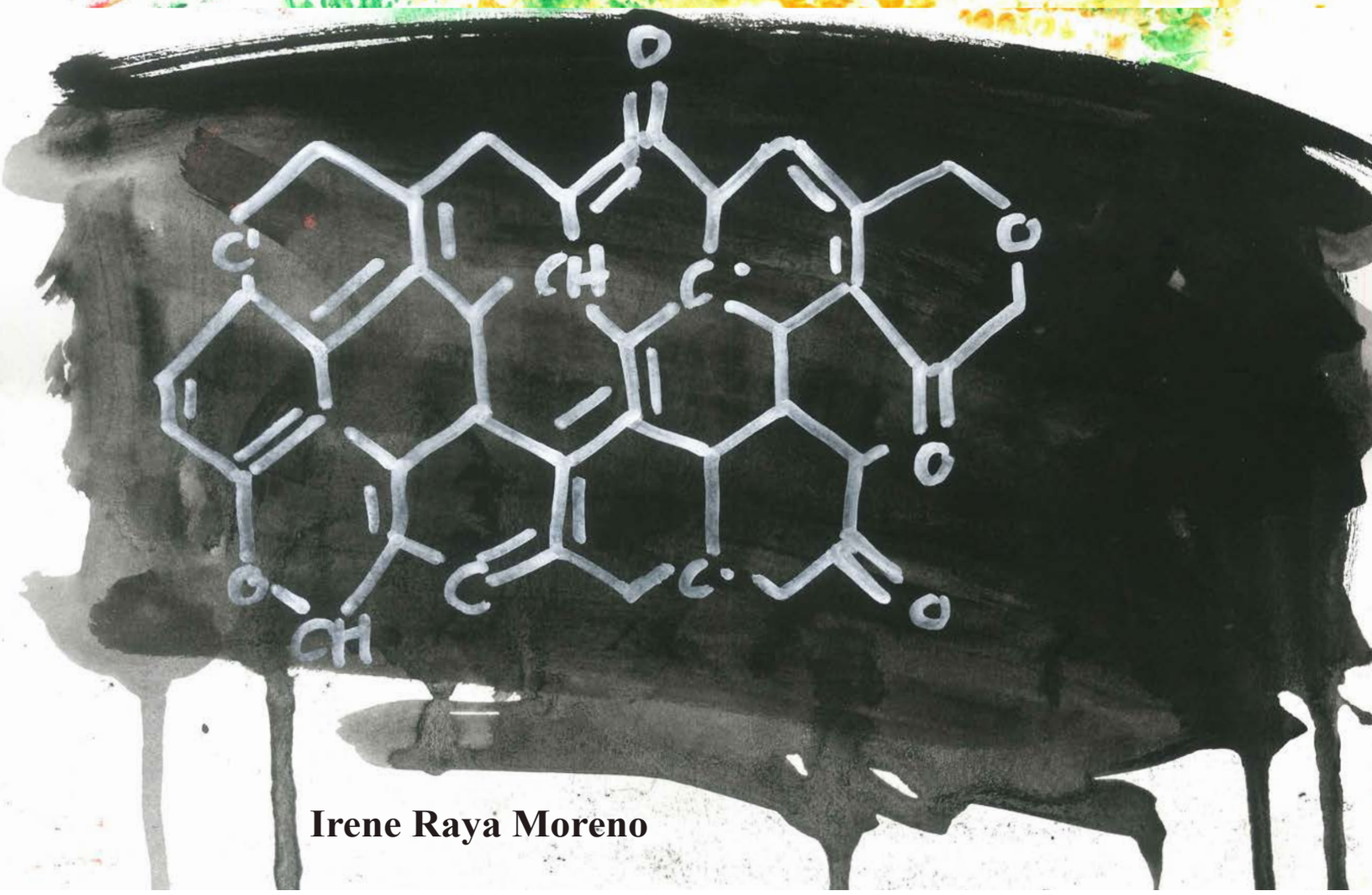

Irene Raya Moreno

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Efectes de l'aplicació de biochar de pi i de blat de moro en el carboni orgànic d'un sòl agrícola Mediterrani

Memòria presentada per Irene Raya per optar al títol de doctor per la Universitat Autònoma de Barcelona

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A mis padres y hermana

"El aspecto más triste de la vida en este preciso momento es que la ciencia reúne el conocimiento más rápido de lo que la sociedad reúne la sabiduría."

Isaac Asimov

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Abstract

The increment of global threats due to climate change, caused by an increase in atmospheric concentration of GHGs, is predicted to have a severe impact on our planet. The use of biochar, obtained from the thermochemical conversion of biomass in an oxygen-limited environment, as a soil amendment has been proposed as one strategy for C-sequestration. Many environmental benefits have been attributed to the application of biochar into soil, including long-term C-sequestration compensating for CO2 emissions. However, biochar effectiveness still remains under debate because effects can vary greatly depending on biochar and soil type. The main objective of this thesis was to assess the effects of two contrasting biochars, from pine wood (PB) and corn cob (ZB) remains, incorporated at a rate of 6.5 g kg-1 on a sandy loam vineyard soil with neutral pH and low organic carbon (OC) content, in field conditions over two years. Specifically, the aims were to evaluate the consequences of the addition of the different biochars on: 1) soil OC resistance to thermochemical processes (Chapter 1 and Chapter 2); 2) the potential OC availability to be mineralized by soil microorganisms (Chapter 3); and 3) physical OC protection by the promotion of aggregates (Chapter 4).

The analytical methods used to evaluate the effects of biochar in soil OC-resistance were: weight loss-on-ignition (LOI), dry-combustion (TOC), strong (sO) and mild (mO) acid potassium dichromate oxidations, acid hydrolysis (AH), peroxide-oxidation (PO) and isotope analysis. Moreover, soil and biochar resistant-OC (ROC) was estimated through a mass balance. Also, soil field samples were collected at the short- and the medium-term (2 and 26 months after the application, respectively), and then incubated in the lab for 250 additional days. The CO2-C released as soil respiration and the CO2-C isotopic signature were assessed after 30 and 250 days of the incubation. Additionally, dissolved-OC was assessed in the field soil samples by hot-water extraction. Regarding physical properties, water-stable aggregates and particulate fraction weight were determined using a wet-sieving apparatus, using distilled water or hexametaphosphate for aggregates disruption. Oxidisable and resistant OC (attributed mainly to native soil and biochar, respectively) inside and outside of aggregates was estimated through a mass balance using mO and TOC. On the other hand, native soil and biochar-OC contribution in ZB biochar-amended soil was estimated by isotope analysis.

The ROC estimated by AH and mO led to similar values in control soil (5 g C kg-1 soil), whereas higher ROC values were obtained in biochar-amended ones (6-12 g C kg-1 soil). Moreover, qualitative biochar detection was achieved by comparing δ13C in amended and non-amended soils regardless of the biochar feedstock origin. However, 35% of ZB biochar-OC was apparently lost over two years, which was attributed to biochar dilution into soil. In addition, in the short-term, negative-priming was observed in amended-soil with PB (made at high temperature) whereas positive-priming was seen in those amended with ZB (produced at lower temperatures) as a result of the highest labile-OC content in ZB biochar compared to PB. However, in the medium-term, slightly negative-priming effects in both biochar-amended soils were found. This could be explained by promotion of physical protection processes preventing priming. This fact was corroborated as higher TOC and BOC amount was observed inside of aggregates in biochar-amended soils compare to controls. It seems that PB tended to be incorporated into aggregates while ZB promoted native soil-OC occlusion. Then, after labile-OC has been exhausted, the promotion of OC occlusion prevented further losses. Therefore, the application of biochar to a Mediterranean agricultural soil increases soil-OC persistence due to innate biochar-OC resistance and OC physical protection, which decrease OC degradation by abiotic and biotic agents.

Resum

El canvi climàtic, produït per l'increment de la concentració de gasos d'efecte hivernacle a l'atmosfera, amenaça la integritat del nostre planeta. En aquestes circumstàncies el biochar, material obtingut a partir de biomassa pirolitzada, s'ha proposat com a una possible mesura per augmentar el segrest de carboni en sòls. L'aplicació de biochar en sòls pot servir com a magatzem de carboni a llarg termini compensant les emissions de CO2. No obstant, l'eficàcia del biochar depèn del tipus de biochar i sòl utilitzats. L'objectiu principal d'aquesta tesi és avaluar els efectes de l'aplicació de biochars de pi (PB) i de blat de moro (ZB) a una dosi de 6.5 g kg-1 en un sòl franco-arenós amb pH neutre i baix contingut de carboni orgànic (CO) en condicions de camp durant dos anys. Els objectius específics són els efectes de l'aplicació de biochar en : 1) la resistència termoquímica del CO del sòl (Capítol 1 i Capítol 2); 2) la disponibilitat del CO pels microorganismes (Capítol 3); 3) la protecció física del CO per oclusió en els agregats (Capítol 4).

Els mètodes utilitzats per estudiar els efectes del biochar sobre la resistència del CO van ser: pèrdua de pes per ignició (LOI), combustió-seca (TOC), oxidació forta (sO) i feble (mO) amb dicromat-potàssic, hidròlisis-àcida (AH), oxidació amb peròxid d'hidrogen (PO) i anàlisis-isotòpic. A més, el CO-resistent del sòl i del biochar es va estimar mitjançant un balanç de masses. També, es van dur a terme dos mostrejos de sòl a curt i llarg termini (2 i 26 mesos), i es van incubar durant 250 dies. El dia 30 i 250 d'incubació va ser determinada la quantitat i la senyal isotòpica del CO2-C respirat. Addicionalment, es va mesurar el CO-dissolt en les mostres de sòl mitjançant el mètode d'extracció amb aigua-calenta. Les propietats físiques van ser avaluades quantificant el pes dels agregats estables amb aigua destil·lada i de la fracció en partícules amb hexametafosfat (per la disgregació dels agregats) utilitzant el wet-sieving apparatus. A més, el CO procedent del sòl natiu i del biochar dins i fora dels agregats es va estimar mitjançant un balanç de masses utilitzant el mO i el TOC. També es va estimar la contribució de CO del sòl i del biochar en els sòls esmenats amb ZB utilitzant el anàlisis-isotòpic. Es van trobar quantitats de ROC similars en els sòls controls estimats mitjançant mO i AH (5 g CO kg-1), mentre que més contingut de ROC es va observar en els sòls esmenats (6-12 g CO kg-1). La presencia de biochar es va detectar en els sòls esmenats mitjançant la comparació del δ13C en sòls esmenats i no-esmenats, independentment de l'origen del biochar. D'altra banda, el 35% del CO del biochar de

ZB dels sòls esmenats es va perdre en dos anys com a resultat de la dissolució del biochar en el sòl. A curt termini, es va observar un priming-negatiu en sòls esmenats amb PB i el contrari en sòls esmenats amb ZB, en resposta al major contingut de CO-làbil del ZB. No obstant, un lleuger priming negatiu es va observar en els dos sòls esmenats a mig termini ja que augmenta la protecció física del CO. Mentre el PB tendeix a ser incorporat en els agregats, el ZB promou l'oclusió del CO natiu del sòl. Al esgotar-se el CO-làbil, el CO queda protegit dins dels agregats. Per tant, l'aplicació del biochar en sòls agrícoles mediterranis augmenta la persistència del CO en el sòl com a resultat de la resistència innata del CO del biochar i la protecció física augmentant el contingut de CO dins dels agregats.

Resumen

El incremento de gases de efecto invernadero en la atmosfera puede tener consecuencias severas para nuestro planeta. El uso de biochar como enmienda, material obtenido a partir de biomasa pirolizada, se ha propuesto como estrategia para el secuestro de carbono en el suelo. Sin embargo, la efectividad del biochar varía mucho dependiendo del biochar y el tipo de suelo. El objetivo principal de esta tesis es evaluar los efectos de dos biochares, de restos de pino (PB) y mazorca de maíz (ZB), incorporados a una dosis de 6.5 g kg-1 en un suelo de viña franco-arenosa con pH neutro y bajo contenido de carbono orgánico (CO), en condiciones de campo durante dos años. Los objetivos específicos fueron la evaluación de: 1) la resistencia del CO en el suelo a los procesos termoquímicos (Capítulo 1 y Capítulo 2); 2) la disponibilidad de CO a ser mineralizada por microorganismos del suelo (Capítulo 3); y 3) protección física de CO por aumento de agregados (Capítulo 4).

Los métodos analíticos utilizados para evaluar los efectos del biochar en el CO resistente del suelo fueron: pérdida de peso por ignición (LOI), combustión-seca (TOC), oxidación fuerte (sO) y suave (mO) con dicromato potásico, hidrólisis-ácida (AH), oxidación con peróxido de hidrogeno (PO) y análisis isotópico. Además, se estimó el CO-resistente del suelo y del biochar a través de un balance de masas. Por otro lado, el suelo se muestreó a corto y medio plazo (2 y 26 meses) y las muestras se incubaron en el laboratorio durante 250 días. Se determinó el CO2-C liberado durante la respiración del suelo y la señal isotópica del día 30 y 250 de incubación. Además, se cuantificó el CO disuelto mediante un extracto con agua caliente. Para evaluar las propiedades físicas, se determinaron los agregados estables en agua destilada y el peso de la fracción particulada con hexametafosfato para la disrupción de los agregados usando el wet-sieving apparatus. El CO oxidable del suelo nativo y del biochar dentro y fuera de los agregados se estimó a través de un balance de masas usando mO y TOC. Por otro lado, mediante el análisis isotópico se estimó la contribución de CO del suelo nativo y del biochar en suelos enmendados con ZB.

Se cuantificaron valores similares de ROC en los suelos control mediante AH y mO (5 g C kg-1), mientras que se obtuvieron valores de ROC más altos en los suelos enmendados con biochar (6-12 g C kg-1). Además, la detección cualitativa de biochar se logró comparando δ13C en suelos enmendados y controles, independientemente del origen del biochar. Sin embargo, el 35% de ZB-CO se perdió durante los dos años de

experimento por dilución del biochar en el suelo. A corto plazo se observó un priming-negativo en suelos enmendados con PB y al contrario en los suelos con ZB debido al mayor contenido de CO-lábil en ZB comparado con PB. Sin embargo, se encontró un priming ligeramente negativo a medio plazo en ambos suelos enmendados con biochar, como consecuencia de una mayor protección física del CO. Mayores cantidades de TOC y BOC se encontraron en los agregados de los suelos enmendados aunque tuvieron lugar dos procesos diferentes, mientras el PB tiende a incorporarse en agregados el ZB promueve la oclusión del CO del suelo nativo. Al agotarse el CO-lábil, el CO-ocluido queda protegido previniendo las pérdidas adicionales por degradación. Por lo tanto, la aplicación de biochar a un suelo agrícola mediterráneo aumenta la persistencia del CO del suelo debido a la resistencia innata al biochar-CO y la protección física del CO, que previene la degradación biótica o abiótica del CO.

Contents

List of abbreviations

AH	Acid hydrolysis (HCl, 100ºC, 17 h)
AHR	Acid hydrolysis-resistant Residue
BOC	Biochar Organic Carbon
C	Carbon
CEC	Cation-Exchange Capacity
DOC_{hw}	Dissolved Organic Carbon estimated by hot water method
IC	Inorganic Carbon
LOI	Loss-On-Ignition (375 ºC, 550 ºC, 950ºC)
LPO	Loss-On-Peroxide-hydrogen oxidation
mO	Mild potassium dichromate Oxidation (K2Cr2O7, 60 ºC, 8 h)
MWD	Mean Weight Diameter
O	Oxigen
OC	Organic Carbon
P	Pine splinter biomass
PB	Pine Biochar
PE	Priming Effect
PO	Hydrogen Peroxide Oxidation (method)
POR	Hydrogen Peroxide Oxidation-resistant Residue
ROC	Resistant Organic Carbon
S	Control soil (with no-biochar)
S+PB	Soil amended with Pine Biochar
S+ZB	Soil amended with Corn Cob Biochar
sO	Strong potassium dichromate Oxidation (K2Cr2O7, 150 ºC, 10 min)
SOC	Soil Organic Carbon
SOM	Soil Organic Matter
TC	Total Carbon
TOC	Total Organic Carbon
Z	Corn cobs biomass
ZB	Corn Cobb Biochar
$\delta^{13}C$	C isotopic signature

SI prefixes

μ micro ($x10^{-6}$)

m milli ($x10^{-3}$)

c centi ($x10^{-2}$)

d deci ($x10^{-1}$)

k kilo ($x10^{-3}$)

M mega ($x\text{-}10^{6}$)

General introduction

Climate change and agrarian ecosystems

One of the biggest environmental challenges at the present is climate change. Since the Industrial Revolution, global atmospheric concentrations of greenhouse gases (GHGs) have increased as a result of human activities (IPCC, 2014). In particular, land use changes such as the conversion of natural to agricultural ecosystems, biomass burning, deforestation, drainage of wetlands and soil cultivation are considered important sources of atmospheric CO_2 (Lal, 2004). Moreover, the combination of human activities in soil (overcultivation, overgrazing and deforestation) and climatic variations (prolonged droughts and floods) in arid, semi-arid, and dry sub-humid areas could cause land degradation. Soil degradation is a gradual process of soil productivity loss and thinning out of the vegetative cover. If it is prolonged over time, topsoil can be eroded in just a few seasons, with detrimental effects on all soil properties. This process, accentuated by human activities, is called desertification, and it can take centuries to build up and recover the soil, with its associated properties, after it has reached a desertification state (United Nations, 2018). Mediterranean ecosystems are considered one of the most vulnerable to suffer desertification in Europe, due to adverse climatic conditions, long soil exploitation periods and a high population in relation to soil production capacity (Metzger et al., 2006). The increment of global threats, such as soil degradation as a result of human bad practices, and climate change caused by an increase in atmospheric concentration of GHGs, is predicted to have a catastrophic impact on our planet (IPCC, 2014) and has thus motivated research about mitigation solutions. A coordinated effort should be carried out at individual, local and global scales to achieve the required improvements. Moreover, it is likely that multiple actions will have to be implemented to have large enough repercussions for mitigation work. For example, according to Lugato et al. (2014), potential soil organic carbon (SOC) sequestration of 101-336 Mt CO_2 eq. and 549-2141 Mt CO_2 eq. could be achieved by 2020 and 2100, respectively, if alternative management practices are implemented on 12 to 28% of European arable land. These alternatives would include the conversion of arable land to grassland, reduced tillage, straw incorporation combined with reduced tillage, ley cropping system and the use of cover crops. Similarly, Sperow et al. (2003) estimated that 60-70 Tg C yr^{-1} could be sequestered in U.S. cropland soils if some different management practices such as no-till, decreased fallow operations, conversion of highly erodible land to grassland, and increased use of

cover crops in annual cropping systems were applied. Moreover, the addition of organic C to soil is recommended as a way to sequester C and improve soil fertility. However, the success of soil amendment in improving carbon storage will depend on the properties of the amendment, soil type and environmental conditions. Specifically, one approach is the use of biochar as a global strategy for C-sequestration and environmental management.

Biochar origin and characterization

The study of biochar, the charcoal made from the pyrolysis of diverse biomass and intended for soil application, began after the discovery of Amazonian dark earths named '*Terra preta'*. The formation of *Terra preta* is attributed mainly to the accumulation of cooking waste (mostly coal and ash, but also fish and mammals bounds) from pre-Columbian inhabitants at Upper Xingu region and central Amazonian (the ancient one is dated 2500 year BP). Therefore, this is a man-made soil, distributed in heterogeneous patches, representing 10% of the Amazonian area (Glaser and Birk, 2012). The majority of Amazonian ecosystems are characterized by low fertility and high risk to soil degradation as a result of overexploitation. Conversely, *Terra preta* is considered a model of sustainable agriculture in the humid tropics, as it is suitable for agriculture and contains large carbon stocks and high nutrient levels. Moreover, *Terra preta* shows better soil properties of pH, nutrient content, nutrient holding capacity and soil organic matter (SOM) stability compared to surrounding soils. Most of these benefits are associated with the high amounts of pyrolysed biomass (char) found in *Terra preta* soils (Glaser and Birk, 2012). When this material is produced and used as a soil amendment it is referred to as biochar, which is a carbon rich product highly resistant to decomposition, originated from biomass lysis in a poor oxygen atmosphere.

In the last decades, several authors have described many environmental benefits attributed to the application of biochar into soil, including the enhancement of nutrient retention, pH, cation exchange capacity (Glaser et al., 2002; Lehmann et al., 2006), soil aggregate stability (Li et al., 2017), bulk density (Suliman et al., 2017), water holding capacity (Blanco-Canqui, 2017; Mukherjee and Lal, 2013), and long-term C sequestration in soil (Shackley et al., 2013). Increased C sequestration can act to compensate for CO_2 emissions (Fang et al., 2014) and thus play a key role in a global C-negative strategy (Cheng et al., 2006; Qin et al., 2016), especially as biochar can persist in soil on a millennial time scale (Cope and Chaloner, 1980; Forbes et al., 2006; Patterson et al., 1987). In addition, the estimated global potential of biochar for annual sequestration of

atmospheric CO_2 is 10^9 t yr^{-1} within 30 years (Sohi et al., 2009). However, the importance of biochar as a carbon storage strategy still remains under debate because it depends on many factors such as soil type (Bird et al., 1999; Lützow et al., 2006), climate, the original feedstock (Fang et al., 2014) and even the pyrolysis temperature used (Singh et al., 2012). Moreover, inconclusive results of biochar contribution to climate change mitigation have so far been found in all locations and for all types of biochar. Also, the verification of biochar suitability as a soil amendment is required through extensive research based on detailed carbon balance and economic analyses taking into account biochar production, transport and field application. However, there are relatively few studies that make a quantitative assessment of biochar-based soil management scenarios with regard to energy, greenhouse gases, and economic perspectives (Sohi et al., 2009).

Production and properties of biochar

Three groups of products are obtained during the pyrolysis process for biochar production: gases, solids, and liquids. Pyrolysis gases comprise a flammable mixture (syngas) suitable for generating energy through heat, while liquids can be used as a combustion fuel (Mohan et al., 2006; Wang et al., 2013) or as raw materials for other chemicals. Finally, the solid product, named biochar when used as a soil amendment, is composed mostly of highly condensed aromatic C (65-90%), mineral compounds (ash) and volatile matter which is principally aromatic-aliphatic removed as smoke during burning, although some is retained and adsorbed into the solid matrix (Krull et al., 2009). The proportion of the resulting liquids, biochar and gases depends on the biomass origin and the conditions during the pyrolysis process (Table 1).

Table 1 Typical product yields (dry basis) for different modes of pyrolysis (Brown, 2012)

Pyrolysis Mode	Temperature (ºC)	Vapor residence time	Liquids (%)	Biochar (%)	Gases (%)
Fast	~500	Short~1 sec	75	12	13
Moderate	~500	Moderate~10-20 sec	50	20	30
Slow	~500	Long~5-30 min	30	35	35
Gasification	>750	Moderate~10-20 sec	5	10	85

The properties of biochar will depend on the feedstock original structure, mineral content (ash) and molecular composition (e.g. hemicelluloses, cellulose and lignin), as all of these substances have different levels of thermostability (Joseph et al., 2012; Krull et al., 2009; Sjöström, 1993). Moreover, the treatment temperature during the pyrolysis will be decisive for biochar properties, because higher temperature during pyrolysis process will result in biochars with greater surface area and a higher level of molecular structure organization, having more hydrophobicity and lower biochar particle size. The unit of carbon organization is denominated crystallite and is composed of graphite-like molecules (Downie et al., 2009). With increasing temperature the stacking of these rings becomes more ordered, from a largely amorphous mass to increasingly conjugated sheets, whereas the greatest degree of ordination (Figure 1) and stacking represents what is called graphite. A high degree of organization is attributed to carbon aromaticity (associated with low H/C ratio) and to physicochemical resistance of biochar. In fact, molar H/C ratios of <0.5 have been used to define biochar, although this number cannot be applied to all biochar, as higher H/C values of burning residues have been described. Also, other influencing factors of the pyrolysis process are the heating rate, pressure and reaction or residence time, which will have an impact on the porosity and the functional groups on the biochar surface. The porosity depends on the ease with which gases can be released from the biochar matrix during pyrolysis, as they can be trapped inside and later solidify, reducing the pore diameter (Downie et al., 2009; Joseph et al., 2012). Therefore, prediction of biochar properties and its classification is very difficult because there is a high number of possible combinations of both feedstock and production types that will define biochar properties.

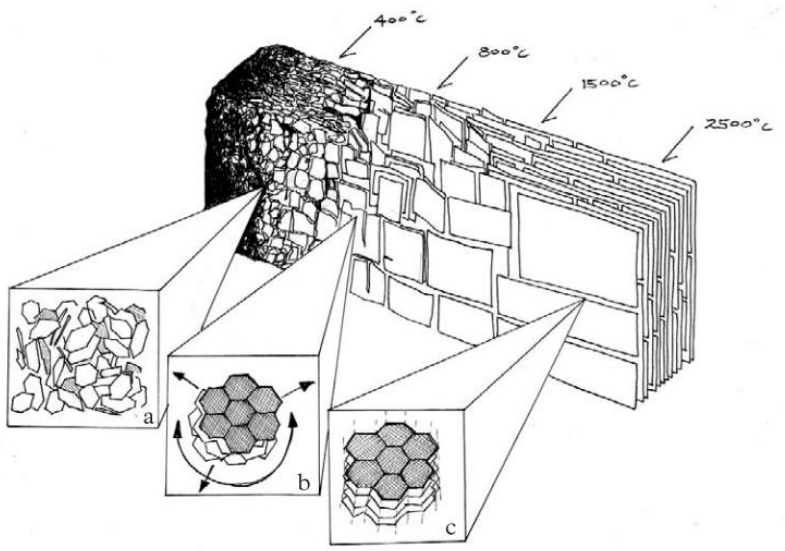

Figure 1 Ideal biochar structure development with rising pyrolysis temperature (Downie et al., 2009)

A simple classification system that includes the type of feedstock and the process conditions has not been yet achieved. However, four main properties have been suggested to characterize biochar, which are: i) percentage of total carbon and volatile content, ii) mineral, nitrogen and sulfur content, iii) surface area and pore-size distribution and, iv) cation-exchange capacity (CEC) and functional groups content (Joseph et al., 2012). Much information about biochar properties and predicted effects of biochar in soil can be extracted from this data. For example, carbon and ash percentage provides a good indication about biochar biomass composition and pyrolysis process conditions. Furthermore, labile carbon which is a very small proportion of biochar is associated with available C for microorganisms that can be easily incorporated into soil microbial biomass, however, some of these C compounds could be toxic, preventing germination or plants growth. Therefore, it is important to define the labile C composition of biochar. Inorganic compounds (mineral, nitrogen and sulphur) might improve crop yields because they are associated with nutrient retention. However, in some cases, negative effects have been attributed to the biochar mineral content such as an increment of leaching substances in some soils as a result of the breakdown of humic and fluvic acid compounds. Otherwise, biochar surface area is positively correlated with pore content, which is a beneficial property in most of soils. While macropores increase water holding capacity, as water can be transported and retained in biochar surfaces, micropores are associated

with adsorption of gases. However, the role of pore distribution in gas-liquid-solid reactions has not yet been reported. In addition, biochar surface charge and total concentration of functional groups is associated with greater CEC which increases interactions with soil particles, water, dissolved organic matter, gases and microorganisms. Ash-rich biochar has more functional groups (carboxylic, phenolic, hydroxyl, carbonyl or quinine C forms), which could be helpful in assisting plant growth by cation retention. Also, the biochar surface could be oxidised by the aging process, increasing functional groups and CEC.

Biochar interaction with native soil organic carbon

One of the most relevant properties of biochar is its chemical stability because it is rich in aromatic C structures (Baldock and Smernik, 2002; Calvelo Pereira et al., 2011; Downie et al., 2009; Singh et al., 2012), and highly resistant to microbial decomposition (Shindo, 1991; Cheng et al., 2008). However, biochar contains a minor labile organic fraction that consist mostly of carbohydrates, proteins and fatty acids that have not been completely charred (Downie et al., 2009). This minor organic fraction alone might not contribute to efficient C sequestration in soil due to its fast turnover rates, although it may play a key role in soil structure owing to physical protection processes that might increase its residence time in soil (Plaza et al., 2016). Thus, carbon sequestration in biochar-amended soils will depend on biochar innate chemical stability (carbon resistance), physical localization (carbon protection) and its availability to be decomposed by microorganisms (carbon availability). Therefore, the resistance of biochar against decomposition could be assessed by proxy methods based on chemical, physical and biological measurements (Strosser, 2010; von Lützow et al., 2007).

Mechanisms for carbon stabilization in soil

There are several processes that explains organic carbon persistence in soils:

a) Carbon resistance

Various attempts have been made to study resistant organic carbon in biochar amended soils and to correlate analytical determinations with SOC pools (Strosser, 2010; von Lützow et al., 2007). A variety of thermal and chemical soil analysis methods have been used for the quantification of soil organic matter (SOM) (e.g. dry combustion, loss-on-ignition, and loss-on-hydrogen peroxide oxidation) and soil organic carbon (SOC) (e.g.

dichromate oxidation and acid hydrolysis). Moreover, some of these methods are also useful to discriminate the recalcitrant C fraction (Rovira and Vallejo, 2007). However, the accuracy of these methods for the quantification of these fractions in biochars is unclear due to the diverse composition of carbon pools and their vulnerability to chemical attack. Consequently, the validity of these chemical methods has been questioned (Naisse et al., 2013).

b) Carbon availability

The utility of biochar as a carbon sink has been greatly discussed since some studies showed that it is able to promote native soil organic matter (SOM) decomposition, in the so-called positive priming effect (PE) (Hamer and Marschner, 2005; Kuzyakov et al., 2000; Luo et al., 2011). However, alternative trends, such as neutral and negative priming effects (i.e. the native SOM decomposition slows down) have been also reported elsewhere. Furthermore, changes in the priming signal, over time, have been observed. It is plausible to think that biological community and environmental conditions (climate and soil type), apart from biochar type, will play an important role in this issue.

c) Carbon protection

Biochar could be protected from oxidation when it is involved in organo-mineral association (Glaser et al., 2000), hence biochar occluded in aggregates allows C-resistant fractions to remain protected for longer in the soil. Also, biochar might influence soil aggregation and aggregate stability, further increasing the organic matter protected within aggregates. Therefore, the study of biochar effects on soil structure (aggregate stability) and carbon distribution (inside and outside of aggregates) is required to evaluate the effects of biochar on carbon protection. Moreover, stabilization mechanisms of biochar in soil are still poorly understood as they are dependent on both the type of soil and biochar.

Justification of the thesis

The benefits of biochar application in temperate regions are usually less evident than in acidic unfertile tropical soils. The main reason for this is that, after biochar application, the nutrient content and pH in acidic soils increases, leading to more productive soils, while less or no effect is observed in neutral soils which are the more common in temperate regions. Moreover, the majority of studies into biochar-amended soils are carried out in short-term laboratory experiments with high biochar rates, with only a few

studies having been conducted in field conditions with realistic doses and medium-term observations. However, both, lab and field experiments give different but valuable information. Laboratory experiments enable the testing of a specific soil amendment product with almost all variables controlled for, and the flexibility and the achievement of short term results, makes such experiments more attractive for researchers. Conversely, in the field the limited number of dosages and unavoidable uncontrollable conditions increases variability in results making their interpretation harder. However, even with these difficulties, the results from field experiments are more realistic and are indispensable to account for the possible effects and risks of an agricultural amendment product or practice in a determined area. Consequently, there is a scarcity and urgent requirement for field studies that investigate, in depth, the role of biochar on soil chemical, physical and biological properties in temperate areas (Blanco-Canqui, 2017). Also, experimentation with different types of biochars is necessary because biochar properties are highly dependent on the feedstock and pyrolysis process and, consequently, contrasting results could be obtained following their application in the same soil.

In this thesis the carbon resistance of two very different biochars, applied at a realistic agricultural dose in a two-year field experiment conducted on a Mediterranean soil, was tested by chemical, physical and biological methods, filling the gap in this issue.

Therefore, the main aims were to:

1) Quantify organic **carbon resistance** of pine and corn cob biochars discriminating resistant soil and biochar organic carbon pools by chemical and thermal processes (Chapter 1), and evaluate biochar persistence over two years through a mass balance using chemical and isotopic methods (Chapter 1 and Chapter 2).
2) Assess the effects of pine and corn cob biochar on the potential organic **carbon availability** to be mineralized by soil microorganisms identifying short-term priming effects and the eventual persistence of those effects at medium-term (two years after biochar application) (chapter 3).
3) Evaluate the effects of pine and corn cob biochars on physical organic **carbon protection** assessing the stability of soil aggregates and the implications of biochar in the distribution of organic carbon inside and outside of aggregates after two years of biochar application (chapter 4)

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Chapter 1: Comparing current chemical methods to assess biochar organic carbon in a Mediterranean agricultural soil amended with two different biochars

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Abstract

Several methods have been proposed to quantify biochar C recalcitrance but their suitability is questionable. The aims of this work are: i) to compare the suitability of thermal or chemical oxidation and acid hydrolysis methods to quantify biochar C-pool in a biochar-amended soil, and ii) to calculate the biochar content in the soil through a mass balance derived from the obtained data.

Two contrasted biochars from pine wood and corn cob remains were incorporated at a rate of 5 Mg C ha^{-1} to a sandy loam vineyard soil with neutral pH and low organic carbon content, in field conditions. The analytical methods used to determine the oxidability and hydrolyzation of soil and biochar-C were: i) weight loss-on-ignition (LOI) at three temperatures (375°C, 550°C and 950°C) for the assessment of organic matter, and ii) dry-combustion (TOC), strong (sO) and mild (mO) acid potassium dichromate oxidations, acid hydrolysis (AH) and peroxide oxidation (PO) for the assessment of organic C-pools. mO mainly estimated the easy oxidisable organic fraction of soil. Resistant organic carbon (ROC), estimated as non-hydrolysable organic carbon by AH and as non-oxidisable by mO, led to similar values in control soil (5 g C kg^{-1} soil), whereas different ROC values were obtained in soils amended with biochar (6-12 g C kg^{-1} soil). The suitability of these different methods as proxies to quantify biochar C was verified through a mass balance observing differences between them. PO removes well native soil organic matter, but also attacks partially biochar's fraction, so an underestimation exists. However, mO leaves intact biochar in the amended soil. Summarising, LOI, TOC and mO were the best proxies for biochar-C quantification, especially the last one, somewhat clarifying the debate on this topic.

Abbreviations[1]

[1] *Abbreviations:* PB, pine biochar; ZB, corn cob biochar; S, control soil; S+PB, soil amended with pine biochar; S+ZB, soil amended with corn cob biochar; AH, acid hydrolysis; LOI, loss-on-ignition; LPO, loss-on-peroxide-hydrogen oxidation; mO, mild potassium dichromate oxidation; PO, peroxide-hydrogen oxidation; sO, strong potassium dichromate oxidation; TOC, total organic carbon (by dry combustion); BOC_{TOC}, biochar (of pine or corn cob) organic carbon content estimated by TOC; BOC_{AH}, biochar (of pine or corn cob) organic carbon content estimated by AH; BOC_{LPO}, biochar (of pine or corn cob) organic carbon content estimated by LPO; BOC_{mO}, biochar (of pine or corn cob) organic carbon content estimated by mO; CF, correction factor for mineral losses based on LOI 550°C; ROC_{AH}, resistant organic carbon values calculated from AH; ROC_{mO}, resistant organic carbon values calculated from mO; ROC_{PO}, resistant organic carbon values calculated from PO

1.1 Introduction

Biochar has received much attention in recent years due to its properties as a potential soil amendment (Lehmann and Joseph, 2015). Biochar is a solid carbonaceous material obtained from the thermochemical conversion of biomass in an oxygen-limited environment and intended for use as soil amendment (International Biochar Inititiative, 2012). Furthermore, the application of biochar to soil provides additional environmental benefits, such as long-term C sequestration in soil (Shackley et al., 2013), compensating CO_2 emissions (Fang et al., 2014) and playing a key role in a global C-negative strategy (Cheng et al., 2006; Qin et al., 2016).

Biochar stability could vary depending on biomass feedstock and pyrolysis procedure, which influences aromatic C condensation (Lehmann et al., 2006; Wang et al., 2015). Most biochar C is very stable due to its chemical structure, which is rich in aromatic C structures (Baldock and Smernik, 2002; Calvelo Pereira et al., 2011; Singh et al., 2012), highly resistant to microbial decomposition (Shindo, 1991; Cheng et al., 2008). However, biochar contains a minor labile fraction that consist mostly of carbohydrates, proteins and fatty acids that have not been completely charred (Lehmann and Joseph, 2009). This minor organic fraction alone might not contribute to efficient C sequestration in soil due to its fast turnover rates, although it may play a key role in soil structure owing to physical protection processes that might increase its residence time in soil (Plaza et al., 2016).

While there can be no doubt that the application of biochar to agricultural soils increases the recalcitrant fraction of soil organic carbon (SOC), and hence C sequestration in soil, the most suitable methods for its assessment are still under debate. Various attempts have been made to correlate analytical determinations -chemical, biological and physical methods- with SOC pools (Strosser, 2010; von Lützow et al., 2007) but, in the specific case of chemical methods, their validity has been questioned (Naisse et al., 2013). A variety of current thermal and chemical soil analysis methods have been used for this purpose as dry combustion, loss-on-ignition (LOI), and loss-on-hydrogen peroxide oxidation (LPO) being the most widespread methods to determine soil organic matter (Gustafsson et al., 1997; Mikutta et al., 2005). On the other hand, dichromate oxidation has been widely used to assess soil organic carbon (e.g. Walkley and Black, 1934) and acid hydrolysis (AH) to discriminate the recalcitrant C fraction (Rovira and Vallejo, 2007). However, the accuracy of these methods for the quantification of biochars is unclear due to the diverse composition of carbon pools and their vulnerability to chemical

attack. As an example of this, acid dichromate oxidation has been suggested by several authors as an alternative to test biochar C reactivity in soil (Calvelo Pereira et al., 2011; Knicker et al., 2007). This procedure is a modification of the broadly used classic methods to estimate organic carbon (e.g. Walkley-Black, 1934; Mebius, 1960). The $K_2Cr_2O_7/H_2SO_4$ concentration, temperature and time of digestion are the key parameters which determine the oxidation degree because not all biochar C is always oxidised by dichromate (Rumpel et al., 2006). Calvelo Pereira et al. (2011) proposed acid dichromate oxidation as a method to evaluate the most reactive fraction of biochar, suggesting that resistant C to that oxidation could reflect the most stable fraction and the degree of biochar aromatisation.

The main aims of this field study, carried out in a soil amended with two different biochars, were: i) to evaluate which of the widely used chemical methods for the assessment of soil organic matter (SOM) or SOC are the most suitable for discriminating resistant soil and biochar organic carbon pools, and ii) to calculate the biochar content in soils through a mass balance derived from the available analytical data and confirm if these methods are good proxies to quantify biochar.

1.2 Materials and methods

Table 1.1 summarise and define the acronyms used in this paper.

Table 1.1 List of acronyms used in this paper by alphabetical order.

General acronyms	Definition
Biochars	
PB	Pine biochar
ZB	Corn cob biochar
Treatment	
S	Control soil
S+PB	Soil amended with pine biochar
S+ZB	Soil amended with corn cob biochar
Methods	
AH	Acid hydrolysis (HCl, 100ºC, 17 h)
LOI	Loss-on-ignition (375 ºC, 550 ºC, 950ºC)
LPO	Loss-on-peroxide-hydrogen oxidation
mO	Mild potassium dichromate oxidation ($K_2Cr_2O_7$, 60 ºC, 8 h)
PO	Peroxide-hydrogen oxidation
sO	Strong potassium dichromate oxidation ($K_2Cr_2O_7$, 150 ºC, 10 min)
TOC	Total organic carbon (by dry combustion)
Specific acronyms	**Definition**
BOC_{TOC}	Biochar (of pine or corn cob) organic carbon content estimated by TOC
BOC_{AH}	Biochar (of pine or corn cob) organic carbon content estimated by AH
BOC_{LPO}	Biochar (of pine or corn cob) organic carbon content estimated by LPO
BOC_{mO}	Biochar (of pine or corn cob) organic carbon content estimated by mO
CF	Correction factor for mineral losses based on LOI 550°C
ROC_{AH}	Resistant organic carbon values calculated using AH
ROC_{mO}	Resistant organic carbon values calculated using mO
ROC_{PO}	Resistant organic carbon values calculated using PO

1.2.1 Biochar characterisation

Two biochars were tested in this study, one obtained as a residue of the gasification of mixed pine wood splinters (*Pinus radiata* and *P. pinaster*) at 600-900°C (PB), and the other by slow pyrolysis of corn cobs (*Zea mays*) at 450-500°C (ZB).

Biochar C and H content were determined using a Flash 2000 C.E. Elemental Analyzer (Thermo Fisher Scientific) at 1020°C, N content by a Flash EA 1112 Elemental Analyzer (Thermo Fisher Scientific) at 1020°C, S by ICO-OES spectrometry using a Varian 725-

ES Radial ICP Optical Emission Spectrometer (Varian Inc.). Organic O was estimated by subtraction of the others elements, ash and the mineral O loss from carbonates. Inorganic C was measured by the titrimetric method described by Wang et al. (2014) and used to calculate the organic C (TOC) by subtraction from the total C (TC). Molar concentrations of O, C and H were used to calculate O:C and H:C ratios (Baldock and Smernik, 2002; Knicker et al., 2005). The pH of biochar was measured in a 1:20 w/v water suspension, and electrical conductivity (EC) was measured after filtering the same extract. The ash content was determined as the weight of the residue of combustion by a muffle furnace at 950 °C during four hours. Loss-on-ignition at three temperatures, loss-on-hydrogen peroxide oxidation, total carbon, acid potassium dichromate oxidation, hydrochloric acid hydrolysis and inorganic carbon were also determined in biochar samples by the methods described later.

1.2.2 Site and soil description

The study was conducted in a 20-year-old vineyard grown on a *Fluventic Haploxerept* soil (Soil Survey Staff., 2014) located in Vimbodí-Poblet (Catalonia, NE Spain), on a hillside with a slope of 8%. The parent material is a quaternary alluvial deposit formed by a mixture of slates, sandstone, granodiorite and limestone gravels (64% w/w) mixed in a clay-loam matrix. Average annual rainfall and air temperature for the area are 550 mm and 14.6°C, respectively. The soil can reach a depth of 120 cm and almost all the initial carbonates have been dissolved (around 1% remaining). The field bulk density of topsoil is 1.43 Mg m^{-3}. The fine fraction (< 2 mm) is 36% (w/w). The A_p horizon has a sandy loam texture with a clay content of 15%, a neutral pH (7.2), a low organic carbon content (0.97%), and low cation exchange capacity (7.1 $cmol_c$ kg^{-1}) mainly saturated by calcium.

1.2.3 Experimental design and sampling

Two biochars were applied in a unique dose, to field plots at 5 Mg C ha^{-1}, equivalent to 6.5 g kg^{-1} in the < 2mm soil fraction, in a randomised block design with three treatments (in triplicate): control (S), soil amended with pine biochar (S+PB) or amended with corn cob biochar (S+ZB). This application rate is representative of a realistic application dosage for an agricultural soil (Jeffery et al. 2011). Three plots (10 x 8.8 m^2) were set up per treatment, each with four *Vitis vinifera* rows including ten plants/row. The biochar

was weighted and uniformly applied to the soil surface and then was incorporated to the soil by tilling to a 15-cm plough depth. The vineyard in this study was managed using ecological agriculture practices, and ploughed three or four times per year to control weeds. Two years before, the vineyard was fertilised with composted cow manure, though not fertilised during the experimental period of this study. Agrochemicals had not been added with the exception of the regular Bordeaux mixture treatments for fungal disease control.

Soil samples were collected in July 2013, July 2014 and July 2015 after 2, 14 and 26 months of biochar application, respectively. Each sample was the result of eight random soil cores of 4 dm^3 pooling, taken at a depth of 0-10 cm (A_p horizon) within each plot (approximately 45 kg/sample). Soil samples were sieved to 5 mm in the field to separate gravels, and then air-dried, sieved to 2 mm in the laboratory, and stored at 4°C. A representative portion of each sample was grounded and sieved to 0.02 mm to carry out the analyses as required. Laboratory analyses were carried out in triplicate.

1.2.3.1 Loss-on-ignition (LOI)

Loss-on-ignition (LOI), both of soil and biochar, was measured in a muffle furnace using ground samples, in triplicate, at three temperatures. In the first, samples were subjected to 375°C for 18 h without acid pre-treatment to assess biochar organic content without the soot fraction (LOI 375°C) (Gustafsson et al., 1997; Poot et al., 2009). In the second, the calcined samples were further heated at 550°C for 5 h to remove soot (Gustafsson et al., 1997), hence allowing the complete oxidation of the organic carbon fraction (LOI 550°C). Finally, the samples were subjected to 950°C for 5 h, in order to remove carbonates and other mineral components (LOI 950°C) (Santisteban et al., 2004). LOI 550°C values were corrected subtracting mineral losses due to water and mineral components using a linear regression between LOI 550 °C and TOC when organic C was 0 (Y intercept) in these soil samples (correction factor, CF = 28.42 g kg^{-1}).

1.2.3.2 Loss-on-hydrogen peroxide oxidation (LPO)

Samples were subjected to a slow and progressive oxidation with an excess of hydrogen peroxide (Mikutta et al., 2005). Briefly, 4 g of oven-dried soil samples were placed in a 100 ml Erlenmeyer flask and hydrogen peroxide (33%) was added several times until the oxidation reaction finished (one week was required in this study). Then the remaining residue was dried and accurately weighted to estimate organic matter losses. To estimate

resistant organic carbon to hydrogen peroxide oxidation (PO), the residue was analysed using a Flash EA 1112 Elemental Analyzer at 1020°C.

1.2.3.3 Total carbon (TC), total organic carbon (TOC) and inorganic carbon (IC)

Total carbon content in soil samples was determined by elemental analysis using a Flash EA 1112 Elemental Analyzer at 1020°C. Inorganic C was measured by the titrimetric method described by Wang et al. (2014). TOC of soil samples was calculated as a difference between TC and IC.

1.2.3.4 Oxidation by potassium dichromate

Oxidisable organic carbon with potassium dichromate ($K_2Cr_2O_7$) in acid media was determined using a strong (sO) or a mild oxidation (mO). Strong oxidation was conducted according Nelson and Sommers (1996) with some modifications, briefly 10 ml of 66.7mM $K_2Cr_2O_7$ were dissolved in a concentrated mixture of H_2SO_4/H_3PO_4 (1:1 v/v) and added to a glass tube containing 200-300 mg of grounded soil. The tubes were vortexed (15s) and heated to 150°C in a digestion block for 10 minutes. After cooling, 90 ml of distilled water were added and the excess of dichromate ($Cr_2O_7^{-2}$) was back titrated with 0.2 N ammonium iron (II) sulphate (Mohr's salt) using diphenylamine as indicator. The mild oxidation was conducted according to Rumpel et al. (2006) without HF acid pre-treatment. Briefly, 200 mg of grounded soil was mixed with 5 ml of potassium dichromate solution (0.1M $K_2Cr_2O_7$ / 2M H_2SO_4) and oxidised at 60°C for 8 h. Oxidised C was quantified by titration as already described.

1.2.3.5 Acid hydrolysis (AH)

Biochar recalcitrance has been assessed by its resistance to acid hydrolysis by some authors (Nocentini et al., 2010), as this method is widely used for organic carbon recalcitrance in soils (Plante et al., 2006; Rovira and Vallejo, 2002; Silveira et al., 2008). Acid hydrolysis was carried out according to Rovira and Vallejo (2007) with few modifications: 20 ml of 6M HCl were applied to 500 mg of ground sample for 17 hours at 105°C; the resulting slurry was then vacuum filtered and washed with distilled water using a weighted Duran® nº2 filter crucible. The filtrate was discarded whereas the non-hydrolysed residue remaining in the filter crucible was dried at 105°C for 3h. The organic C resistant to acid hydrolysis was determined by elemental analysis of the residue using a Flash EA 1112 Elemental Analyzer at 1020°C.

1.2.3.6 Mass balance calculations

For each of the different methods used in this work a mass balance was conducted to estimate biochar organic carbon in biochar amended-soils, subtracting the labile and resistant fraction of control soil. The respective equations used could be seen in the appendix section.

1.2.3.7 Data analysis

Before statistical analysis, data were tested for homogeneity of variance using the Shapiro-Wilk test. In each treatment, global significance tests on the effects of each biochar type were performed using Two-Way RM ANOVA tests. Significant differences between treatments in a given sampling time, and between samplings in treatments, were assessed by using the Bonferroni test at a probability level of 0.05. All tests were carried out using R software (R Core Team 2013). See p-values of the analysis in the appendix section.

1.3 Results

1.3.1 Composition and chemical properties of pine and corn cob biochars

Table 1.2 shows the main chemical composition and properties of the two biochars tested. As expected, high pH and relatively low soluble salts values were obtained. The organic carbon content was high and similar, and the inorganic C content (carbonates) was low in both biochars. Both met the criteria of organic C content and H/C molar ratio <0.7 (International Biochar Inititiative, 2012), although the H/C ratio was slightly lower in pine than in corn cob biochar while O/C ratio was the same. Corn cob biochar showed higher N and H concentration, related to biochar raw material and pyrolysis type. Slightly higher S values were observed in pine than corn cob biochar. Organic matter estimated by LOI had a similar thermochemical profile and ash content was approximately 9% in both biochars.

Regarding biochar C chemical stability estimated by different methods, noticeable differences were observed between them. More specifically, when strong oxidation was conducted, 74% of TOC was quantified in pine biochar whereas just 30% was measured in corn cob biochar. Carbon losses by mild dichromate oxidation (mO) were approximately 6% of TOC in both biochars. Resistant C determined by acid hydrolysis

was around 9% of TOC. Loss-on-peroxide oxidation organic matter (LPO) was slightly higher in corn cob than in pine biochar even though values were minimal in both materials (<2.5 % of TOC).

Table 1.2 Elemental analysis, molar ratios and chemical properties of biochar from pine wood (PB) and corn cob (ZB). Total carbon (TC), Total nitrogen (TN), hydrogen (H), Total organic oxygen (O), O/C and H/C molar ratios, electrical conductivity (EC), ash, pH, sulphur (S), weight loss-on-ignition (LOI) and weight loss-on-peroxide oxidation (LPO), inorganic carbon (IC), organic carbon destroyed by strong potassium dichromate oxidation (sO), or mild potassium dichromate oxidation (mO), and organic carbon resistant to acid hydrolysis (AH).

Biochar feedstock		**Pine (BP)**	**Corn cob (ZB)**
pH (water, 1:20 w:v)		11.5±0.04	10.3±0.04
EC (dS m^{-1} 25°C)		0.69±0.02	2.54±0.5
TC (g kg^{-1})		793.4	785.8
IC (g kg^{-1})		4.0±0.07	2.7±0.06
TN (g kg^{-1})		0.20	6.80
H (g kg^{-1})		12.2	19.1
S (g kg^{-1})		1.48	0.64
O (g kg^{-1})		90.15	89.36
Ash (g kg^{-1})		91.9	91.1
H/C		0.19	0.29
O/C		0.11	0.11
	375°C	885.9±0.2	891.7±0.3
LOI (g kg^{-1})	550°C	892.1±0.3	897.9±0.2
	950°C	905.9±0.2	917.7±0.2
LPO (g kg^{-1})		0.95±0.87	19.55±3.84
sO (g kg^{-1})		590.64±32.57	235.27±40.09
mO (g kg^{-1})		46.82±1.59	43.69±3.64
AH (g kg^{-1})		84.05±2.5	65.66±8.46

1.3.2 Soil organic matter content by loss-on-ignition (LOI) and loss-on-hydrogen peroxide oxidation (LPO)

Soil LOI values are shown in Table 1.3. As expected, significant differences were detected between control soil and that amended with biochars in LOI at 375°C *(p= 0.002)*, 550°C (*p=0.002)* and 950°C (*p=0.002)* (Table 1.3). However, no significant differences between sampling times were found, meaning that no relevant quantitative changes had occurred along two years after biochar application.

Regarding LPO, no significant differences were detected between control soil and soil amended with biochars (*p=0.143*). When LPO was compared to LOI 375ºC (Table 1.3), it was clear that approximately half of SOM was oxidised by hydrogen peroxide in control soil, but less in biochar amended soil.

Table 1.3 Soil organic matter content estimated by loss-on-ignition (LOI) at different temperatures and loss-on-peroxide hydrogen oxidation (LPO) in a soil treated with pine (S+PB) and corn cob (S+ZB) biochar compared to control (S) at three sampling times (2, 14 and 26 months) after biochar application. Mean values and standard deviation of three replicates are shown. Values sharing capital letters indicate the lack of significant differences between sampling times within treatments ($p<0.05$), while equal small letters imply the lack of differences between treatments within times ($p<0.05$). If letters are not given, no significant differences were observed.

Biochar treatment	LOI 375°C ($g\ kg^{-1}$)	LOI 550°C ($g\ kg^{-1}$)	LOI 950°C ($g\ kg^{-1}$)	LPO ($g\ kg^{-1}$)
2 months				
S	27.4±1.34 a	41.67 ± 1.31 a	50.22± 1.39 a	11.67±1.36 A
S+PB	34.99±3.46 b	50.02±3.79 b	58.84±4.01 b	14.51±1.30 A
S+ZB	36.80±1.86 b	52.15±1.55 b	61.60±1.38 b	12.85±1.30 A
14 months				
S	28.18±0.92 a	43.17±1.32 a	51.96±1.59 a	11.68±0.53 A
S+PB	35.75±1.05 b	51.47±1.37 b	60.45±1.63 b	13.67±0.82 A
S+ZB	33.78±2.47 b	50.72±3.18 b	60.09±3.37 b	13.01±0.57 A
26 months				
S	28.50±0.80 a	42.70±1.0 a	51.88±1.35 a	15.24±0.58 B
S+PB	34.43±2.58 b	49.29±2.56 b	58.85±2.82 b	15.44±1.98 B
S+ZB	33.20±0.98 b	48.77±1.36 b	56.30±1.47 b	15.83±0.86 B

1.3.3 Total carbon (TC), total organic carbon (TOC) and inorganic carbon (IC) in soil

No significant variations in TC or TOC in the soil samples content were detected over time in S, S+PB and S+ZB treatment (Table 1.4). The inorganic C content was very low

and no significant differences were found between treatments. Since both biochars contributes with an even low carbonate content compared to soil (Table 1.2 and 1.4) it should mostly come from the latter. As expected, significant differences in TOC were detected between control soil and soil amended with biochars ($p=<0.001$).

Table 1.4 Soil total carbon (TC), inorganic carbon (IC), and total organic carbon (TOC), in a soil treated with pine biochar (S+PB) and corn cob biochar (S+ZB) compared to control (S) at three sampling times (2, 14 and 26 months) after biochar application. Mean values and standard deviation of three replicates are shown. Values sharing capital letters indicate the lack of significant differences between sampling times within treatments ($p<0.05$), while equal small letters imply the lack of differences between treatments within times ($p<0.05$). If letters are not given, no significant differences were observed.

Biochar treatment	**Total Carbon (TC)** ($g\ kg^{-1}$)	**Inorganic Carbon(IC)** ($g\ kg^{-1}$)	**Total Organic Carbon (TOC)** ($g\ kg^{-1}$)
2 months			
S	10.72±0.79 a	0.94±0.25	9.77±0.54 a
S+PB	17.98 ±2.67 b	1.12±0.52	16.87±2.56 b
S+ZB	21.33 ±1.50 b	1.12±0.95	20.21±2.37 b
14 months			
S	11.41 ± 0.86 a	1.43±0.99	9.99±1.03 a
S+PB	18.97±0.40 b	1.33±1.18	17.65±1.21 b
S+ZB	18.53±2.98 b	1.35±0.37	17.15±3.31 b
26 months			
S	10.29±0.73 a	0.49±0.29	9.80±0.85 a
S+PB	16.81±0.89 b	0.84±0.88	15.97±1.23 b
S+ZB	16.60±1.03 b	0.96±1.11	15.64±2.03 b

1.3.4 Strong and mild dichromate oxidation of soil organic matter

Potassium dichromate strong oxidation (sO) values accounted for approximately 90% of the total C concentration (Table 1.5). Therefore, this method was sensitive to the increase in C content after biochar addition ($p=0.002$). No significant differences were observed between 2, 14, 26 months, either for soil amended with pine or corn cob biochars, once again showing the lack of variation in the whole organic carbon content occurring over the two-year period.

When soil samples were submitted to mild oxidisation (mO), a less aggressive method for determining the labile C fraction (Table 1.5), no significant differences were observed between treatments at any time ($p=0.798$). In control soils oxidisable carbon estimated by mO represented approximately 50% of TOC whereas in biochar-amended soils only 30% of TOC.

Table 1.5 Organic carbon resistant to a strong and mild $K_2Cr_2O_7$ oxidation of a control soil (S) and soil treated with pine (S+PB) or corn cob (S+ZB) biochar at 2 months, 14 months and 26 months after biochar application. Mean values and standard deviation of three replicates. Values sharing capital letters indicate the lack of significant differences between sampling times within treatments ($p<0.05$), while equal small letters imply the lack of differences between treatments within times ($p<0.05$). If letters are not given, no significant differences were observed.

Biochar treatment	Strong oxidation (sO) ($g\ kg^{-1}$)	Ratio sO/TOC	Mild oxidation (mO) ($g\ kg^{-1}$)	Ratio mO//TOC
2 months				
S	9.32±0.93 ABa	0.95±0.07	5.97±0.60	0.61±0.03
S+PB	15.38±1.85 b	0.91±0.06	5.78±0.14	0.35±0.06
S+ZB	15.43±0.41 b	0.77±0.07	5.98±0.05	0.30±0.04
14 months				
S	11.59±1.68 Aa	0.95±0.07	4.66±1.05	0.46±0.07
S+PB	16.07±1.68 b	0.91±0.05	5.14±0.82	0.29±0.03
S+ZB	14.16±1.86 ab	0.83±0.06	5.43±0.42	0.33±0.07
26 months				
S	7.55±1.18 Ba	0.77±0.08	5.32±0.38	0.54±0.02
S+PB	13.70±1.84 b	0.85±0.05	5.72±0.58	0.36±0.04
S+ZB	14.43±0.93 b	0.93±0.09	4.75±0.33	0.31±0.04

1.3.5 Organic carbon recalcitrance estimated by acid hydrolysis (AH)

The non-hydrolysable C content was dependent on the biochar addition and significant differences were detected (*p=0.001*) between control soil and soil amended with biochars at any time, but not between pine and corn cob biochar (Figure 1.1).

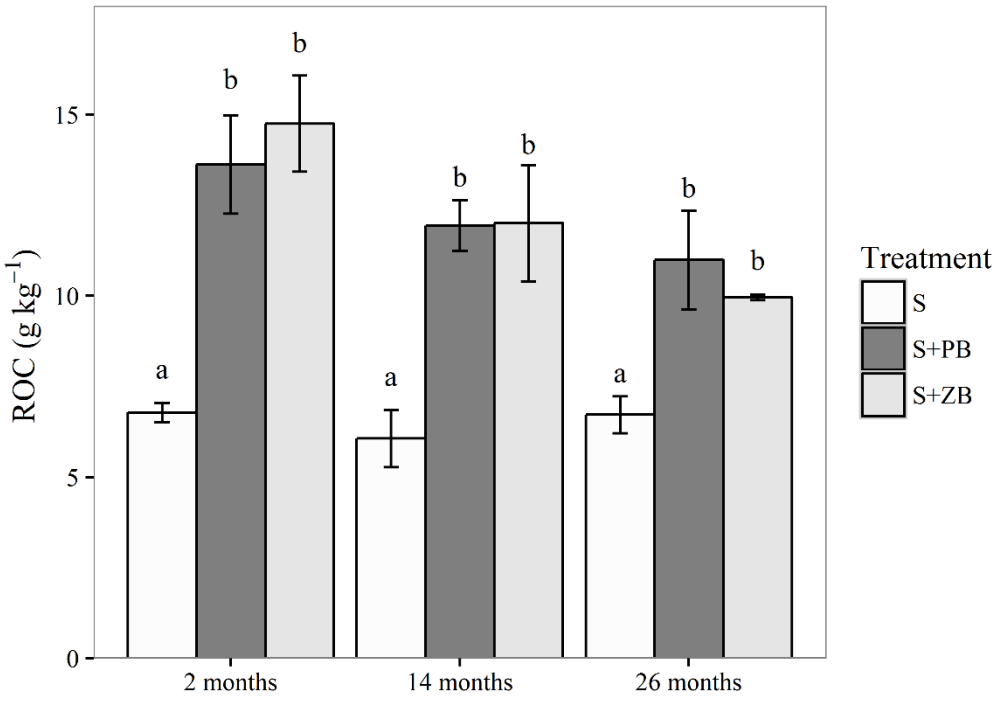

Figure 1.1 Resistant organic carbon (ROC_{AH}) to acid hydrolysis (AH) of a control soil (S) and soil treated with pine (S+PB) or corn cob (S+ZB) biochar, 2 months, 14 months and 26 months after biochar application. Values sharing capital letters indicate the lack of significant differences between sampling times within treatments (*p<0.05*), while equal small letters imply the lack of differences between treatments within times (*p<0.05)*. If letters are not given, no significant differences were observed.

1.3.6 Comparative resistance of organic carbon (ROC) to chemical reagents

The concentration of C in the residue of peroxide oxidation (S: 1.56 g kg^{-1}; S+PH: 5.6 g kg^{-1}; S+ZH: 7.36 g kg^{-1}) and acid hydrolysis (Figure 1.1), measured by elemental analysis, were used to determine ROC. The ratio ROC/TOC estimated by three different methods was calculated (data not shown). ROC_{PO} was less than 20% of TOC in control soil, and less than 40% in biochar-amended one. On the other hand, ROC_{mO} was lower than ROC_{AH} in control soil, while in biochar-amended soil ROC measured by mO and AH was approximately 70% of TOC. However, no significant differences were observed over time (data not shown).

If we compare methods, peroxide oxidation gives lower proportion of resistant C than mO or AH (Figure 1.2). In any case, soil amended with both types of biochar showed higher ROC values compared to the control soil regardless of the method used (Figure 1.2).

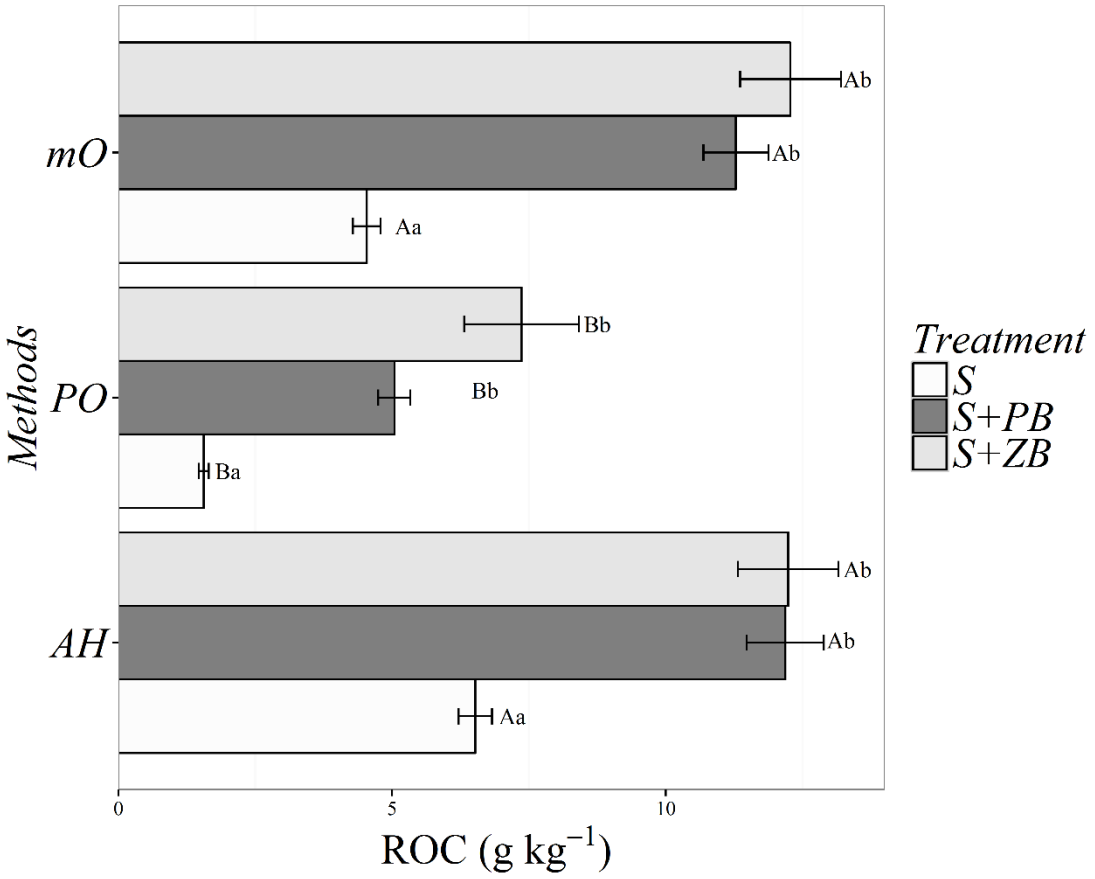

Figure 1.2 Resistant organic carbon (ROC) content in a biochar-amended soil estimated by three methods: acid hydrolysis (AH); loss-on-peroxide hydrogen oxidation (LPO) determined as differences between TOC and LPO; mild dichromate oxidation (mO) determined as differences between TOC and mO of control soil (S) and biochar-amended soil with pine (S+PB) and corn cob (S+ZB) biochar. Mean values and standard deviation of three replicates at three sampling times (2, 14 and 26 months) after biochar application are shown. Values sharing capital letters indicate the lack of significant differences between ($p<0.05$) methods within treatments, while equal small letters imply the lack of differences between treatments within the same methodology ($p<0.05$). If letters are not given, no significant differences were observed.

1.3.7 Biochar organic carbon (BOC) quantification by different methods

As can be seen in Table 1.6, lower significant values of BOC were obtained when estimation was performed using PO data (explained in appendix section) compared to the other methods. However, similar results were obtained using TOC, LOI and mO, agreeing

with the nominal dose of biochar C applied (5 Mg C ha^{-1}). A moderate underestimation is obtained with AH method.

Table 1.6 Pine (PB) and corn cob (ZB) biochar organic carbon (BOC) content in amended soil estimated by different methods (TOC, LOI, LPO, mO and AH). Nominal biochar dose, 5 Mg C ha^{-1}soil. Mean values and standard deviation of three replicates of three sampling times are showed. Differences within a row, followed by the same small letter, are not significant between methods ($p<0.05$). No significant differences between biochar type ($p<0.05$) were observed (within a column).

Biochar treatment	BOC_{TOC} (Mg C ha^{-1}soil)	BOC_{LOI} (Mg C ha^{-1}soil)	BOC_{PO} (Mg C ha^{-1}soil)	BOC_{mO} (Mg C ha^{-1}soil)	BOC_{AH} (Mg C ha^{-1}soil)
Pine (PB)	5.39±0.58 a	4.96±1.64 ab	2.67±0.67 b	5.21±1.37 a	4.37±1.63 ab
Corn cob (ZB)	6.03±1.83 a	5.01±1.76 ab	4.47±2.43 b	5.98±2.13 a	4.41±2.13 ab

1.4 Discussion

In spite of a very different feedstock composition and pyrolysis procedure, no great chemical composition differences were detected between both biochars. In our study, pine biochar showed a slightly lower H/C ratio than corn cob biochar. A lower H/C ratio suggested a more fused aromatic structure (Baldock and Smernik, 2002; Knicker et al., 2005). This aromatic structure might confers biochars a high degree of stability in terms of resistance to biological (Sun and Lu, 2014), chemical (Rovira and Vallejo, 2002) and physical decomposition (Li et al., 2016).

1.4.1 Suitable methods for total organic matter or carbon quantification

Elemental analysis was a reliable method to determine all sources of carbon (TC) in soil, making it possible to estimate total organic carbon (TOC) when inorganic carbon (IC) was subtracted.

Weight loss-on-ignition at 375°C corresponds mainly to organic matter although some crystalline water or mineral losses are included (Santisteban et al., 2004); whereas weight loss at 550°C overestimates organic matter due to dehydration of clays, hydroxides and other minerals (Heiri and Lotter, 2001). Weight loss between 550°C and 950°C estimated mainly CO_2 released from carbonates, crystalline water and volatile mineral elements

losses (approximately 1% of weight losses in this soil), showing no significant differences between treatments. At each ignition temperature a correction factor (CF) for mineral losses is needed. In this work, the CF for LOI at 550ºC represents the half of the weight losses at this temperature with a value of 28.42 g kg^{-1}.

Biochar loss-on-ignition depends on the feedstock and pyrolysis process used in biochar production. Brändli et al (2009) observed that biochar is not completely thermo-oxidised at 375ºC, suggesting the higher the temperature applied to obtain biochar, the higher the temperature needed for its complete combustion. However, Gelinas et al (2001) found biochar to combust completely at 375°C. In contrast, Brändli et al. (2009) reported that higher temperatures were required. In our study differences in OM quantification at three different temperatures (LOI 375°C, 550°C and 950°C) were less than 2% in both pine and corn cob biochars. Even though, we recommend the use of LOI 550°C subtracting mineral losses (CF) to ensure that all biochar organic matter in amended soils is completely thermo-oxidised. Moreover, Santisteban et al. (2004) pointed out LOI 550ºC as a qualitatively reliable method to detect biochar presence and possible losses in the field. Furthermore, Koide et al. (2011) considered it as an appropriate method to quantify biochar in the field.

1.4.2 Suitable methods for the discrimination of resistant organic carbon

Hydrogen peroxide was a good oxidant agent for removing native OM from soil samples, leaving the most resistant carbon, for example biochar (Liang et al., 2006; Mikutta et al., 2005). This method was easy to implement although long oxidation times are required and accurate weighting is needed to reduce gravimetric errors. Hydrogen peroxide oxidise a small fraction of both biochars (Table 1.2) due to the high amount of resistant carbon in these materials. The high hydrophobicity of pine biochar (data not shown), could reduce peroxide oxidation reactions. The hydrophobicity can protect biochar from chemical attack (von Lützow et al., 2007), which might at least partly explain the lower oxidability of pine biochar compared to the corn cob type (Table 1.2). Moreover, biochar's physical characteristics could change in the laboratory after exposition to hydrogen peroxide (Huff and Lee, 2016), and in the field by weathering, for example, by wetting and drying cycles in soil such as the reported increase in hydrophilicity of hydrophobic coal (Spokas et al., 2014; Velasco-Molina et al., 2016). Considering these observations, biochar organic carbon could be underestimated using this method (Table 1.6).

Total organic carbon quantification with dichromate strong oxidation method was lower than TOC estimated by elemental analysis, because dichromate did not oxidise all organic carbon present in the sample (Brändli et al., 2009). Biochar organic carbon (BOC) oxidation depends on temperature, reaction time, concentration of reagents and the degree of carbonisation of the biochar (Knicker et al., 2007). Several years ago, similar methods based on dichromate oxidation (Nelson and Sommers, 1996) were widely used for organic C quantification in soils due to the capacity of dichromate to oxidise resistant molecules such as lignins, pectins, tannic acids, and humic acids (Ball, 1964). However, Rumpel et al (2006) used a similar method for biochar quantification in soil because of dichromate's inability to oxidise some resistant molecules from biochar and black carbon. Furthermore, these authors also applied a hydrofluoric acid pre-treatment to liberate the organic matter occluded in microagregates or in the interlaminar spaces of clays. However, the hydrofluoric acid was not used in our experiment so an underestimation of oxidisable organic matter could exist. Nevertheless, as biochar was quantified by subtracting that of the control, mass balance is comparatively correct. Our results in biochar-amended soil also support the view that the partial oxidation produced by mO only quantifies labile OC, for the most part from native soil organic matter in soil samples. On the other hand, sO estimated 90% of TOC in soil amended with pine biochar while 80% of TOC in that amended with corn cob biochar owing to the latter being less dichromate-oxidisable (see Table 1.2). Such results indicate an underestimation of biochar carbon due to: i) only partial oxidation of resistant organic carbon and, ii) reduced chemical attack capacity associated with biochar buoyancy when mixed with dichromate solution, since some particles remained on the Pyrex tube walls after agitation and digestion.

Easily oxidisable C quantified by mO was approximately 50% of the TOC of the soil, as the ratio mO/TOC of control soil treatment indicated (Table 1.5). Yet it should be noted that this method is also suitable for indirect estimation of the resistant carbon fraction of soil. All that said, mild oxidation is a suitable proxy for estimating the amount of resistant organic carbon added with biochar amendments by subtraction from the TOC.

Acid hydrolysis had also been used to estimate the resistant organic carbon fraction in the soil samples as it removes labile compounds such as carbohydrates and proteins by disruption of glycosidic and peptide bonding and leaves others such as alkyl and aryl chains (Leavitt et al., 1996; von Lützow et al., 2007). In our soil samples, ROC/TOC ratio estimated by acid hydrolysis showed higher values, mainly in control soil, compared to

the rest of the methods. This behaviour could be explained due to acid hydrolysis is unable to break lignin molecules (Leavitt et al., 1996), thus increasing the amount of resistant carbon in the soil residues. Moreover, according with Greenfield et al. (2013) carbohydrate destruction (by hydrolysis) derived components can be converted into new and more stable forms compounds (e.g. furfurals) which increase its resistance to acid hydrolysis.

1.4.3 Estimation of ROC in amended soils

Resistant organic C-fraction (ROC) was estimated and compared using three different methods: i) ROC_{PO} in the residue of peroxide oxidation; ii) ROC_{mO} remaining after mild dichromate oxidation and iii) ROC_{AH} in residue of acid hydrolysis. Soil amended with biochar showed higher ROC values compared to the control soil, estimated by PO, mO and AH, indicating that biochar increased the ROC pool in soil (Figure 1.2). Comparing ROC_{PO}, ROC_{mO} and ROC_{AH} to TOC ratio in soil control, it was observed that hydrogen peroxide was able to oxidise more soil organic carbon than mild dichromate oxidation or acid hydrolysis giving a lower ROC ratio value. Apparently, the ROC_{AH}/TOC ratio was similar in control soils and those treated with biochar, so one might reasonably think that biochar is partially sensitive to acid hydrolysis attack or that a portion of soil organic matter (such as lignin) was not hydrolysed, although in absolute values AH results (Figure 1.1) were higher in biochar-treated soils. Nevertheless, it was found that biochar application nearly doubled the amount of ROC in soil (Figure 1.2). ROC content in soil obtained by mO and AH was very similar, in contrast with ROC estimated by peroxide oxidation which was lower. This finding supports the study of Mikutta et al. (2005), in which different chemical agents were compared, with hydrogen peroxide achieving the most efficient oxidation in removing labile soil organic matter and leaving the most resistant ones. Several authors have described the efficacy of the PO method for non-resistant soil organic matter oxidation (Huff and Lee, 2016; Liang et al., 2006; Mikutta et al., 2005), leaving the most resistant SOM pool intact (Liang et al., 2006). However, when these methods were compared it was observed that PO was the most efficient method used in this study to remove native soil organic matter, although a small fraction of biochar was partially oxidized. Also, mild dichromate oxidation method (mO) was assumed to quantify mostly the native soil organic carbon fraction, leaving biochar intact (Knicker et al., 2007; Naisse et al., 2013; Rumpel et al., 2006; Velasco-Molina et al., 2016), which has been confirmed in our study.

1.4.4 Suitability of chemical methods for the quantification of biochar in amended soils

The estimation of biochar content in amended soils based on chemical parameters that measure the concentration of organic C variables relies on a good knowledge of the applied biochars and its homogenous application. In agricultural soils requires effective tilling operations and a moderate spatial variability of soil properties. When trying to apply this strategy to soils affected by wildfires, charcoal quantification could be a handicap.

In our work, the ideal method should (i) oxidize all soil native organic matter, and (ii) leave biochar intact. No one of these methods used met completely these assumptions, but obtained estimations of biochar organic carbon in soil reasonably agree with biochar nominal application (5 Mg C ha^{-1}), with exception of PO method (Table 1.6). This is due as hydrogen peroxide oxidation removes well soil organic matter, but also partially attacks biochar, so an underestimation exists. However, mild dichromate oxidation leaves quite intact biochar in amended soil. For that reason, based on our results, mild oxidation can be considered the best proxy to quantify indirectly biochar organic carbon in amended soils.

Furthermore, our results provide evidence that, during two years after biochar application in the field, no noticeable changes or losses of biochar organic carbon in soil were found, thus supporting the efficiency of biochar as a strategy to C storage in soil.

1.5. Conclusions

The estimation of biochar using a mass balance in amended soils needs the combination of (i) harsh methods to quantify the total organic matter or carbon in amended and control soils, and (ii) milder, less aggressive methods to quantify (labile) native soil organic matter combined with total organic matter or carbon determination. As this regard, loss-on-ignition and TOC were suitable methods to biochar's quantification when a control soil was available. However, mild dichromate oxidation combined with TOC was the most adequate procedure for biochar resistant organic carbon determination also comparing with a control soil. Special attention will need to be paid to acid hydrolysis and loss-on-peroxide oxidation which might underestimate the biochar amount.

Therefore, as mild oxidation leaves practically intact biochar organic carbon, it can be considered the best proxy to indirectly quantify biochar in amended soils.

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Chapter 2: Changes in ^{13}C abundance in a soil amended with pine and corn cob biochars by different chemical processes

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Abstract

The best methods to assess biochar concentration in soil and/or its resistance have yet to be refined, since many of them are unable to distinguish biochar carbon from that of native organic matter. The use of stable isotope signatures has been proven useful for such discrimination, although there are uncertainties to this approach.

This study was carried out under field conditions, in a sandy loam vineyard soil with neutral pH and low organic carbon content, where two different biochars (from C_3 pine wood and C_4 corn cobs) were incorporated at a rate of 5 Mg C ha^{-1}. The aim of the study was to assess the changes in carbon $\delta^{13}C$ in a real scenario by 1) studying how pyrolysis affect $\delta^{13}C$ of plant biomass 2) assessing how two chemical methods used to estimate carbon resistance (peroxide oxidation and acid hydrolysis) affect $\delta^{13}C$ of amended-soil; 3) measuring any variation in the $\delta^{13}C$ of this soil-amended over a two-year field study.

While residues from acid hydrolysis were 1-2‰ ^{13}C depleted, those from peroxide oxidation were 2-6‰ ^{13}C enriched. Qualitative biochar detection was achieved comparing amended and non-amended soils $\delta^{13}C$ regardless biochar feedstock metabolic pathways origin. Corn cob biochar quantification by partitioning using stable isotopes was underestimated circa 10% when soil samples were subjected to these two chemical procedures. Over two years, 35% of biochar was apparently lost which was attributed to biochar dilution in soil. Our data suggest that isotopic signature analyses could be a good proxy to evaluate changes in SOC resistance.

2.1 Introduction

Biochar is a carbon-rich and highly recalcitrant material obtained by biomass pyrolysis intended to be applied to soil (International Biochar Initiative, 2017). Biochar has been proposed as a potential soil amendment (Lehmann and Joseph, 2015) because it enhance soil properties such as nutrient retention, water holding capacity, soil pH and cation exchange capacity (Glaser et al., 2002; Lehmann et al., 2006). Studies of biochar as soil amendment in many different agricultural systems have been carried out with different effects on soil properties and crop yield (Jeffery et al., 2011; Marks et al., 2016). Biochar is highly resistant to decomposition and interaction of biochar with organic molecules in soil may lead its preservation contributing to soil C sequestration (Lehmann et al., 2006; Woolf et al., 2010). However, the feasibility of the biochar technology as a carbon storage strategy still remains under debate because it depends on many factors such as: i) carbon and energy balance from biochar production and transport process (e.g transport of the feedstock, drying needs, chipping needs, transport of the biochar to the site of application) ii) interaction between soil type (Bird et al., 1999), climate, original feedstock (Fang et al., 2014) and even the pyrolysis process and temperature used (Singh et al., 2012) after application of biochar into soil. Evidences found by some authors suggests that pyrogenic C can persist in soil on a millennial time scale (Cope and Chaloner, 1980; Patterson et al., 1987), although the half-life for natural oxidation of resistant carbon (which contains biochar) has been calculated to be <100 years by other authors (Bird et al.,1999). The uncertainties of such projections are high since most studies infer residence time from pot trials and laboratory studies usually conducted for short-term periods, highlighting the need for longer-term experiments for greater insight into biochar's effect under field conditions.

The persistence of carbon in biochar has traditionally been assessed by proxy methods based on physical, chemical and biological measurements (Strosser, 2010; von Lützow et al., 2007), with those measuring chemical resistance being the most widely used approach. Regarding the chemical methods, permanganate (Plaza-Bonilla et al., 2014) or dichromate oxidation (Herath et al., 2014; Knicker et al., 2007) and acid hydrolysis (Rovira and Vallejo, 2007; Rumpel et al., 2007), followed by carbon quantification in the respective residues, are the most commonly used due to their ease of implementation. However, completely equivalent residues after chemical processes were not obtained when applied to pure biochars or to biochar-amended soils (Raya-Moreno et al., 2017).

For example, hydrogen peroxide oxidation (Mikutta et al., 2005; Plante et al., 2005; Schumacher, 2002) is more efficient removing the native soil organic matter (SOM) than other chemical processes and hence facilitates the quantification of remaining biochar carbon, but it is less practical due to the long reaction time and the need for high accuracy in weighting. This is why the carbon isotopic signal, combined with chemical recalcitrance methods, has been used for a more accurate estimation of the relative contribution of biochar and native SOM to the total soil recalcitrant carbon pool, whenever the $\delta^{13}C$ values of each component are previously known (Werth and Kuzyakov, 2010; Whitman et al., 2014).

The particular isotopic signal of a biochar depends on: i) the specific biomass used, e.g. regarding the plant metabolism, since C_3 plants (Calvin cycle) have an isotopic signal range between -34‰ and -20‰ and C_4 (Hatch-Slack cycle) and have an isotopic signal range between -17 ‰ and -9‰ (Bernoux et al., 1998; De la Rosa et al., 2008; O'Leary, 1981; Peterson and Fry, 1987); ii) the plant parts used as feedstock (roots, stems, leaves) regarding their dominant molecular composition (Bird and Ascough, 2012), since it has been proven that the $\delta^{13}C$ increase as: alkanes < lipids < bulk leaf matter (Collister et al., 1994). Cellulose rich tissues have higher $\delta^{13}C$ than woody tissue because woody plants are rich in lignin, with lower $\delta^{13}C$ values (Schleser et al., 1999); iii) changes due to the pyrolysis process where selective isotope loss will depend on many factors (e.g. temperature, feedstock composition, proportion of oxygen presence) (Purakayastha et al., 2016; Bird et al., 2012).

Carbon fractioning techniques and, more specifically, chemical methods evaluating organic carbon resistance in soils , e.g. non-oxidisable C (dichromate, hydrogen peroxide) and non-hydrolysable C (acid-hydrolysis), can change the isotopic signals of the resulting residues which represent the carbon resistant fraction of SOM, as shown by Leavitt et al. (1996), who reported ^{13}C depletion in soil residues after acid hydrolysis.

Regarding the stability of carbon isotopic signature over time, the variation in isotopic signals is hard to predict since two opposite mechanisms might be at work: i) the isotopic discrimination of heterotrophic metabolism, which uses pathways that might prefer ^{12}C, increasing $\delta^{13}C$ of SOM (Ascough et al., 2011; Ehleringer et al., 2000); and ii) the selective microbial decomposition of plant compounds depending on molecular complexity because some resistant compounds are rich in ^{12}C (lignin over cellulose).

For all these reasons, organic carbon isotopic composition and its variation over time in a given soil is directly related to SOM origin and turnover (Martin et al., 1990), and

therefore provides information on SOM dynamics (von Lützow et al., 2007). However, what happens to the biochar ^{13}C signature in amended soils over time has received little attention. Due to its high recalcitrance, one might expect the $\delta^{13}C$ not to vary in years.
The aim of this field study, conducted on a vineyard soil amended with pine vs. corn cobs biochars and over a two-year period, was to assess the suitability of isotopic and chemical methods for biochar quantification in amended soils, namely: 1) to study changes in isotopic signature caused by pyrolysis process of pine and corn cob biomass; 2) to evaluate the effects of chemical fractioning methods (peroxide oxidation or acid hydrolysis) on the biochar-carbon isotopic signature, and; 3) to assess changes of carbon isotopic signature over time (two years).

2.2 Material and methods

2.2.1. Site and soil description

The experiment was carried out in *Fluventic Haploxerept* soil (Soil Survey Staff., 2014) in Vimbodí-Poblet (Catalonia, NE Spain) at latitude 41°22'38.3"N and longitude 1°04'28.8"E. It is located on a gentle slope (8%) and has been farmed as a vineyard for 20 years. The annual average of total vine biomass was 1.6 kg ha^{-1}.The area has a Mediterranean-type climate with a 14.6°C average annual air temperature and 550 mm of rainfall. The parent material is made up of stony quaternary alluvial deposits consisting of a mixture of slates, sandstones, granodiorites and limestone gravels (65% w/w) mixed in a clay-loam matrix. The bulk density of the topsoil (0-10 cm) is 1.43 Mg m^{-3}. The soil has a sandy loam texture with a clay content of 15%, neutral pH (7.2), low organic carbon content (9.7g kg^{-1}), and low cation exchange capacity (7.1 cmol kg^{-1}) with a nearly complete base saturation degree dominated by calcium. Traces of carbonates (ca 1%) were detectable in the soil profile. N (Kjeldahl), available K, and P (Olsen) were 0.07%, 170 and 12 mg kg^{-1}, respectively.
The vineyard had been cultivated following ecological agriculture regulations, and ploughed three to four times per year to control weeds. Plots were previously amended with compost manure but were not fertilised during the experimental period of this study (two years). No agrochemicals were added other than Bordeaux mixture treatments for fungal pest control.

2.2.2. Biochar characterisation

Biochar from corn cobs (*Zea mays*) was produced by slow pyrolysis at 450-500°C (hereinafter referred to as ZB), while pine wood biochar was obtained from a mixture of *Pinus radiata* and *P. pinaster* chips by gasification at 600-900°C (hereinafter referred to as PB). For their characterisation, both chars were ground and dried at 105ºC for 12h. The organic matter content was then estimated by ignition at 550ºC. A FlashEA 1112 (Thermo Electron) elemental analyser was used for biochar C, H and N determination. Inorganic C was measured following Wang et al., (2014) and used to estimate organic carbon by subtraction from the total C (Table 2.1). Organic O was estimated by subtraction of the other elements, ash and the mineral O loss from carbonates. Molar concentrations of organic O, C and H, were used to determine O:C and H:C ratios (Krevelen, 1961) (Table 2.1). Carbon resistance to peroxide oxidation (C_{POR}) or acid hydrolysis (C_{AHR}) was also determined in biochar samples by the methods described in section 2.4 (Table 2.1). Isotopic signature of biochars and respective feedstock materials was assessed by placing them in tin capsules for complete combustion in a FlashEA 1112 (Thermo Electron) elemental analyser at 1020°C coupled to a Delta V Advantage (Thermo Electron) isotope ratio mass spectrometer (IRMS) for $\delta^{13}C$ determination. The pH of biochars was measured in a 1:20 w/v water suspension and electrical conductivity (EC) was measured in the same extract after filtration with Whatman #42 filter. Both biochars were grounded and sieved to 2 mm before field application. Subsamples of both biochar were sieved with a mesh of 0.2 mm to calculate the biochar fine particle size fraction. Granulometric analysis of biochars showed that the fraction >0.2 mm in PB and ZB were 42.7% and 93.8% respectively.

Table 2.1 Elemental analysis, molar ratios and chemical properties of biochar from pine wood (PB) and corn cob (ZB). pH, electrical conductivity (EC), loss on ignition (LOI), total carbon (TC) , inorganic carbon (IC), total nitrogen (TN), hydrogen (H), sulphur (S), total organic oxygen (O), O/C and H/C molar ratios, , ash, weight loss-on-peroxide oxidation (LPO) and organic carbon resistant to acid hydrolysis (AH).

Biochar feedstock	Pine (BP)	Corn cob (ZB)
pH (water, 1:20 w:v)	11.5±0.04	10.3±0.04
EC (dS m^{-1} 25°C)	0.69±0.02	2.54±0.5
LOI (g kg^{-1})	892.1±0.3	897.9±0.2
TC (g kg^{-1})	793.4	785.8
IC (g kg^{-1})	4.0±0.07	2.7±0.06
TN (g kg^{-1})	0.20	6.80
H (g kg^{-1})	12.2	19.1
S (g kg^{-1})	1.48	0.64
O (g kg^{-1})	90.15	89.36
Ash (g kg^{-1})	91.9	91.1
H/C	0.19	0.29
O/C	0.11	0.11
LPO (g kg^{-1})	0.95±0.87	19.55±3.84
AH (g kg^{-1})	84.05±2.5	65.66±8.46

2.2.3. Experimental design and sampling

In 2013, both biochars were applied in field plots at a single rate equivalent to 5 Mg C ha^{-1}, which was intended to represent a realistic agricultural scenario (Jeffery et al. 2011). Each plot had an area of 10 x 8.8 m^2 with four rows of vine and ten plants per row, a density of 4545 vine per ha^{-1}. Plots were distributed in a random block design with three different treatments: control soil (S), pine biochar-amended soil (S+PB), or corn cob biochar-amended soil (S+ZB), in triplicate. Biochar was uniformly applied in a single application to the soil surface and then thoroughly incorporated into the soil in two tilling cycles at 15 cm depth in spring 2013. Soil samples (0-10 cm) were collected 2, 14 and 26 months after biochar application. At each sampling time, eight soil cores of 4 dm^3 at a 0-10 cm depth were randomly taken in each plot and pooled into a single composite sample.

The soil samples were then sieved to 5 mm in the field to remove gravel, and then air-dried, sieved to 2 mm in the laboratory, and stored at 4°C. A representative portion of each sample was milled to <0.02 mm as required for analysis.

Beginning in 2013 and before harvesting, the leaf area was measured and shoots were collected to quantify annual biomass in five randomly selected vines per row. The biomass per surface unit of leaves or shoots was calculated as the product of the sum of all rows of vine biomass and the field area.

2.2.4 Assessment of total carbon content, isotopic signature and chemical fractioning

To carry out the isotopic analysis, 1 mg of feedstock of biochars and 35 mg of soil (whole soil sample and respective residues of peroxide oxidation and acid hydrolysis), finely ground and dried for 24h at 105ºC, were placed in tin capsules. In samples of acid hydrolysis residue, 5 mg of vanadium oxide were added to catalyse and facilitate total combustion. The elemental C (TC) and C isotopic signature ($\delta^{13}C$) were determined using a FlashEA 1112 (Thermo Electron) elemental analyser at 1020°C coupled to a Delta V Advantage (Thermo Electron) isotope ratio mass spectrometer (IRMS). The $\delta^{13}C$ was calculated from the carbon isotope ratios of each sample and that of the VPDB (Vienna Peedee belemnite), in thousandths (‰) according to Eq 2.1.

Eq 2.1. Isotopic signature quantification

$$\delta^{13}C\ (‰)\ sample = \left(\frac{{}^{C^{13}}/_{C^{12}}\ sample}{{}^{C^{13}}/_{C^{12}}\ VPDB} - 1 \right) \times 1000$$

Total C and isotopic signatures were measured in: 1) vine leaves and shoots (V), the biochar feedstocks (pine splinters (P) and corn cobs (Z)); 2) the corresponding biochars; 3) the original soil samples (meaning not hydrolysed or oxidised) from each treatment (S, S+PB and S+ZB); 4) the hydrogen peroxide oxidation-resistant residue (POR) and the acid hydrolysis-resistant residue (AHR), obtained as described below.

POR was obtained by adding in excess of 33% hydrogen peroxide for a progressive and complete oxidation of soil samples (Mikutta et al., 2005). Briefly, hydrogen peroxide was progressively added to 4 g of oven-dried ground soil samples placed in a 50 ml Erlenmeyer, several times until sample reaction ceased (one week for the soil samples of this study). The remaining residue was then dried and accurately weighted to estimate the mass loss by peroxide oxidation (LPO), and finely ground. AHR was obtained according

to Rovira and Vallejo (2007) with few modifications: 20 ml of 6 M HCl was applied to 500 mg of a soil sample for 17 hours at 105°C; afterwards hydrolysis samples were vacuum filtered and washed with distilled water using weighted glass filter crucibles (10-15µm nominal pore size). The filtrate was discarded whereas the acid hydrolysis-resistant soil sample remaining in the crucible was dried at 105°C for 3h.

2.2.5 Quantification of the carbon fraction attributable to biochar (f_B) by isotope partitioning

Only ZB was considered to quantify the current biochar organic carbon fraction in the treated soil due to its natural ^{13}C enrichment as C_4 plant, hence clearly different from the native soil organic matter coming mainly from vines (C_3 plants). The stable isotopic partitioning through a two-compartment model proposed by Werth and Kuzyakov (2010) and Whitman et al. (2014) was used for this purpose. It states that the $\delta^{13}C$ of the biochar-amended soil should range between that of the biochar and that of the native organic matter depending on the partial contribution of each component. The biochar-C percentage in a soil sample was estimated by multiplying the corn cob biochar carbon fraction $\left(f_{ZB}(\%)\right)$ (Eq 2.2) by the total soil carbon (TC). The expected biochar-C contents were then compared with the nominal application rate (5 Mg C ha^{-1}), by transforming the carbon concentrations (in %) to Mg C ha^{-1} considering an incorporation depth of 15 cm and a bulk density of 1.43 Mg m^{-3}. The same procedure was used for the acid hydrolysis and the hydrogen peroxide oxidation residues in the corn cob biochar-amended soil samples.

Eq 2.2. Biochar-C fraction (%)

$$f_{ZB}(\%) = \left(\frac{\delta^{13}C_{S+ZB} - \delta^{13}C_S}{\delta^{13}C_{ZB} - \delta^{13}C_S}\right) \text{x } 100$$

f_{ZB}: fraction of total carbon (TC) attributable to corn cob biochar, in the biochar-amended soil, or in the corresponding acid hydrolysis or hydrogen peroxide residues

$\delta^{13}C_{S+ZB}$: $\delta^{13}C$ of corn cob biochar-amended soil (S+ZB), or of the corresponding acid hydrolysis or hydrogen peroxide residues

$\delta^{13}C_S$: $\delta^{13}C$ of control soil (S), or of the corresponding acid hydrolysis or hydrogen peroxide residues

$\delta^{13}C_{ZB}$: $\delta^{13}C$ of corn cob biochar (ZB), or of the corresponding acid hydrolysis or hydrogen peroxide residues

2.2.6 Data analysis

Before the statistical analysis, data were tested for normality using the Shapiro-Wilk test. For isotopic signature analysis, soil amended with biochar was compared with control soil separately, S with S+PB or S with S+ZB. Significance tests of the effects of biochar type were performed comparing isotopic signature of S+PB and S+ZB with S using two-way repeated measures ANOVA tests. After that, S+PB and S+ZB were compared with S within a sampling time using the Bonferroni test at a probability level of 0.05. For carbon analyses, global significance tests of the effects of biochar amendment (S, S+PB, S+ZB) were performed using two-way repeated ANOVA tests. Significant differences between biochar amendment within a sampling time, and between samplings within a biochar amendment, were assessed by using Bonferroni test ($p<0.05$). For isotopic signature and carbon analyses significant differences between chemical treatment (peroxide oxidation and acid hydrolysis), within each biochar amendment (S, S+PB, S+ZB) and sampling times, were assessed using the Bonferroni test at a probability level of 0.05. Also, significant differences between biochar amendments, within each chemical treatment and sampling time, were assessed using the same test. All tests were carried out using R software (R Core Team, 2013).

2.3 Results

2.3.1 Feedstock and pyrolysis type influence on the isotopic signature of biochar and biochar-amended soils

2.3.1.1 Comparison of the isotopic signal of the vine biomass, native soil organic matter, biochar and respective feedstocks

The annual average soil litter input of above-ground vine biomass during the study years was 176.7 g m^{-2} for leaves, and 77.9 g m^{-2} for shoots (which were incorporated as chopped stems). No significant differences were observed between the $\delta^{13}C$ of wine shoots (-26.61‰) or leaves (-26.68‰) and that of soil organic matter (-26.74‰), which is consistent with the fact that the vine biomass was the main source of organic matter in this soil (Table 2.2) as no compost was applied during experiment duration. On the other hand and as expected, clear differences were observed in the isotopic signature of pine wood (-26.61‰, C_3) and corn cob (-12.17‰, C_4) ($p=<0.001$), either as raw or pyrolysed materials, but no differences were observed between vine and pine wood feedstocks

(Table 2.2). As a result of pyrolysis, the $\delta^{13}C$ of biochar was lower in both pine (-1.42‰; *p=0.01*) and corn cob (-0.95‰; *p=0.02*) compared with respective feedstocks (Table 2.2). Thus, the isotopic signatures of both biochars (-13.12‰ for corn cob biochar, and -28.03‰ for pine wood biochar) were different from that of the vine biomass (*p<0.001*) (Table 2.2).

Table 2.2 Isotopic signature ($\delta^{13}C$) of native soil organic matter, the plant biomass (PB), acid hydrolysis residue (AHR), peroxide hydrogen oxidation residue (POR) and respective biochars.

Material		$\delta^{13}C$ (‰)
Plant biomass	Wine plant shoots	-26.61
	Wine plant leaves	-26.68
	Pine wood (P)	-26.61
	Corn cobs (Z)	-12.17
Soil	Whole organic matter	-26.74
Biochar and residues of chemical attack	Pine wood biochar (PB)	-28.03
	AHR	-28.01
	POR	-27.97
	Corn cobs biochar (ZB)	-13.12
	AHR	-13.1
	POR	-13.6

2.3.1.2 Total carbon and $\delta^{13}C$ of biochar amended soil

As expected, the differences in total carbon content of the whole sample between control and biochar-amended soils were significant (*p=0.001*) (Figure 2.1), but no significant differences were detected over time in S and S+PB treatments (Table 2.3). Also as expected, isotopic signature of corn cob biochar-amended soil (S+ZB, $\delta^{13}C$= 20.9) was significantly higher than control soil (S, $\delta^{13}C$=26.7) (*p=<0.001*), while significantly lower were found for pine biochar-amended soil (S+PB, $\delta^{13}C$ =17.9) (*p=0.008*) even differences were smaller (Figure 2.2). No changes were observed over time in S and S+PB isotopic signature, whereas in S+ZB it decreased significantly over two years from -19.83 to -21.95 (*p=<0.001*) (Table 2.3).

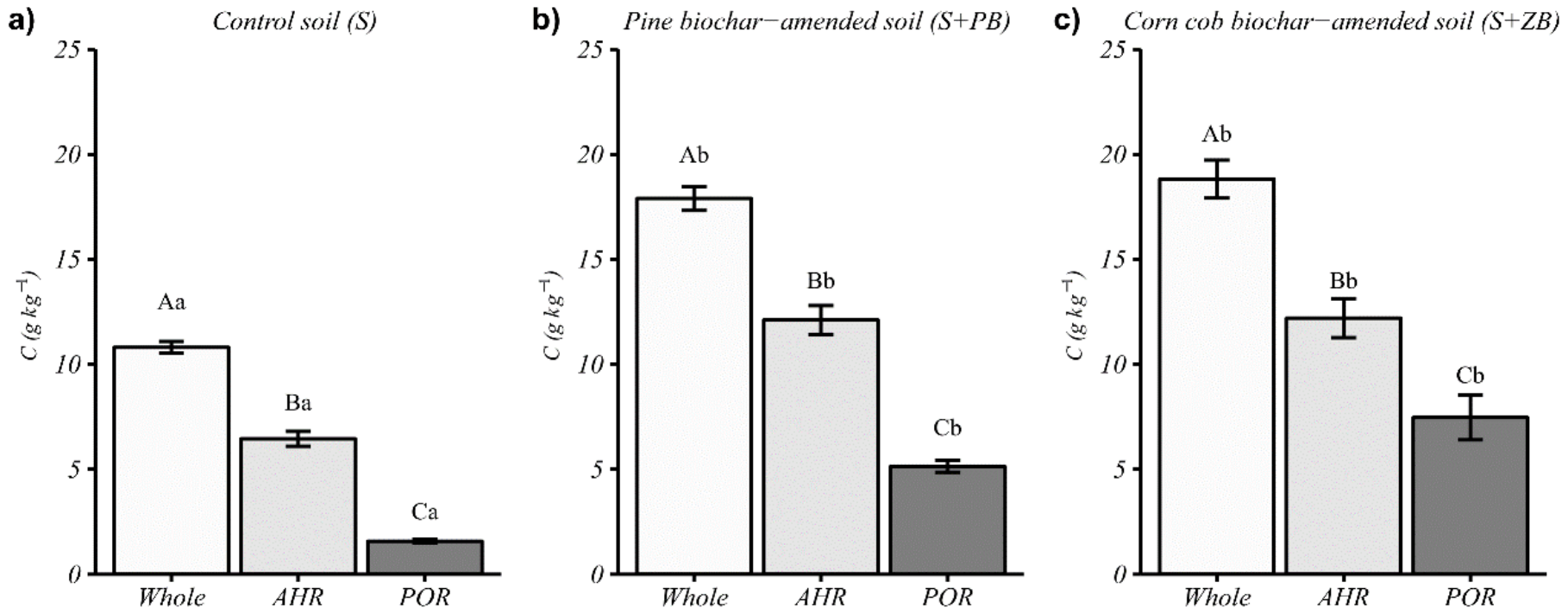

Figure 2.1 Total carbon content (g kg^{-1}) on the whole sample of the: a) control soil (S), b) soil amended with pine biochar (S+PB) and c) soil where corn cobs biochar was applied (S+ZB), as well as the carbon content of the corresponding chemically resistant fractions to acid hydrolysis (AHR) or peroxide oxidation (POR), respectively. Bars correspond to the mean of all sampling times values together with the corresponding standard deviation (n=3). Capital letters indicate significant differences between chemical treatment (AHR or POR) within each biochar amendment ($p<0.05$), while equal small letters indicate the lack of differences between biochar amendments within the same chemical treatment ($p<0.05$).

Table 2.3 Total carbon content (TC, g kg^{-1}) and $\delta^{13}C$ of the whole sample for the control soil (S), soil amended with pine biochar (S+PB) and soil amended with corn cobs biochar (S+ZB), as well as the carbon content of the corresponding chemically resistant fractions to acid hydrolysis (AHR) or hydrogen peroxide oxidation (POR), respectively. Data correspond to the mean and standard deviation over the three sampling times (n=3). Values sharing the same capital letters do not ($p<0.05$) differ between chemical procedures (whole, AHR, or POR) for a specific amendment treatment (i.e., control soil). Values sharing the same small letter do not significantly differ ($p<0.05$) between amendments (S, S+PB and S+ZB) for a specific chemical treatment (i.e., whole). If letters are not given, no significant differences were observed.

		Whole soil		AHR		POR	
Treatment	**Sampling times (months)**	**TC (g kg^{-1})**	**$\delta^{13}C$**	**TC (g kg^{-1})**	**$\delta^{13}C$**	**TC (g kg^{-1})**	**$\delta^{13}C$**
S	2	10.7±0.7 a	-26.7±0.1 a	6.7±0.6 a	-28.4±0.1 a	1.6±0.1 a	-20.0±0.8 a
	14	11.4±0.9 a	-26.7±0.2 a	6.0±1.7 a	-28.6±0.2 a	1.4±0.3 a	-21.5±1.0 a
	26	10.3±0.7 a	-26.7±0.0 a	6.7±0.8 a	-28.2±0.4 a	1.8±0.3 a	-20.8±1.2 a
S+PB	2	18.0±2.7 b	-27.3±0.1 b	13.3±2.5 b	-28.6±0.1	5.3±1.2 b	-25.3±0.8 b
	14	18.9±0.4 b	-27.2±0.2 b	12.0±1.0 b	-28.6±0.1	4.8±0.7 a	-25.9±0.2 b
	26	16.8±0.9 b	-27.1±0.2 b	11.0±2.4 a	-28.6±0.2	5.3±0.7 a	-26.2±0.2 b
S+ZB	2	21.3±1.5 Ab	-19.8±0.3 Ab	14.7±2.3 b	-20.8±0.3 Ab	10.7±2.2 Ab	-14.2±0.2 b
	14	18.5±3.0 ABb	-21.0±0.7 Bb	12.0±3.0 b	-22.3±1.1 Bb	7.0±2.7 ABb	-14.8±0.3 b
	26	16.6±1.0 Bb	-21.9±0.1 Cb	9.9±0.1 a	-23.3±0.7 Cb	4.7±0.7 Ba	-15. 9±0.2 b

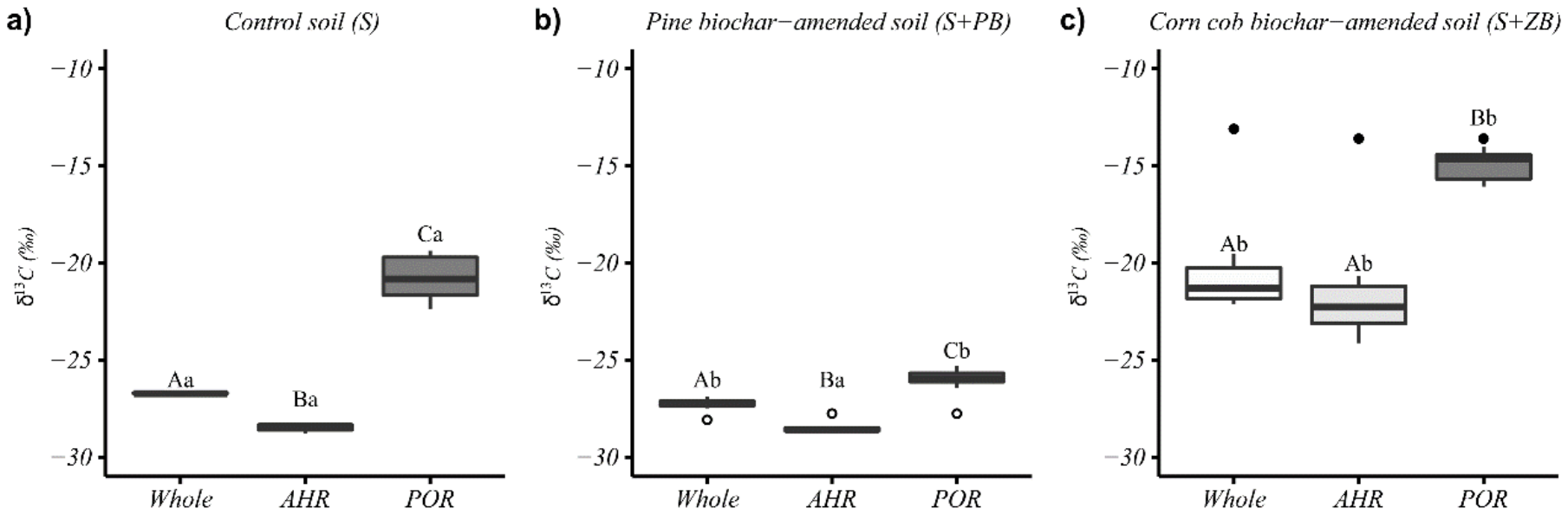

Figure 2.2 $\delta^{13}C$ (‰) of the: a) control soil (S), b) pine biochar-amended soil (S+PB) and c) corn cobs biochar-amended soil (S+ZB) – whole soil samples-, and their corresponding acid hydrolysis (AHR) and peroxide oxidation (POR) residues. Pine (with open circles) and corn cob (with closed black filling circles) biochar and their corresponding acid hydrolysis and peroxide oxidation residues are represented. Boxplots correspond to the mean values together with the bars representing the standard deviation over the three sampling times (n=3). Values sharing the capital letter indicate the lack of significant differences between chemical attack method within each biochar amendment ($p<0.05$), while equal small letters indicate the lack of differences (AHR or POR) ($p<0.05$).

2.3.2 Effect of chemical fractioning on the isotopic signal of biochars and biochar-amended soils

2.3.2.1 $\delta^{13}C$ of biochar chemically resistant fractions

For both pine and corn cob biochars, no significant differences were observed between the original biochar $\delta^{13}C$ and the corresponding chemically resistant fractions (Table 2.2). Namely, the $\delta^{13}C$ of the peroxide oxidation residue was -27.97 in PB and -13.6 in ZB, and that of the acid hydrolysis residue was -28.1 in PB and -13.1 in ZB, which are no different from those of the respective biochars as reported in Table 2.2, showing the lack of change in biochar isotopic signature through such chemical processes in these very different types of biochars.

2.3.2.2. Carbon chemically resistant fraction in biochar-amended soil samples

The amount of total carbon fraction resistant to acid hydrolysis found in S+PB and S+ZB (ca. 12 g kg^{-1} soil) was approximately twice that of the S treatment ($p=<0.001$) (Figure 2.1), while the percentages of carbon resistant to acid hydrolysis in relation to total carbon were similar in all cases (around 60%). Furthermore, the amount of carbon resistant to acid hydrolysis showed non-significant differences in any treatment over time (Table 2.3). Carbon resistant to hydrogen peroxide oxidation was significantly higher in S+PB and S+ZB compared to S (Figure 2.1) after two months of biochar application ($p=<0.001$) (Table 2.3). The percentage of C-resistant to peroxide oxidation relative to total carbon were 14.7% in S, 28.7% in S+PB and 39.7% in S+ZB, reflecting the relative enrichment in resistant C in biochar treatments upon oxidation. The amount of carbon chemically resistant to hydrogen peroxide oxidation showed non-significant differences in S and S+PB treatment over time (Table 2.3) whereas significant differences were observed in S+ZB treatment (from 50.27% to 28.29%, $p=0.035$).

2.3.2.3. Isotope signature of the carbon chemically resistant fraction in biochar-amended soil samples

The $\delta^{13}C$ of acid hydrolysis-resistant residue (AHR) from soil samples was lower than that from the non-hydrolysed ones (1-2‰ in all treatments) (Figure 2.2). Besides, the isotopic signature of the AHR residue of corn cob biochar-amended soil ($S+ZB_{AHR}$) was

significantly higher than controls (S_{AHR}) (*p=<0.001*), whereas no significant differences were observed between S_{AHR} and $S+PB_{AHR}$. Moreover, a significant decrease in $\delta^{13}C$ in $S+ZB_{AHR}$ treatment was observed over time (from -20.85 to -23.34*, p=0.001*) (Table 2.3).On the other hand, greater $\delta^{13}C$ values were observed in the resistant fractions to hydrogen peroxide oxidation (POR) compared to the corresponding whole soil samples (Figure 2.2). In general, data showed that peroxide oxidation attack had a strong isotope effect in all treatments. In one side, the isotopic signature of $S+ZB_{POR}$ was significantly higher (*p=<0.001*) than control (S_{POR}) whereas that of $S+PB_{POR}$ was significantly lower (*p=<0.001*) than S_{POR}.

2.3.3 Biochar quantification by carbon isotopic partitioning and its changes over two years period

Biochar quantification in soil samples using carbon isotopic partitioning was only possible in corn cob biochar treatment as it comes from C_4 plant metabolism and differ greatly with native organic matter which manly came from C_3 plants.

Mean corn cob biochar quantification through isotopic partitioning in the different treatments produced 6.31, 4.88 and 3.98 Mg C ha^{-1} in the whole soil, and the acid hydrolysis and peroxide oxidation residues, respectively, the latter consisting of the chemically resistant-C fraction, hence representing 63% and 77% of the biochar applied, respectively (Figure 2.3). An overestimation of the biochar-C was obtained in the soil samples of the first sampling time (two months after biochar application). Moreover, a significant decrease in biochar-C concentration was detected over time (*p=<0.001*), in both, in the whole soil samples and their respective AHR and POR resistant fractions (Figure 2.3).

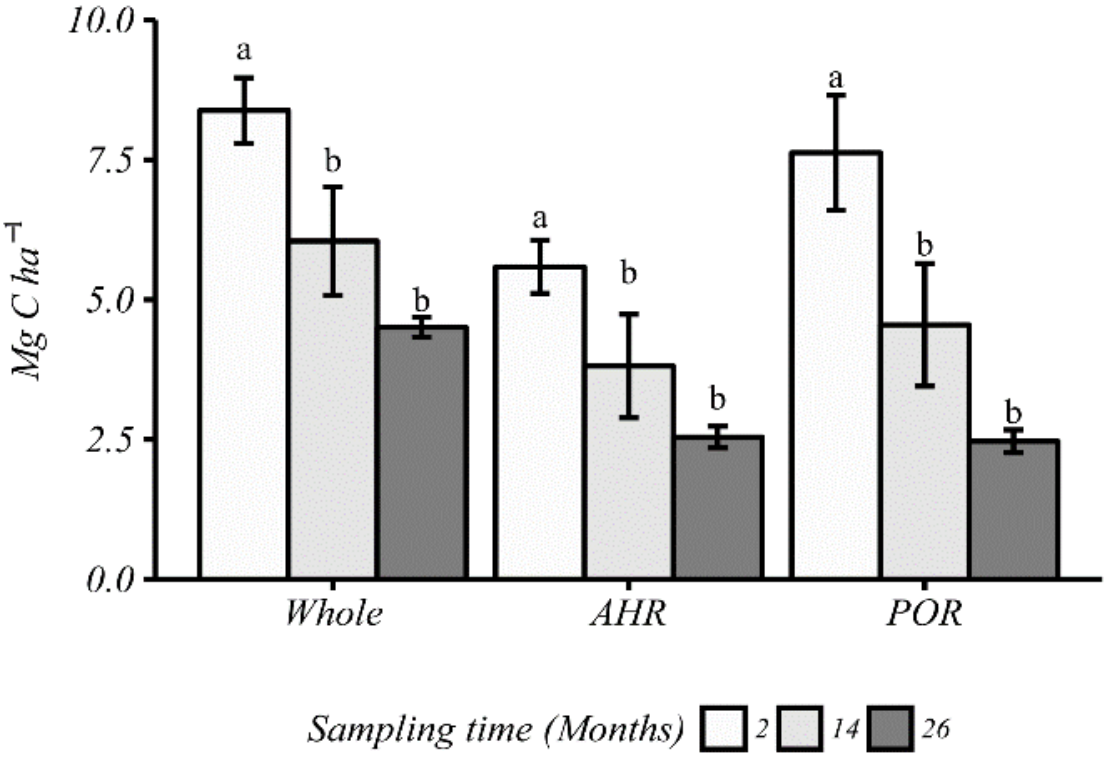

Figure 2.3 Isotopically estimated biochar-C content (Mg C ha^{-1}) in the corn cob biochar-amended soil (S+ZB), and in the chemically resistant fractions to acid hydrolysis (AHR) or peroxide oxidation (POR), at three sampling times (2, 14, and 26 months after biochar application). Bars correspond to the mean values shown together with the corresponding standard deviation (n=3). Values sharing small letters indicate the lack of significant differences between sampling times within treatments ($p<0.05$).

When the biochar-C fraction, out of total carbon, was calculated (f_{ZB}, %), a higher biochar-C proportion in peroxide oxidation residue was observed compared to whole soil ($p<0.001$), but only a slightly higher, non-significant at $p<0.05$, proportion of biochar-C in acid hydrolysis residue compared to whole soil (Table 2.4).

Table 2.4 Biochar C fraction (f_{ZB}, %) in a corn cob biochar-amended soil estimated in the whole soil sample and respective fractions resistant to acid hydrolysis (AHR) or to peroxide oxidation (POR), calculated from carbon isotopic data at three sampling times. Data correspond to the average of the three replicates together with the corresponding standard deviation (n=3). Values sharing capital letters do not ($p<0.05$) differ between sampling times for a specific chemical treatment (i.e., whole) to quantify C fraction, while values sharing small letters indicate do not ($p<0.05$) differ between methods for specific sampling times ($p<0.05$).

	Sampling times (months)	**Biochar C-fraction (f_{ZB}) (%)**
Whole soil	2	45.83±3.03 Aa
	14	35.92±5.78 Ba
	26	28.67±1.13 Ca
Acid hydrolysis residue (AHR)	2	49.41±1.96 Aa
	14	39.92±7.31 Ba
	26	33.17±4.61 Ca
Peroxide oxidation residue (POR)	2	91.72±2.80 Ab
	14	83.68±4.35 Bb
	26	68.08±2.79 Cb

2.4 Discussion

2.4.1 Feedstock and pyrolysis effects as limiting factors for biochar quantification in soils

The isotopic signatures from the C_3 plants studied were around -27 ‰ (vine leaves and shoots, native organic matter and raw and pyrolysed pine biomass) while $\delta^{13}C$ from raw and pyrolysed corn cob biomass (C_4 plant) was around -13 ‰. The fact that $\delta^{13}C$ of the control soil was consistent with a C_3 metabolism origin (Table 2.2) supports its origin from vine leaves and shoots as the main source of organic matter in this soil, which were

buried regularly by tillage, in accordance with the known continued use as a vineyard over the previous 20 years. Therefore, it was assumed that compost applied before experiment started was rapidly decomposed and did not affected to $\delta^{13}C$. Regarding the two different biochars used in this study (Table 2.1), while it is a known fact that pyrolysis is a strong thermo-chemical transformation of organic matter with selective losses of elements (H, N, O) with a consequent enrichment in C, less is known about the impact of pyrolysis on isotopic signature. Likewise, our results showed a slight decrease in the $\delta^{13}C$ between the original plant biomass and the respective char (1.42‰ and 0.95‰ for pine and corn cob respectively, Table 2.2). This results are in agreement with Bird and Ascough (2012) who attribute more ^{13}C losses in pyrolysis at temperatures higher than 300 °C as a result of loss of isotopically heavier cellulose and C=C bond formation.

As expected, biochar application significantly increased the soil total carbon content (Figure 2.1) in biochar-amended soils, and changed the isotopic signature when corn cob biochar was used, due to the C_4 metabolism of this plant (S+ZB, Figure 2.2.a and 2.2.c). However, a small but significant difference was still found between the $\delta^{13}C$ of control soil (S) and the pine biochar-amended one (S+PB), despite the fact that the organic matter derived from plants of the same metabolic pathway, due to the slight smaller delta^{13}C of the pine biochar amendment, compared with that of the soil (Figure 2.2, Table 2.2). Hence, qualitative biochar detection was possible in both biochar-amended soils (S+PB and S+ZB) regardless of their signature.

2.4.2 Impact of chemical fractioning on isotopic signature of carbon resistant

Traditionally, recalcitrant carbon in a soil and biochar-amended soils might be estimated by the carbon fraction resistant to acid hydrolysis or hydrogen peroxide oxidation, or other chemical processes such as potassium dichromate oxidation (Calvelo Pereira et al., 2011; Raya-Moreno et al., 2017). Mikutta et al. (2005) and Plante et al. (2005) observed that pyrolysed biomass is relatively resistant to hydrogen peroxide oxidation and hence proposed this as a good proxy for recalcitrant carbon. Similarly, acid hydrolysis has been used to isolate recalcitrant organic matter due to its capacity to remove carbohydrates and proteins (Paul et al., 1997), leaving other more resistant compounds such as lignin, suberin, cutin and waxes (von Lützow et al., 2007). However, the appearance of "*de novo synthesis of nonhydrolysable*" compound of ^{13}C-depleted material dominated by aromatic, alkyl and carbonyl moieties was observed by Greenfield et al. (2013). We also

observed a higher removal capacity in hydrogen peroxide oxidation of native organic carbon than by acid hydrolysis (Figure 2.1).

The biochar expected impact on isotopic signature in amended soils has not been yet reported in the biochar literature. In our study, a contrasting effect of chemical processes on carbon isotopic signature was observed in the isolated biochar materials and the biochar-amended soils. While neither acid hydrolysis nor peroxide oxidation changed the $\delta^{13}C$ of the resistant residue of pure biochars (Table 2.2), it changed in the biochar-amended soils (Figure 2.2), suggesting that such chemical methods have a differential impact on native organic matter rather than on biochar. Changes in $\delta^{13}C$ produced when whole samples were compared with their respective acid hydrolysis and hydrogen peroxide oxidation was attributable to a selective isotopic attack only to native organic matter, since (i) no isotopic changes were measured between the biochars studied and its respective AHR and POR residues (Table 2.2) and (ii) higher carbon losses (Figure 2.1) in control soil chemical residues compared to biochar treatments were found. This effect was partially described by Leavitt et al. (1996), who reported that residues from acid-hydrolysis are ^{13}C depleted due to a selective chemical processes by ^{13}C-rich molecules such as hemicellulose and proteins, and the coupled enrichment in ^{12}C-rich molecules such as lignin. Also, Greenfield et al. (2013) found that AHR residue of senescent maize was more ^{13}C-depleted compare to respective "nonhydrolysed" biomass. This trend is also in agreement with Hobbie and Werner (2004), who highlighted that differences in the isotopic signature between plant tissues as related to their main composition (e.g. C_3 woody plants are rich in ^{12}C compounds because its high content of lignin or lipids). Regarding the increase in $\delta^{13}C$ by oxidation, O'Leary (1981) reported the preferential ^{12}C losses during SOM oxidation. Some authors have pointed out that peroxide oxidation attacks compounds such as polysaccharides, with black carbon derived compounds being resistant to this oxidation (Calabi-Floody et al., 2011; Plante et al., 2005). The effects of this chemical oxidation process is somewhat comparable to that of biological decomposition, since microorganisms prefer use ^{12}C than ^{13}C during litter decomposition (Glaser, 2005; Werth and Kuzyakov, 2010).. Moreovoer, no quantitative and qualitative changes over time were observe in control soils because organic carbon inputs from litter and arable layer were relatively homogeneus at deeper < than 15 cm as a result of repetitive tilling over many years. Yet, carbon oxidation reduced substantially the carbon amount and increased significantly isotopic signature of soil organic carbon. It partially explains why the isotopic signature of POR from biochar-amended soil (S+PB and S+ZB)

were similar to their respective biochars due to an important amount of native soil carbon was lost, while biochar organic carbon remains in soil, during peroxide oxidation .

2.4.3 Medium-term validity of isotopically-based biochar quantification in soil

2.4.3.1 Temporal changes in soil isotopic signature

Over time, a decrease in $\delta^{13}C$ and carbon content in whole soil samples, acid hydrolysis and peroxide oxidation residues of the S+ZB samples was observed (Table 2.3), but not in S+PB treatment due to native soil organic matter and pine biochar $\delta^{13}C$ similarity (Table 2.3). Ehleringer et al (2000), Fernandez et al (2003), and Glaser et al (2005) suggested that the observed ^{12}C depletion in aged soil organic matter could be explained by microbial preferences during litter decomposition (^{12}C against ^{13}C). Similarly, Werth and Kuzyakov (2010) and Ehleringer et al (2000) proposed that microorganisms incorporated heavy ^{13}C into their biomass and released the lighter ^{12}C. Nevertheless, in our experiment the expected enrichment of ^{13}C was not observed over time, meaning that microbial degradation of SOM cannot be the main explanation. Some studies have failed to find a ^{13}C enrichment after C_4 plant material decomposition (Henn and Chapela, 2000), due to low content of ^{12}C molecules compared with C_3 plants, but this explanation does not apply to our study since $\delta^{13}C$ decreased over time.

Since biochar is a very stable and recalcitrant material, making it hard to detect any change in isotopic signal in a two-year field experiment, changes in the isotopic signature can only be associated with the SOM mineralisation process or by the transport of biochar particles to deeper soil layers by eluviation, or by a simple mechanical dilution produced by tilling. Many authors have described an increment of SOM mineralization or priming effect after biochar application (Wang et al., 2016; Zimmerman et al., 2011). But in this case, an increment of ^{13}C would be expected. For this reason a priming effect is not the main explanation for carbon amount diminution. Regarding biochar eluviation, some authors such as Bird et al. (1999), have suggested that biochar is divided into finer particles by the weathering process through its exposure to environmental factors such as temperature, precipitation and/or UV exposure (Spokas, 2010), which leads to its physical breaking and facilitating further surface oxidation processes (Spokas et al., 2014). Once this occurs, the fragmented biochar particles can be eluviated more easily into deeper soil layers, but water-soluble compounds can also be released by weathering and leached (Bird and Ascough, 2012; Preston and Schmidt, 2006). Even this process could have

partially occurred in our soil, the low density of the finer biochar particles, moderate rainfall of the area, and the relative short study period (two years) make internal particle transport less plausible as the main explanation for the $\delta^{13}C$ decrease. Thus, the simple mechanical dilution, i.e. a thorough incorporation of the biochar with each tilling event in a bigger soil volume than initial, could be the main explanation for the trends observed in this study. Even if this statement is plausible, further research about biochar mobilisation in deeper layers should be carried out to validate this explanation.

2.4.3.2 Verification of the isotopically-based biochar quantification approach

For the isotope-based biochar quantification, an isotopic fractionation theory of a simple two-compartment model was adopted, considering the $\delta^{13}C$ of the main organic pools in soil (SOM and biochar in this case) to be significantly different (Bernoux et al., 1998). Thus, we only applied this model to the data from corn cob biochar-amended soil. Even the two-compartment model was suitable for application to our S+ZB soil samples with a mix of C_3 (SOM) and C_4 (corn cob biochar) biomass, some limitations in carbon quantification could exist. The most relevant uncertainties that might hinder a suitable biochar carbon were: 1) possible external C incorporation from herbs growing in the field; 2) non homogeneous application of organic amendment before initiated the experiment; 3) biochemical composition (lignin, cellulose, etc.); 4) changes in $\delta^{13}C$ of SOM due to decomposition; and 5) biochar-C dilution into the soil.

Mean corn cob biochar C-fraction corresponded to 42.5% of the total soil carbon (Table 2.4), which is equivalent to 8.74 g kg^{-1} of C increment in S+ZB treatment relative to control soil (Figure 2.1). Similarly, corn cob biochar C-fraction estimated in AHR samples comprised 40% of the total soil carbon of this treatment (Table 2.4). The similarity of these results could be explained by a high proportion of non-hydrolysable components of SOM, such as lignin, which decreases the $\delta^{13}C$ in AHR samples, masking an expected isotopic signature similar to corn cob biochar (ZB). Moreover, the proportion of biochar C in POR samples was higher since biochar C is more resistant to peroxide oxidation than native organic matter (Table 2.4). In this way, biochar-C_{AHR} was more than 60% of the total biochar-C while biochar-C_{POR} accounted for more than 75%. Therefore, our results highlight that when these two chemical treatments are used, biochar-C tend to be underestimated (Figure 2.3). Conversely to Raya-Moreno et al (2017) findings where less than 10% of biochar-C was vulnerable to those chemicals procedures.

When the biochar application rate was calculated, an overestimation was obtained (Figure 2.3) in whole soil compared to the nominal 5 Mg ha^{-1} applied. This pattern could be the consequence of an unavoidable non-homogeneous biochar application in the vineyard as a consequence of the presence of vine rows where tilling cannot be carried out. The same pattern was obtained when data of AHR or POR samples were used. Hence, the results of our study suggest that, when the abovementioned limitations are not cause for concern, the suitability of isotope-based biochar quantification approaches enable, in whole soil samples, an accurate estimation of the inaccurate biochar vineyard field application rates.

2.5 Conclusions

Besides the known influence of the original feedstock on carbon isotopic signature, pyrolysis modifies the isotopic signal of the resulting biochars.

The chemical fractioning methods used for the assessment of carbon recalcitrance also change the isotopic signature: oxidation with hydrogen peroxide strongly increase $\delta^{13}C$, while acid hydrolysis decrease it slightly in soils amended with biochar.

Over time, carbon isotopic changes in a soil amended with corn cob biochar over two years seem to be more explained by mechanical dilution into the soil than as a result of decomposition and mobilisation to deeper layers of biochar particles.

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Chapter 3: Biochars addition in a Mediterranean agroecosystem: short-term divergent priming effects

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Abstract

The aim of this work was to assess the resistance of biochar to microbial decomposition and its effects on native SOM decomposition. The study was performed under field conditions in a vineyard on a sandy loam Mediterranean soil with neutral pH and low organic carbon content, amended at 6.5 g biochar kg^{-1} with two biochars obtained from pine (PB) and corn cob (ZB), and monitored over two years. Soil field samples were collected in the short- and the medium-term (2 and 26 months after the application, respectively), and then incubated in the lab for 250 additional days. The CO_2-C released as soil respiration and the CO_2-C isotopic signature were assessed after 30 and 250 days of the incubation. Additionally, dissolved organic carbon, was assessed in the original field soil samples by hot water extraction (DOC_{hw}). Such approach aimed to identify possible PE and to assess the persistence of any PE over two-years.

In the short-term, priming effects of the two biochars depended on the feedstock and pyrolysis temperature used for its production, with negative priming in wood biochars produced at high temperature and positive in grass biochars produced at low temperatures. The underlying mechanism for the short-term positive priming is the highest labile OC content in ZB biochar compared to PB. In the medium-term, the strong initial priming effects were attenuated to a slightly negative priming in both biochars, after the exhaustion of labile carbon fraction and the expected promotion of physical protection processes preventing priming.

3.1 Introduction

Biological activity of soils is often estimated by the CO2 release mostly resulting from the organic matter decomposition either in field conditions (Major et al. 2010) or laboratory incubations (Pell et al. 2006). Organic amendments generally stimulated soil biological activity depending on its origin and stability degree (i.e. their resistance to decomposition). Fresh organic wastes are rich in easily biodegradable substances that induce high CO2 emissions when applied to soil and can strongly modify soil microbiota communities (Fontaine et al. 2003). On the contrary, stable organic amendments such as mature compost contribute to soil organic matter content increases and to enhance microbial activity. When the priority is to increase soil organic carbon stocks, stable organic materials such as biochar are preferred (Nayak et al. 2015).

Biochar results from the thermochemical carbonisation of biomass in low oxygen concentration conditions and used as a soil amendment (Intergovernmental Panel on Climate Change, 2014). Its highly aromatic and condensed structure confers high resistance to abiotic and biotic degradation (Zimmerman 2010). Those properties explain the expected carbon sequestration by biochar application into soil (Nayak et al. 2015). However, the utility of biochar as a carbon sink has been largely discussed since some studies have shown that is able to promote native soil organic matter (SOM) decomposition (Kuzyakov et al. 2000; Hamer and Marschner 2005; Luo et al. 2011), in the so-called positive priming effect (PE). This trend has been also observed in other organic or mineral amendments (such as biochar, fresh biomass or other organic or inorganic materials), but alternative trends, such as neutral and negative priming effects (i.e. the native SOM decomposition slow down) have been also reported elsewhere (Bingeman et al. 1953; Dalenberg and Jager 1989; Fontaine et al. 2003; Cheng 2009).

Priming effect in biochar amended soil depends on many factors such as biochar type, and the particular microorganism community and physicochemical properties of the receiving soils. Moreover, a negative correlation biochar temperature production and PE has been described (Luo et al. 2011; Bruun et al. 2014; Wang et al. 2016a). High temperature biochars are associate to a negative PE (Cross and Sohi 2011; Malghani et al. 2013), something that has been explained by their elevate amount of polycyclic aromatic compounds (McBeath et al. 2014), and their high sorption capacity (Ahmad et al. 2014), that decrease organic matter (OM) decomposition and availability, respectively. Conversely, low temperature biochars have more easily degradable carbon structures that

stimulate microorganism's activity therefore inducing a positive PE (Wang et al. 2016b; Luo et al. 2017). Other authors have not find this relationship between pyrolysis temperature and biochar priming effect (Herath et al. 2015), indicating that other factors might influence PE in other scenarios, such the nature of the biochar feedstock (Hilscher et al. 2009; Zimmerman et al. 2011; Singh et al. 2014) ecotoxicological risks (Kloss et al. 2011); pH (Luo et al. 2011; Bruun et al. 2014; Sheng et al. 2016); aggregate composition (Kimetu et al. 2009; Liang et al. 2010), clay (Bruun et al. 2014; Wang et al. 2016a) and carbon content (Kimetu et al., 2009, Maestrini et al. 2015); or the presence of plants (Whitman et al. 2014); or environmental factors such as temperature (Fang et al. 2014) all them potentially influencing native SOM decomposition rates.

However, priming effect is not a permanent state, as it depends on some properties that fluctuate over time, such as microorganisms's OM accessibility (Kuzyakov et al. 2000). In addition, the application of fresh OM promotes fast decomposition and a "r strategy" in the microorganism communities present (Fontaine et al. 2003). After this, the remaining and more recalcitrant OM is then decomposed at a slower rate by more "K strategy" microorganisms' communities that increase their dominance over time. This partly explains why, in general, a short term positive PE and latter negative PE is found after the addition of biochar (Maestrini et al. 2015), and therefore, the duration of the experiment is important for the results interpretation (Wang et al. 2016a). The initial increment in carbon decomposition comes from biochar labile C compounds rather than from the native SOM as would be expected under a true positive PE (Cross and Sohi 2011), despite that, little fraction of biochar carbon that can be degraded. As an example, Wang et al. (2016a) reported that the labile and recalcitrant biochar carbon have a mean residence time of about 108 days (ca. 3%) and 556 years (ca. 97%), respectively. Similarly, in a two years isotopic field study in a savannah Oxisol carried out by Major et al. (2010), only 2.2% of black carbon applied was lost by respiration over the experimental period. The distinction between decomposition coming from biochar or native SOM pools can be achieved by two-component studies using isotope techniques (Werth and Kuzyakov 2010). These techniques allow determining respired CO2 contribution from biochar and native SOM by isotope partitioning estimations. Three-component studies have been also used for this purpose, adding a third isotopic signature to the assessment, such as in biochar amended soils also amended with plants biomass (Keith et al. 2011; Kerré et al. 2016; Cui et al. 2017), with organic wastes (Hamer and Marschner 2005), or with cultivated plants (Whitman et al. 2014). Most of these studies

have shown that biochar supress decomposition of added biodegradable materials that are different to native SOM. An explanation is that biochar might provide physical protection of this newly added OM by increasing soil aggregation (Kerré et al. 2016).

However, very little have been done in biochar-amended Mediterranean agroecosystems and under field conditions, with soil chronically low organic matter content and with discontinuous decomposition along the year, concentrated in the wet seasons. Furthermore, most studies on this topic are short-term incubation studies that prevent doing long-term projections (Maestrini et al. 2015).

The aim of this work was assessing the resistance of two contrasted biochars to microbial decomposition and how its application influence native SOM decomposition, under Mediterranean conditions, and in the short- and the medium-term. The study was performed in a vineyard amended with two contrasted biochars and over a two-year period. The experiment is based on laboratory incubations of field-collected samples, using CO2-C stable isotopes as tracers, in order to: 1) identify significant and contrasting PE effects for the studied biochars in the short-term; and 2) the eventual persistence of those effects in the medium-term (after two-years).

3.2. Material and methods

3.2.1 Site and soil description

This study was conducted in a *Fluventic Haploxerept* soil (Soil Survey Staff., 2014) in a 20-year-old Mediterranean vineyard located at Vimbodí i Poblet (Catalonia, NE Spain). Its organic carbon and total nitrogen contents were low (9.7 and 0.89 g kg^{-1} soil, respectively, see Raya-Moreno et al., 2017 for more details). Microbial biomass-C, estimated as the ATP / biomass-C ratio, calculated as described in Jenkinson and Oades (1979) was also low (49.9 mg C kg^{-1} soil). The vineyard was managed under ecological agriculture practices, fertilized with composted cow manure two years before the start of this study, but no fertilization was carried out during the experimental period. The only fresh organic matter input was hence vineyard pruning remains and leaves. Similarly, no agrochemicals were applied with the exception of the Bordeaux mixture treatments for fungal disease control. The vineyard was ploughed three to four times per year to control weeds.

3.2.2 Biochar characterisation

Two different biochars were tested, one obtained by a gasification process at 600-900°C of mixed pine wood splinters (*Pinus radiata* and *P. pinaster*), and the other by slow pyrolysis of corn cobs (*Zea mays*) at 450-500°C, herein named PB and ZB, respectively. Prior to field application, both biochars were sieved to 2 mm, and grounded before analysis. $\Delta^{13}C$ of ZB and PB were 28.03‰ and 13.12‰, respectively (see Raya-Moreno et al., 2017 for more details on the biochars properties).

3.2.3 Experimental design and sampling

Plots each consisting on a 10 x 8.8 m^2 area and containing 40 *Vitis vinifera* plants were randomly distributed on the vineyard. Three different treatments (in triplicate) were applied: control soil (S), soil amended with pine biochar (S+PB), or amended with corn cob biochar (S+ZB). Biochar was applied at a rate of 5 Mg C ha^{-1} of biochar (corresponding to 6.5 g kg^{-1} in the <2 mm soil fraction) at the range of many experimental biochar trials, as reported by Jeffery et al., 2011, and applied once. The biochars were uniformly distributed on the soil surface and then incorporated to the arable layer by two successive plows at a 15-cm depth. Soil samples from each plot were collected in July 2013 and July 2015, after 2 and 26 months of the biochars application. Each sample corresponded to a composite one, made of eight 4 dm^3 soil cores randomly taken (approximately 45 kg per sample) and corresponding to the top 10 cm of the arable layer. Soil samples were sieved to 5 mm in the field to separate gravels, then again sieved to 2 mm in the lab, and stored at 4°C. A representative portion of each sample was grounded and sieved to 0.02 mm to carry out some of the analyses. Laboratory analyses were carried out in triplicate.

3.2.4 Carbon dioxide released from soil samples

The organic carbon evolved as CO_2 in the soil samples was monitored over 250 days in the lab. To do so, field fresh soil samples were carefully re-moistened to 50% of its maximum water-holding capacity according to Pell et al. (2006). Then, 40 g of moistened soil sample were placed in a 150 ml-polyethylene container in turn placed into a 1 liter jar together with a CO_2 trap. The trap consisted of 5 ml of 0.2M sodium hydroxide (NaOH) in a 50 ml polyethylene cup. An additional cup with a deionized water was putted into the jars to keep saturated humidity. The jars were hermetically sealed and stored in a

dark room at 25°C during 250 days. A control jar without soil samples was used to correct the CO_2-C background concentration. The NaOH solution was periodically replaced in a geometric periodicity (more frequently in the initial months and more spread in time in the latest, see Appendix B.1). The CO_2–C evolved from microbial respiration was measured directly in the 50 ml polyethylene container through back-titration with 0.05M HCl, using phenolphthalein as indicator, after adding 15 ml of 0.05M $BaCl_2$ solution. Each soil sample was analysed in triplicate.

For the assessment of the isotopic $\delta^{13}C$ signature of the CO_2–C evolved from the same soil samples, additional replicates were prepared as previously described and placed in the dark at 25°C during 250 days in a 1 L jar sealed with a lid with a rubber septum that allowed the two sampling (after 30-d and until 250-d of incubation) of 10 ml of one month cumulative air with a syringe, then transferred to vacutainers, and injected to an isotope ratio mass spectrometer (IRMS) (MAT253, Thermo Electron Corporation, Bremen, Germany) in continuous flow mode, through the sample introduction system GasBench II (Thermo Electron Corporation, Bremen, Germany).

3.2.5 Hot water extractable carbon

Dissolved organic carbon (DOC_{hw}) was extracted following Ghani et al (2003) with some modifications. Briefly, eight grams of fresh soil (kept at 4°C) were weighed into 50 ml PTFE centrifuge tubes and extracted with 20 ml of distilled water (1:2.5 w:v) by shaking for 1 hour at room temperature on a vertical shaker at 30 rpm. Then, samples were left for 1 h at 80°C in a hot-water bath, shacked for 10 min, and then centrifuged at 10000 rpm for 10 min. After centrifugation, supernatants were decanted and filtered in filter paper Whatman® #40. This step was done twice in parallel, one to quantify the dissolved organic carbon and the other to determine the isotope signature.

Dissolved organic carbon (DOC_{hw}) was determined by potassium dichromate oxidation as follows: an aliquot (4 ml) of the supernatant was placed in a Pyrex tubes and oxidized in a mixture of 0.5 ml of 66.7 mM $K_2Cr_2O_7$ and 4 ml of a biacid mixture (H_2SO_4/H_3PO_4 on a 1:1 v/v basis). The tubes were vortexed for 15 s and heated to 150°C in a digestion block for 10 minutes. After cooling, the solution was transferred to an Erlenmeyer flask and rinsed with 90 ml of distilled water. The excess of dichromate ($Cr_2O_7^{-2}$) was back titrated with 33.3 mM of ammonium iron (II) sulphate (Mohr's salt) using diphenylamine as indicator.

For the carbon isotope signature analysis of the DOC_{hw}, the hot water extracts were filtered through a hydrophilic glass fibre filter of 0.7 µm pore size and transferred to ceramic evaporation capsules to be concentrated at 80°C until ca. one mililiter remains. During this process, ceramic capsules were gently agitated to concentrate the sample. Concentrated extract was transferred to a tin capsule for liquids and heated at 80ºC until sample was completely dry. The isotopic signature of the DOC_{hw} was determined using a Flash EA 1112 (Thermo Electron) analyzer at 1020°C.

3.2.6 Quantification of the carbon contribution attributable to biochar (f_B) and soil (f_S) by isotope partitioning

The stable isotopic partitioning was assessed through a two-compartment model, according to the approach of Werth and Kuzyakov (2010) and Whitman et al. (2014), in order to quantify the relative percentage of evolved CO_2-C attributable to soil and biochar. This approach is based on the assumption that the $\delta^{13}C$ of the biochar-amended soil ranges between that of the biochar and that of the native organic matter depending on the partial contribution of each component, estimated as follows:

Eq 3.1. Biochar-C fraction (%)

$$f_B(\%) = \left(\frac{\delta^{13}C_{S+B} - \delta^{13}C_S}{\delta^{13}C_B - \delta^{13}C_S}\right) \text{ x } 100$$

f_B: evolved carbon fraction attributable to pine or corn cob biochar, in the biochar-amended soil (S+PB or S+ZB)

$\delta^{13}C_{S+B}$: $\delta^{13}C$ of evolved CO_2 from pine or corn cob biochar-amended soil (S+PB or S+ZB) in each sampling time at each incubation periods

$\delta^{13}C_S$: $\delta^{13}C$ of evolved CO_2 from control soil (S) in each sampling time along two incubation periods

$\delta^{13}C_B$: $\delta^{13}C$ of pine or corn cob biochar (PB or ZB)

The percentage of native soil-C fraction evolved along incubation was quantified subtracting biochar-C fraction in each sampling time at each incubation periods in biochar amended soils (Eq 3.2.). The amount of evolved native soil CO_2-C was calculated by multiplying the native soil carbon fraction $(f_S(\%))$ (Eq 3.2) by the total carbon evolved in each sampling time along two incubation periods in biochar amended soils.

Eq 3.2. Native soil-C fraction (%)

$f_S(\%) = 100\text{-}f_B(\%)$

f_B: evolved CO_2-C fraction attributable to pine or corn cob biochar, in the biochar-amended soil (S+PB or S+ZB) in each sampling time at each incubation periods

f_S: evolved CO_2-C fraction attributable to native soil, in the biochar-amended soil (S+PB or S+ZB) in each sampling time at each incubation periods (Eq 3.1)

The amount of evolved CO_2-C from control soil (S) was subtracted to the amount of evolved CO_2-C of native soil-C in biochar amended soils (S+PB or S+ZB) to quantify priming-C effects in each sampling time at each incubation period.

3.2.7 Data analysis

Before statistical analysis, data were tested for normality using the Shapiro-Wilk test. For evolved CO_2-C and DOC_{hw}, global significance tests of effects of biochar amendment (S, S+PB, S+ZB) were performed using a two-way ANOVA repeated measure tests. Significant differences between biochar amendment within a sampling time, and between samplings within a biochar amendment, were assessed by using Bonferroni test at a probability level of 0.05. For isotopic signature analysis, the values of soil amended with biochar (S+PB and S+ZB) were compared with control soil (S) separately. Significance tests of effects of biochar type were performed comparing the isotopic signature of S+PB and S+ZB with S using Two-Way ANOVA RM tests. After, S+PB and S+ZB were compared with S within a sampling time using Bonferroni test at a probability level of 0.05. All the tests were carried out using R software (R Core Team 2013).

3.3 Results

3.3.1 CO_2-C evolved from soil incubation

In the 250 d incubations of the July 2013 samples, the initial CO_2-C production was high during the first 30 d of incubation, and then slow down, while this trend was not observed in the 2015 (see Appendix C.1 in the supplementary materials). Figure 3.1 shows the mean CO_2-C release day^{-1} in the 0-30 d and the 90-250 d period of the incubation, showing that respiration rates were higher in the 2-month sampling (July 2013) than in the 26 month sampling (July 2015). In the July 2013 samples, the CO_2-C evolved in 30 days from S+ZB samples was the only significantly higher than control (S) and also than the S+PB treatment ($p<0.001$), and no differences were observed in the 90-250 days

period. In the July 2015 samples, no differences between treatments were observed. Globally, releases as total CO2-C release during the incubations decreased nearly a half in 2015, two years after the biochar application (Table 3.1).

Regarding percentage of CO_2-C to TOC generally, significantly higher percentage was observed in S compared to biochar-added plot, whereas similar percentages were found in both biochar-amended soil ($p<0.01$) (Table 3.1). When the percentage of TOC released as CO_2-C along 250 incubation days was calculated, we observed that the percentage was nearly twice higher in biochar-plots than in controls, either in the 2013 and the 2015 samplings ($p<0.01$) (Table 3.1).

Table 3.1 Soil total organic carbon (TOC) and percentage of CO_2-C to TOC in a soil treated with pine biochar (S+PB) and corn cob biochar (S+ZB) compared to control (S) at two sampling times (July 2013 and July 2015) after biochar application. Mean values and standard deviation of three replicates are shown. Values sharing small letters indicate the lack of significant differences between treatments within sampling times ($p<0.05$).

Biochar treatment	Total Organic Carbon (TOC) ($g\ kg^{-1}$)	CO_2-C/TOC (%)
July 2013		
S	9.77±0.54 a	6.6 a
S+PB	16.87±2.56 b	3.9 b
S+ZB	20.21±2.37 b	5.3 a
July 2015		
S	9.80±0.85 a	3.7 a
S+PB	15.97±1.23 b	2.5 b
S+ZB	15.64±2.03 b	2.5 b

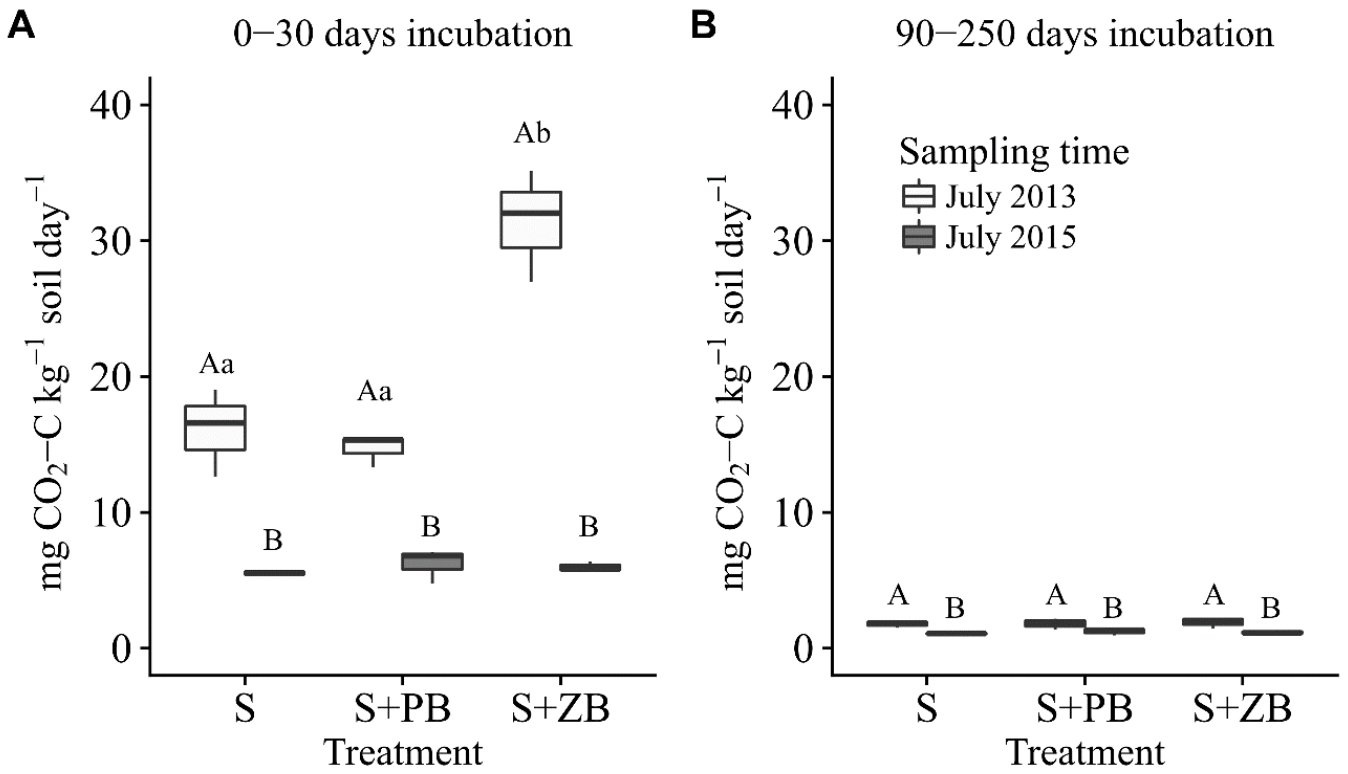

Figure 3.1 Mean CO_2-C day $^{-1}$ produced along the incubation of the field samples collected in July 2013 and 2015 (2 and 26 months after the biochar application): A) between 0 and 30 days of incubation, and B) between 90 and 250 days of incubation, in the control soil (S), and in soil treated with pine (S+PB) or corn cobs (S+ZB) biochar. Values sharing capital letters indicate the lack of significant differences between sampling times within treatments ($p<0.05$), while equal small letters imply the lack of differences between treatments within times ($p<0.05$). No significant differences were found when letters are not present

3.3.2 Dissolved organic carbon

The two biochar in this study had contrasting hydrophobicity (data not shown), with PB being highly hydrophobic compared to ZB, more hydrophilic. In agreement, the dissolved organic carbon (DOC_{hw}) concentration of PB was 0.95 g kg^{-1} of biochar and that of ZB 19.55 g kg^{-1}. When the DOC_{hw} was measured carried out in soils, the values ranged between 100 and 170 mg kg^{-1} (Figure 3.2), with significantly higher values in ZP biochar plots compared to PB biochar(*p=0.01*). Moreover, no significant differences in DOC_{hw} were observed between sampling times (July 2013 and July 2015) within each treatment (Figure 3.2).

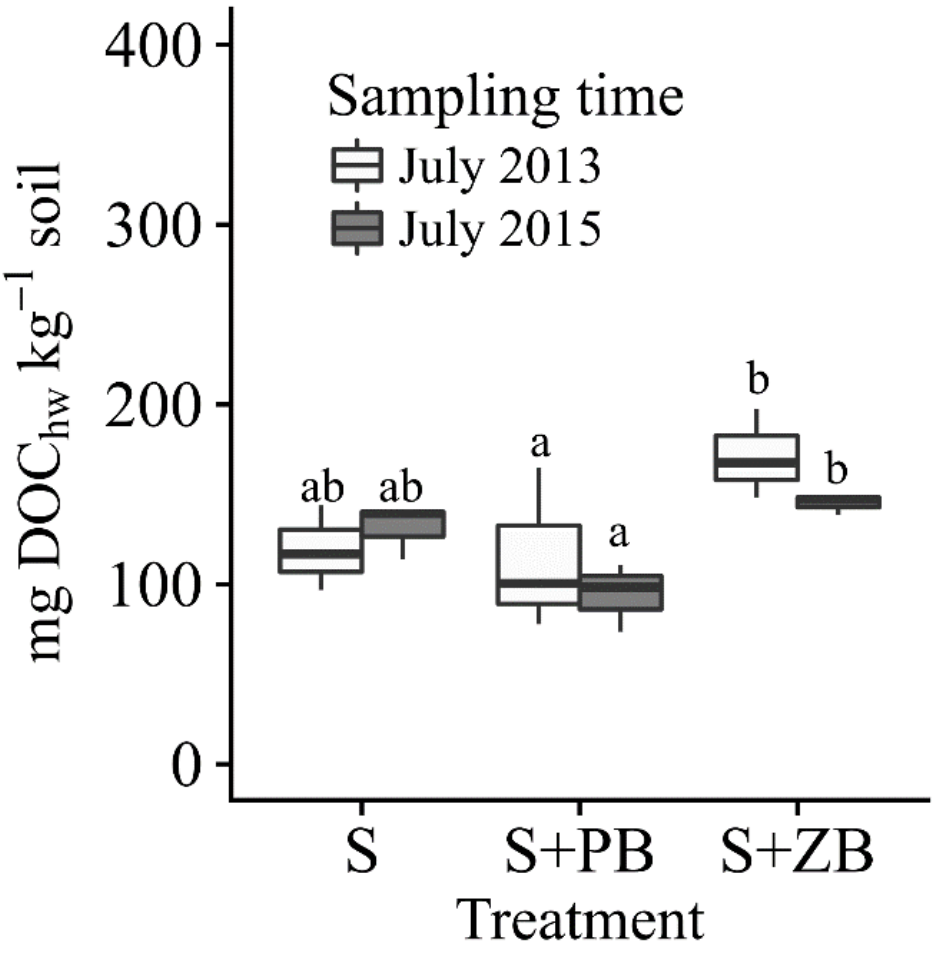

Figure 3.2 Hot water dissolved organic carbon (DOC_{hw}) content of a control soil (S) and soil treated with pine (S+PB) or corn cob (S+ZB) biochar, 2 months (July 2013) and 26 months (July 2015) after biochar application. Values sharing small letters imply the lack of differences between treatments within sampling time (*p<0.05)*. No significant differences were observed between sampling times within each treatment

When the ratio between the CO_2-C released over 30 days to DOC_{hw} was calculated, we observed that two months after biochar addition (July 2013 samples), the carbon release as CO_2 was nearly three times the DOC_{hw} whereas similar amounts were found two years after (July 2015) (Figure 3.3). The CO_2-C/DOC_{hw} ratio values between sampling times were clearly significant ($p<0.001$), but no differences between treatments were observed.

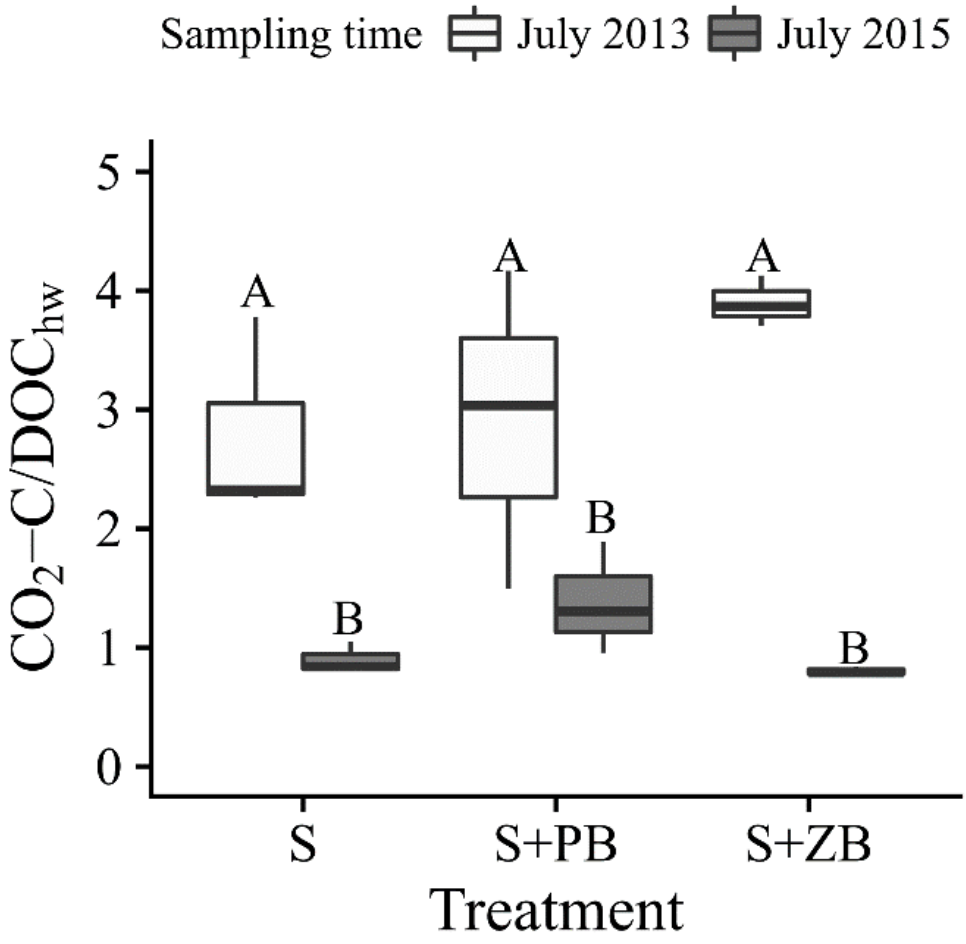

Figure 3.3 Ratio between cumulative CO_2-C respired in 30 days and DOC_{hw} of the control soil (S) and soil treated with pine (S+PB) or corn cob (S+ZB) biochar, 2 months (July 2013) and 26 months (July 2015) after biochar application. Values sharing capital letters imply the lack of differences between sampling time within treatments (*$p<0.05$)*. No significant differences were observed between treatments within sampling times

3.3.3 Carbon isotopic signature

The isotopic signature of hot water dissolved organic carbon (DOC_{hw}), and that of the CO_2-C released in the July 2013 and the July 2015 samples was assessed.

3.3.3.1 $\delta^{13}C$ of the dissolved organic carbon

Similar $\delta^{13}C$ values were found for the DOC in all treatments (Figure 3.4). However, slightly higher values were observed in S+PB compared with S ($p=0.02$), but no significant differences were detected between S and S+ZB in spite of this biochar has a higher $\delta^{13}C$ (-13.12‰ for ZB biochar). The $\delta^{13}C$ range agrees with the dominance of dissolved organic carbon derived from C_3 plants, even in the S+ZB treatment. Significant differences were observed between $\delta^{13}C$ of DOC_{hw} and that of whole soil C in all treatments.

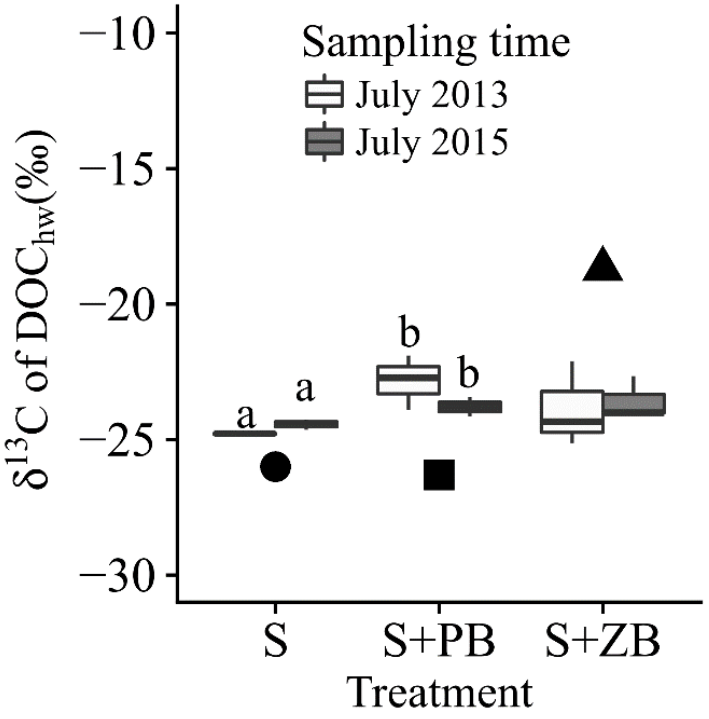

Figure 3.4 $\delta^{13}C$ of hot water dissolved organic carbon (DOC_{hw}) of a control soil (S) and soil treated with pine (S+PB) or corn cob (S+ZB) biochar, 2 months (July 2013) and 26 months (July 2015) after the biochar's application. Circles represent the mean $\delta^{13}C$ of whole control soil (S), square represent S+PB soil $\delta^{13}C$ and triangle represent S+ZB soil $\delta^{13}C$. Values sharing small letters imply the lack of differences between treatments within sampling times ($p<0.05$). No differences between sampling times were observed

3.3.3.2 $\delta^{13}C$ of the released CO_2-C

Regarding the $\delta^{13}C$ of the CO_2 released from the samples, no significant differences were found between treatments (S and S+PB or S and S+ZB) (data not shown). Similarly, no significant differences were observed between the $\delta^{13}C$ of CO_2 respired along the first month or until 250 days of incubation from soil samples collected in July 2013, being around -23‰. However, looking at the mean $\delta^{13}C$ values, there is trend for higher mean values in July 2015 in the S and S+ZB treatments (below -20‰) when compared to those of July 2013, while the opposite tendency was observed in S+PB (Figure 3.5), where $\delta^{13}C$ in 2015 samples were lower compared to 2013. The same trend was also suggested by the $\delta^{13}C$ values of the 30-d or the 250-d incubation from soil samples taken in July 2015 of S and S+ZB treatments were compared. No apparent changes were observed in S+PB soil samples in July 2015.

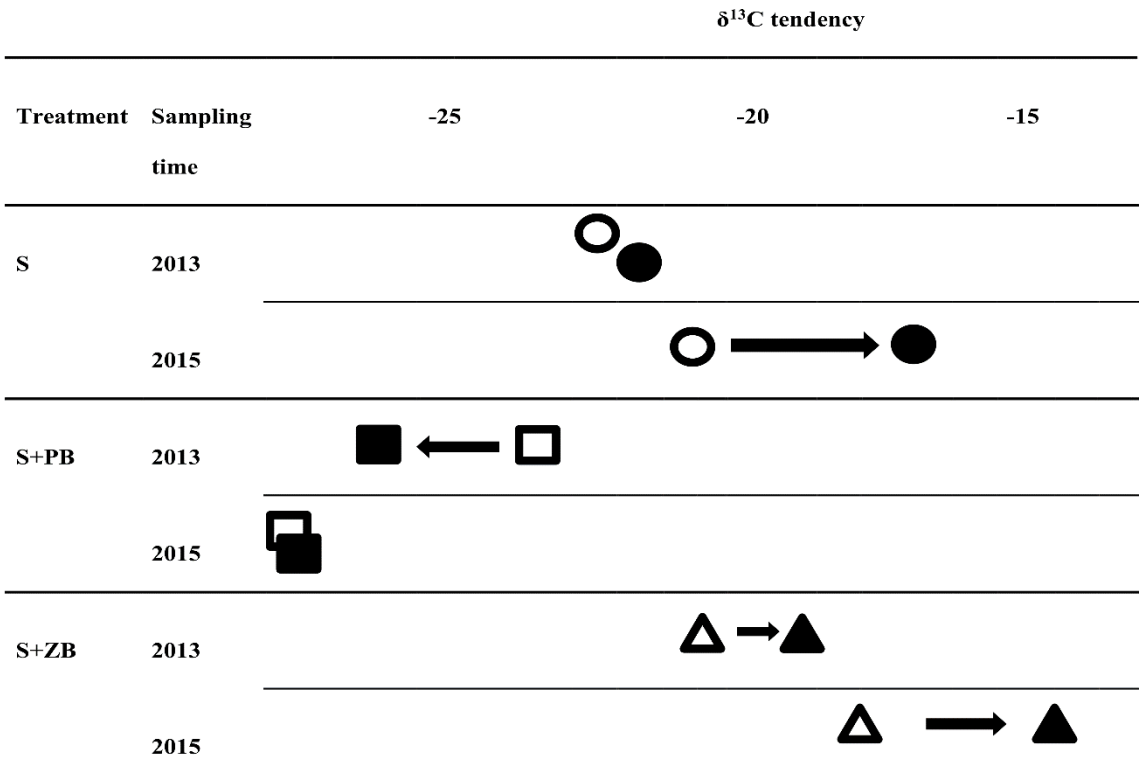

Figure 3.5 Changes of $\delta^{13}C$ tendency of evolved CO_2-C from the control soil (S) and soil treated with pine (S+PB) or corn cob (S+ZB) biochar at two sampling times (July 2013 and July 2015) measured after 30 days (white symbols) or after 250 days (black symbols) incubation. Circles, squares, and triangles correspond to the isotopic signature of control soil (S), and the S+PB, and S+ZB treatments, respectively

3.3.4. Priming effects of biochar on native soil organic C

Using the $\delta^{13}C$ mean values in Figure 3.5, and by using Eq. 3.1, we estimated that the relative contribution of each biochars to the CO_2-C released, which was relatively low and similar (17% and 18% in S+PB and S+ZB, respectively) in the 30-d incubation of the July 2013 soil samples. In addition, the CO2-C fraction from both biochars tended to increase during the laboratory incubation from day 30 to the day 250, either in the July 2013 (from 17% to 59% in S+PB and 18% to 29% in S+ZB) and the July 2015 samples (from 77% to 79% in S+PB and 36% to 71% in S+ZB). When the relative contribution was expressed as true CO_2-C release, calculated as the product of f_B (Eq 3.1) and evolved CO_2-C (Figure 3.1), we observed a decrease from the 30-d incubations (ca. 0.15 mg of biochar-C kg^{-1} day^{-1}) to the 250-d incubations (ca. 0.02 mg of biochar-C kg^{-1} day^{-1}) in both biochars.

During the 30-d incubation of the 2013 samples, native C-mineralization in S+ZB (obtained by subtracting the CO2-C released in controls to the CO_2-C derived from native organic matter in biochar treated samples, the last estimated by using the Eq 3.2) was higher than control soil (positive priming) while the opposite tendency was found in S+PB (negative priming) (Figure 3.6). Conversely, in the 30-d incubations from the 2015 samples, a lower native-C mineralisation (negative priming) was observed in both biochar amended soils compared to control soil. However, negligible changes between control and biochar treatments were found in the 250-d incubations at any sampling time (Figure 3.6).

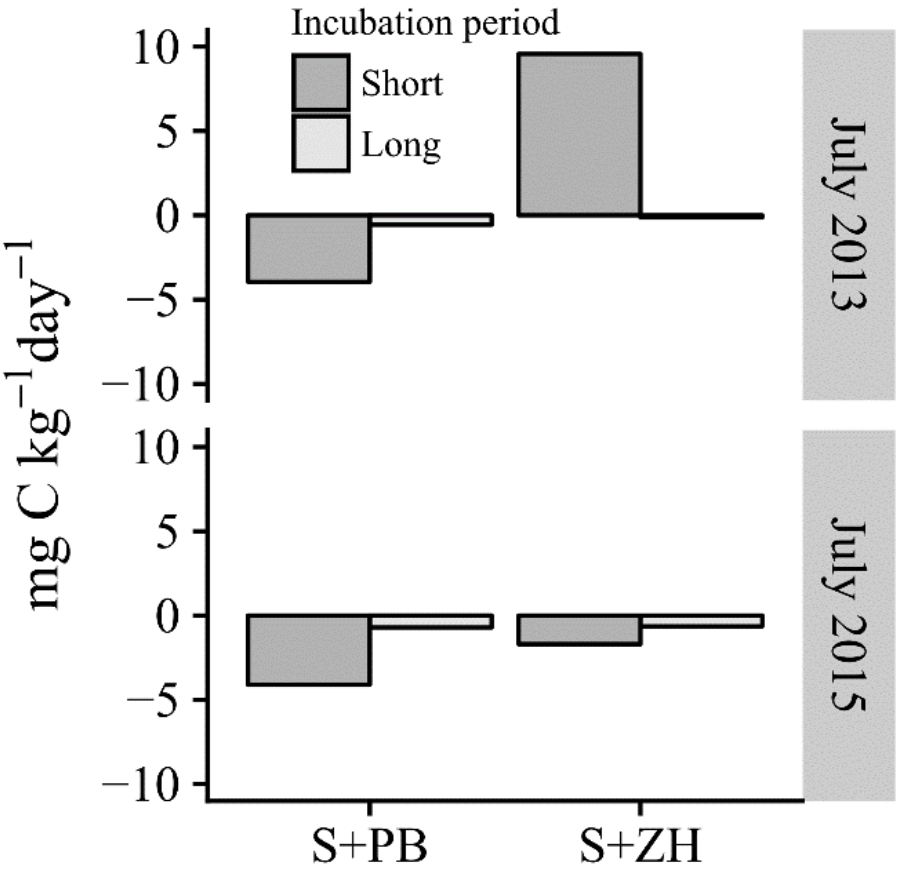

Figure 3.6 Mineralisation balance of organic C in biochar treatments (S+PB, S+ZB) as regards to control soil (mg CO_2-C kg^{-1} soil). Data obtained from the cumulated CO2-C in two incubation times (30-d and 250 d) of the field samples taken in July 2013 and July 2015 (2 and 26 months after the biochars addition)

3.4 Discussion

The addition to soils of fresh organic matter, such as plant debris, compost or manure, promotes the fast grow of specialist microorganisms that results in an initial increment of carbon mineralization (Fontaine et al. 2003) reflected as higher CO_2-C release. However, this is not always the case for biochar amendments, considered a very stable source of organic-C. The evolved CO_2-C in soils and soils amended with biochar ranged between 1.89-28.57 mg kg^{-1} day^{-1} (Shindo 1991; Luo et al. 2011; Bieganowski et al. 2013) or 0.5-8 mg kg^{-1} day^{-1} (Luo et al. 2011; Naisse et al. 2014; Herath et al. 2015), respectively, with the results from our study close to this range (0.94-35.15 mg kg^{-1} day^{-1}). Despite this expected low contribution of biochar carbon to CO_2-C releases, a small fraction of labile

biochar components is prone to decompose and could increase global CO_2-C. This could occur without affecting native soil organic carbon, as reported by Cross and Sohi (2011), or enhance or decrease its decomposition, in the so-called priming effects (PE). In our study, we found that despite around 17% of the CO2-C released came from the biochars labile fraction, two contrasted priming effects were observed in both of the biochars assessed.

Prior studies have documented such priming effects, in a phenomena that has been mostly related with the soil and biochar properties and the time scale over which measurements are made (Zimmerman et al. 2011), with most studies reporting short-term priming effects (Maestrini et al. 2015). However, very little has been reported about biochar effects on Mediterranean soils under field conditions and in the medium-term. In this study, we addressed this knowledge gap by assessing the resistance to decomposition of two different biochars and how their application affects native SOM decomposition over two years by using soil incubations and isotopic techniques.

3.4.1 Biochar origin and production temperature explain the short-term priming effects observed

Priming effects in biochar amended soils, defined as the change of native organic carbon decomposition as a result of biochar application have been widely reported in the current biochar literature (Maestrini et al. 2015; Wang et al. 2016a), with both positive or negative effects, i.e. increases or decreases in the native SOC decomposition. Different factors could affect priming, being biochar type one of the most influential (Cely et al. 2014; Maestrini et al. 2015; Wang et al. 2016a). In one hand, the addition of labile carbon resulting of biochar application could induce an increment of microbial activity promoting SOC decomposition, but on the other hand, the sorption of other organic compounds or changes in chemical (e.g. pH) and physical (e.g. increment of soil aggregation) soil properties could suppress SOC mineralization (Maestrini et al. 2015). In our study, both negative and positive priming was observed after 30-d of incubation of the 2013 samples in pine biochar plots (S+PB) and the maize biochar plots (S+ZB), respectively (Figure 3.6). The positive priming observed in ZB was coupled to a higher carbon mineralization in S-ZB plots (Figure 3.1), that was twice that of control or S+PB plots, which mostly comes from native soil organic matter mineralization, with CO_2-C coming from biochar only accounting for around 17% of the total released. In contrast, the opposite trend was observed in S+PB in the same sampling, with clear negative priming. According to Zimmerman et al. (2011) and Hilscher

et al. (2009), positive priming is expected in grass-derived biochars, but also in biochars produced at low temperatures (Cely et al. 2014; Maestrini et al. 2015), something that agrees with our study, since ZB corresponds to a corn cob biochar produced by slow pyrolysis at 450-500°C and the pine wood biochar (PB) was produced at high temperatures (600-900°C). This feedstock and production temperature effect has been reported in other similar studies, showing suppression in native organic carbon losses of hardwood and pine biochars by Zimmerman et al. (2011) and Hilscher et al. (2009), respectively. Contrasting with this generalized trend, Singh et al. (2014) reported positive priming of a *Pinus ponderosa* biochar. Other authors have described negative priming effects using grass materials (Whitman et al. 2014; Kerré et al. 2016). So, in summary, our results highlight that the short-term priming effects depend principally on the feedstock and pyrolysis temperature used for its production, in agreement with other studies (Cely et al. 2014; Maestrini et al. 2015; Wang et al. 2016a).

3.4.2 Short-term priming effects observed and underlying process

A variety of processes mechanisms proposed to explain the priming effects reported after biochar addition. In a recent meta-analysis, Maestrini et al. (2015) identified the labile fraction of biochar as the main explanation for positive PE, due to its capacity of trigger soil microorganisms' activity in turn speeding up the use of native organic matter. In the same study, the negative PE was mostly associated to an enhanced physical protection of native organic matter promoted by biochar (through adsorption to biochar or the promotion of aggregation). This is why in our study, biochar positive priming potential was assessed by measuring some proxy properties that have been associated to a higher content in labile organic matter: i) the labile carbon fraction in biochars measured by calculation the carbon isotopic signature of the CO_2 released in soil-biochar mixtures; ii) the hot soluble carbon, a measure of the most labile fraction of biochar and soil-biochar mixtures.

Regarding the labile fraction of biochar, in a previous study (Raya-Moreno et al. 2017) we showed that the ZB contained a higher content in labile carbon compared to PB as measured by using four chemical methods (strong and mild potassium dichromate oxidation, peroxide oxidation, and acid hydrolysis, see Table 1.2 in chapter 1). This agrees with the more fast decomposition rates in ZB-amended soils during the 30-d incubation (0.9g kg^{-1} of biochar day^{-1}, see section 3.2.6) of the initial field samples, when compared to the PB-amended soils (0.06g kg^{-1} of biochar day^{-1}, see section 3.2.6). Those values are similar to those reported by Whitman et al. (2014) in a 12-w greenhouse experiment, where biochar loss during the first week was 0.6g kg^{-1} of biochar day^{-1}. Those biochar decomposition rates are higher than those reported in other studies, such as Cui et

al. (2017), whom reported a mean biochar decomposition rate of 0.17g kg^{-1} in a *Oryza sativa* biochar, and Keith et al. (2011), who found similar values (0.12 g kg^{-1}).

Another measure of carbon lability is hot water dissolved carbon (DOC_{hw}) of biochars, highly correlated with biomass and activity of microbes (Ghani et al. 2003; Strosser 2010). The DOC_{hw} in biochars was higher in ZB (9.5±3.30 g C · kg biochar) compared to PB (0.29±0.07 g C · kg biochar) (unpublished data), agreeing with the chemical stability measurements reported in the previous paragraph. In agreement, in biochar plots, the significant increment of DOC after biochar application reported in other studies (Jones et al. 2011, Mukherjee and Zimmerman 2013) was only shown in ZB-amended plots (Figure 3.2). This at least mostly arises from the higher DOC_{hw} content in this biochars but also to its positive priming effect that promotes the content in native organic matter decomposition by microbial activity products. Alternatively, the higher hydrophobicity of PB compared to ZB, or a higher adsorption of DOC_{hw} on PB surface might also explain the lack of DOC_{hw} increases in PB-amended soils compared to controls (Luo et al. 2011; Ahmad et al. 2014; Kerré et al. 2016) by a physical protection mechanism. The limited release from S+PB plots was also reflected by its differential DOC_{hw} $\delta^{13}C$ compared to S and S+ZB observed (Figure 3.4), resulting in limited capacity of DOC release and negative priming in S+PB. Moreover, the similarity of $\delta^{13}C$ of DOC_{hw} in S and S+ZB treatments was feasible, since in the last case most of the DOC_{hw} came from native organic matter due to positive priming.

Alternative explanations have been provided for priming effects, such as the plausible CO_2-C release from carbonates originated during or after biochar production (Bruun et al. 2014), but this mechanism unlikely in our biochar due to its relatively low carbonate content (PB: 4,0 g kg^{-1}; ZB: 2,7 g kg^{-1}) and the neutral/basic pH of the soil that prevents any fast carbonate dissolution.

3.4.3 Persistence of priming effects in the medium-term

In the medium-term (after 26 months of biochar application), a slight negative priming tendency was observed in both S+PB and S+ZB after a 30-d incubation (Figure 3.6). This agrees with the expected long-term predominance of negative priming effects, as suggested in the meta-analysis by Maestrini et al. (2015), and probably due to the predominance of organic matter physical protection processes promoted by biochar (i.e. adsorption promoted by its porous structure) that cause a reduction in microbial activity. As an example, Zimmerman et al. (2011) observed that priming was suppressed during later incubation stages in soils amended with a grass type biochar. This might explain why the initial strong positive priming caused by ZB disappeared in the medium-term (Figure 3.6). The low amount of organic carbon mineralised after the incubation of the medium-term samples support the hypothesis of a reduced labile organic carbon availability resulting from the low carbon inputs along the two years of the field test, due to the organic

fertilization suppression during the experiment and the limited inputs from wine plant leaves, branches and roots, shown by the strong reduction in CO_2-C between field sampling times (Figure 3.1). In contrast, DOC remained without significant changes (Figure 3.2), which seeing Figure 3.3 can only be explained by a succession of microorganism's to communities able to use carbon sources other than DOC along all the experimental period and with a lower use of DOC.

In addition to the change in priming tendencies observed between samplings and increase in the relative biochar-C contribution to the CO_2 released, with higher relative values in the medium-term sampling than that observed in the short-term sampling for both biochars (from 18% to 36% in S+PB and 17% to 77% in S+ZB), but nevertheless, the amount of degraded biochar was very low. Therefore, similarly to results described by Wang et al. (2016a), we found that decomposition rate of biochar decreased with experimental duration, since in all cases biochar decomposition rate in medium incubation test was less than 0.1 g kg^{-1} of biochar day^{-1}.

3.4.4 Isotopic signature differential evolution during the 250-d incubations

For both sampling times, a trend to increased $\delta^{13}C$ was observed in 30-d vs the 250-d incubations in S and S+ZB samples (Figure 3.5), that could be attributed to a two stage process. First, the selective preferential use of ^{12}C resulting from microbial metabolism together with a ^{13}C enrichment in microbial biomass results in initially high $\delta^{13}C$ in C-CO_2. After this, and as ^{12}C source gets exhausted, this death microbial biomass was decomposed, increasing $\delta^{13}C$ of the evolved CO_2 (Glaser 2005). For this reason, $\delta^{13}C$ of incubation air increases along time (known that limited fresh organic matter inputs are present in the plots). Conversely, the opposite $\delta^{13}C$ tendency was observed in S+PB samples, for which a partial PB degradation could explain the trend for a decreased $\delta^{13}C$ of S+PB due to the natural ^{12}C richness of this biochar (Figure 3.5). However, such trends are supported not statistically since no significant differences were detected between treatments in isotopic signature values. This is probably related to the fact that: i) a simple two-compartiment model was applied, i.e. only biochar and whole native soil organic matter isotopic ratios were taken into account without considering that other organic components could be afecting this measure (as fresh shoots, leaves, and microbial biomass); ii) the evolved CO_2-C was a very small C portion of whole SOM pool. Therefore, a more detailed study including other present organic carbon sources contribution and considering higher sample volumes with more representative CO_2-C emissions could have allowed to statistically validating those trends.

3.5 Conclusions

- Priming effects were observed in a Mediterranean agricultural soil amended with a pine wood and a corn cob biochars, with clear and contrasted short-term effects depending on the biochar concerned, and with a strong attenuation of such effects to slightly negative priming after two years.
- In the short-term, priming effects of the two biochars depended on the feedstock and pyrolysis temperature used for its production, with positive priming in wood biochars produced at high temperature and negative in grass biochars produced at low temperatures.
- The underlying mechanism for the short-term positive priming is the highest labile organic carbon content in ZB biochar compared to PB.
- In the medium-term, the strong initial priming effects were strongly attenuated to slight negative priming in both biochars, as expected after the exhaustion of the more labile carbon fraction in the more labile biochar and the biochar promotion of physical protection processes preventing priming.

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Chapter 4: Organic carbon protection in soil aggregates in a biochar amended soil

Irene Raya-Moreno

Abstract

Biochar application into soil has been proposed as a tool for sequestering atmospheric carbon because of its carbon-rich structure, high resistance to decomposition and beneficial properties in soil such improvement of soil structure and stability. This study intended to determine the effects of biochar of Pine (PB) and corn cob (ZB) in soil structure, the specific objectives of this study were 1) to quantify the effects of biochars on soil aggregation stability and, 2) to quantify the implications of biochars in the distribution of organic carbon inside and outside of different aggregates fraction, in a *sandy loam* vineyard soil after 31 months of biochar application. Water-stable aggregates and particulate fraction weight were determined using a wet-sieving apparatus with distilled water and hexametaphosphate for aggregates disruption. Moreover, oxidisable and resistant OC (attributed mainly to native soil (SOC) and biochar (BOC), respectively) inside and outside of aggregates was estimated through a mass balance using mild oxidation (mO) with dichromate and elemental organic carbon content (TOC). Also, SOC and BOC contribution in corn cob biochar-amended soil was estimated by isotope analysis. No significant differences in water-stable aggregates was observed between biochar-amended and control soils. However, a tendency to increasing total organic carbon and BOC was observed inside of aggregates of biochar amended soils. Moreover, significant higher SOC inside of aggregates was observed in soils amended with ZB compare with control. Therefore, in our study was observed some clues that indicates that a portion of BOC was incorporated to aggregates and that ZB promotes SOM occlusion into aggregates.

4.1 Introduction

Biochar is a carbon-rich and highly recalcitrant material obtained by biomass pyrolysis intended to be applied to soil (International, 2017). Studies about biochar used as soil amendment have been performed in many different agricultural systems with different effects on soil properties and crop yield (Jeffery et al., 2011; Marks et al., 2016). Some of the benefits obtained after biochar application are enhancement of soil aggregate stability (Li et al., 2017), bulk density (Suliman et al., 2017), water holding capacity (Blanco-Canqui, 2017; Mukherjee and Lal, 2013) and carbon storage (Du et al., 2017). Its potential positive effect to soil aggregate stability is generally claimed in the biochar literature, although not demonstrated until recently (Gul et al., 2015).

The importance of soil structure into soil functionality has been corroborated. One of the key factors that participate in soil structure is soil aggregation that is associated to many physical soil properties such as an increment of soil porosity (Blanco-Canqui, 2017; DeLuca et al., 2015), enhancement of biochemical processes (Gupta and Germida, 2015) and also, is closely linked to soil organic matter stabilization (Kong et al., 2005). Soil organic matter (SOM) plays an important role affecting soil physical, chemical and biological properties. Its dynamic is complex to understand because there are many mechanisms involved, as SOM could be stabilized and protected from decomposition when are: i) occluded within soil aggregates (physical protection), ii) interacted with mineral particles (physicochemical protection), iii) preserved by stable molecular structures, being biochemically stabilized through the formation of recalcitrant SOM compounds (biochemical protection) (Lützow et al., 2006; Six et al., 2002; Sollins et al., 1996).

Many theories about soil aggregation processes have been formulated during the last century (Six et al., 2004), for example, Emerson (1959) suggest that SOM is cross-linked with clay through Al, Fe and hydrogen bonds in acidic soils, furthermore, Edwards and Bremner (1967) attribute microaggregates formation to association of clay (C) particles (C-P-C) or organic matter (OM) complexes (OM-P-OM) or both (OM-P-C) through polyvalent metal (P). However, there are other factors involved in soil aggregation, such as, soil fauna (Shipitalo and Protz, 1989), soil microorganisms/fungi (Degens, 1997), environmental variables (Denef et al., 2001), roots (Angers and Caron, 1998) and inorganic binding agents (Clough and Skjemstad, 2000; Imhoff et al., 2002).

Biochar used as amendment in soil could affect soil aggregation through interaction with SOM, minerals and microorganisms modifying bulk density and porosity, and therefore, soil hydraulic properties (Brockhoff et al., 2010). Stabilization mechanisms of biochar in soil are still poorly understood. Biochar might influence soil aggregation and aggregate stability, could also be protected from oxidation when is involved in organo-mineral association as proposed by Glaser et al. (2000), hence biochar occluded in aggregates allow that C-recalcitrant fraction remains protected during more time in soil. Some evidences indicate that biochar enhance soil aggregation as positive interaction between biochar, fungi (Warnock et al., 2007), organic matter and clay minerals (Kimetu and Lehmann, 2010; Solomon et al., 2012) has been described. For example, Solomon et al. (2012) observe intimate associations between black C and mineral and OM, being biochar the cross-linking agent. Moreover, Brodowski et al. (2006) find that black carbon is preferentially located inside aggregates and might act as a binding agent. In the same direction, some studies where no-aggregation improvement is found, a portion of occluded biochar organic carbon is detected (Grunwald et al., 2017; Zhang et al., 2015). The amount of biochar occluded and the effect of biochar in soil aggregation will depend on its interaction with clay minerals and native organic matter (Kimetu and Lehmann, 2010), however, it will depend on biochar type (Ajayi and Horn, 2017), feedstock and pyrolysis process (Ojeda et al., 2015), and soil type (Kelly et al., 2017, Grunwald et al., 2017). An increment of aggregate stability in soil-biochar mixture combinations (Blanco-Canqui, 2017) is find in pot incubations (Herath et al., 2013; Liu et al., 2012; Ouyang et al., 2013), grow chamber (Kelly et al., 2017), glasshouse incubations (Sun and Lu, 2014) and field experiments (Fungo et al., 2017; Ma et al., 2016; Obia et al., 2016), but no effect is observed in other works (Blanco-Canqui, 2017; Grunwald et al., 2017; Kelly et al., 2017; Sun and Lu, 2014; Zhang et al., 2015). Also, contrasting results of biochar aggregation promotion in large or small soil fractions has been described. Du et al. (2017), Ma et al. (2016) and Ouyang et al. (2013) observe an increment of macroaggregates (250–2000 μm) while Rahman et al. (2018) find an improvement in microaggregates and silt+clay fraction soil. Nevertheless, Fungo et al (2017) suggest that, in biochar-amended soils, native SOC moves from larger to aggregates of smaller-size. Further, Herath et al. (2014a) observe an increase of occluded soil organic carbon (SOC) in biochar amended soils cultivated with growing plants, increasing SOC protection to mineralization. Otherwise, Ojeda et al. (2015) no observe differences on occluded organic carbon into biochar-amended soil compare with control whereas mostly of biochar was recovered in

the free fraction , therefore , them attribute the biochar resistance to decomposition to chemical stability of biochar. This is in agreement with Fungo et al (2017) and Grunwald et al (2017) findings, as biochar is mostly recovered in soil free fraction. Then, the main factors attributed to biochar persistence are intrinsic biochar resistance to decomposition (Ojeda et al., 2015) and its occlusion in aggregates which limits (exclude) biotic access (Solomon et al., 2012). In addition, environmental conditions also affect soil aggregation process, for example, Rahman et al (2018) find that drying and wetting cycles enhance soil aggregation in biochar amended soils. Moreover, Kelly et al (2017) and Ouyang et al (2013) observe more aggregation after biochar application in weathered and organic matter rich soils. Otherwise, the effects of biochar on aggregation could be linked to its surface charge characteristic which are affected by soil pH (Cheng et al., 2006), oxidation process by aging (Archanjo et al, 2015) and also by interaction with microorganisms and SOM (Warnock et al., 2007). Therefore, after the addition of biochar to soil, a rapid aggregate stabilization by physicochemical processes could be expected.

Several methods have been used to evaluate soil aggregation being the most popular the quantification of water-stable aggregates (WSA) with wet sieving apparatus (Bourget and Kemp, 1956). Also, two useful methods for native soil and biochar organic carbon (OC) discrimination are chemical and isotope methods. In the first term, acid dichromate oxidation is a chemical method improved by Calvelo Pereira et al. (2011) to evaluate the most reactive fraction of biochar, suggesting that resistant C to that oxidation could reflect the most stable fraction and the degree of biochar aromatisation. According with Raya-Moreno et al. (2017), the combination of elemental C and mild dichromate oxidation analysis is a good proxy to discriminate native SOC and biochar. In addition, the distiction between biochar and native OC pools can be achived by two-component models using isotope techniques (Werth and Kuzyakov, 2010). These techniques allow determine OC contribution from biochar (BOC) and native SOC inside and outside of aggregates by isotope partitioning estimations. The combination of water-stable aggregates quantification with chemical and isotope methods could be a powerful tool to evaluate effects of biochar application in SOM and BOC distribution, and therefore evaluate the role of biochar in carbon storage function by physical protection.

Benefits of biochar application in a temperate regions usually are less evident than in acidic tropical soils. Moreover, most of biochar-amended soils studies are carry out by short-term incubations and only a few studies are conducted in field conditions. Thus, go

deeply into the role of biochar in soil physical properties such aggregation in temperate areas is required.

The purpose of this study was to evaluate the effects of two different biochar on soil aggregation, in a vineyard soil after 31 months of biochar application. It was hypothesized that biochar addition will result an increase of soil aggregates stability and occluded carbon into these aggregates. The specific objectives of this study were: i) to quantify the effects of two biochar on the stability of soil aggregates; ii) to estimate the implications of biochar in the distribution of organic carbon inside and outside of different aggregates fractions (5000-250 μm, 250-53 μm, <53 μm).

4.2 Materials and methods

4.2.1 Site and soil description

The study area was a 20-year-old Mediterranean vineyard located at Vimbodí-Poblet (Catalonia, NE Spain) in a *Fluventic Haploxerept* soil (Soil Survey Staff, 2014). Soil organic carbon (9.7 g kg^{-1} soil) and total nitrogen (0.89 g kg^{-1} soil) content were low. Soil texture was determined as sandy loam by USDA classification. Apparent density was 1.56±0.03 g cm^{-3} and stoniness was 63% of bulck soil. The proportion of sand (2000-50μm), silt (50-2μm) and clays (<2μm) was 58%, 27% and 15% of fine soil , respectively (see in Chapter 1 for more information).

No fertilization has been carried out during the experimental period although two years before this study started, plots were amended with composted cow manure. The vineyard has been managed according to the ecological agriculture practices. Therefore, with the exception of the *Bordeaux* mixture treatments for fungal disease control, no agrochemicals were applied. The vineyard was ploughed three or four times per year to control weeds. Vineyard pruning and natural leave fall have been the only fresh organic matter input.

4.2.2 Biochar origin and characterisation

Biochar of mixed pine wood splinters (*Pinus radiata* and *P. pinaster*) obtained by a gasification process at 600-900°C (PB), and biochar of corn cobs (*Zea mays*) produced by slow pyrolysis at 450-500°C (ZB) were tested. Both biochars were grounded and sieved to 2 mm before field application. Subsamples of both biochar were sieved with a

mesh of 0.2 mm to calculate the biochar fine particle size fraction. Granulometric analysis of biochars showed that the fraction >0.2 mm in PB and ZB were 42.7% and 93.8% respectively. Water holding capacity was 53.3% in PB while 187.6% in ZH. PB and ZB apparent density were 0.19 and 0.34 g cm^{-3}, respectively. More biochar properties are described in Raya-Moreno et al, 2017.

4.2.3 Experimental design and sampling

Plots of 10 x 8.8 m^2 containing 40 *Vitis vinifera* (4 rows of 10 plants) plants were randomly distributed on the vineyard. Three different treatments (in triplicate) were conducted: control soil (S), soil amended with pine biochar (S+PB), or amended with corn cob biochar (S+ZB). In the range reported by Jeffery et al (2011), 5 Mg C ha^{-1} of biochar (corresponding to 6.5 g C kg^{-1} in the <2 mm soil fraction) was applied into soil in a single dose. The biochars were uniformly distributed on soil surface and then incorporated into the arable layer by tilling two times at 15-cm plough depth. Soil samples from each plot were collected in January 2016, 31 months after biochar application. Each sample corresponded to a composite one, made of eight randomly distributed soil cores, each one of 4 dm^3 (approximately 45 kg per sample), corresponding to the top 10 cm of the A_p horizon. Soil samples were sieved to 5 mm in the field to separate gravels, and then stored at 4°C.

4.2.4 Wet sieving

According with Ojeda et al. (2015), a sample of 1 or 4 g of the <5 mm fraction of soil stored at 4°C were placed on 53 and 250 µm sieves, respectively, by triplicate. Sieves were putted on the Wet Sieving Apparatus (Eickeclkamp®) and immersed in distilled water during 10 min. After this period two procedures in parallel were carried out to obtain:

1) Water sieved soil fraction (WSF), which corresponds to the sum of water stable aggregates fraction (macroaggregates or microaggregates) + particulate fraction (free). It was obtained as follow: sieves containing soil samples pre-immersed in water (4 g or 1 g on 250 and 53 µm sieves, respectively) were immersed in cans filled with distilled water for 1 minute at the slow oscillation program of Wet Sieving Apparatus. Then after, without removing sieves and cans, the fast oscillation program was applied for 3 min. Finally, sieves with resistant

aggregates were dried at 40°C for 72 hours and cans with disrupted soil particles at 105°C for 24 hours.

2) Particulate fraction (free): the sieves with soil samples were placed in cans filled with a dispersing solution (2% sodium hexametaphosphate) for 1 minute with the slow oscillation program. After, without removing sieves and cans, it was applied the fast oscillation program for 9 minutes. Every three minutes, the sample retained by the sieve was shacked with a glass wand to disrupt mechanically the aggregates. Then sieves were washed with abundant distilled water to remove the dispersing solution. Finally, sieves with the retained soil particles were dried at 40ºC for 72 hours.

A representative portion of the size-fractions samples >250µm, <250µm >53µm and <53µm, before and after hexametaphosphate dispersion, were finely grounded and sieved to 0.02 mm.

4.2.5 Total organic carbon (TOC) and $\delta^{13}C$

Previously to elemental analysis, soil and soil fractions were pre-treated with hydrochloric acid to remove traces of inorganic carbon. Then, total organic carbon (TOC) content in soil samples was determined by elemental analysis using a Flash EA 1112 Elemental Analyzer at 1020°C.

To carry out the isotopic analysis of carbon, 1 mg of biochar and 35 mg of soil (whole soil and respective soil fractions samples of >250µm, > 53µm and <53µm before or after particle dispersion) were placed in tin capsules. The C isotopic signature ($\delta^{13}C$) were determined using a FlashEA 1112 (Thermo Electron) elemental analyser at 1020°C coupled to a Delta V Advantage (Thermo Electron) isotope ratio mass spectrometer (IRMS). The $\delta^{13}C$ was calculated as the carbon isotope mass ratios of each sample and corrected with that of the VPDB (Vienna Pee Dee Belemnite), in thousandths (‰) according to Eq 4.1.

Eq 4.1 Isotopic signature quantification

$$\delta^{13}C\ (‰)\ \text{sample} = \left(\frac{{C^{13}}/{C^{12}}\ \text{sample}}{{C^{13}}/{C^{12}}\ \text{VPDB}} - 1 \right) \times 1000$$

The proportion of TOC derived from biochar in each soil size fraction (>250µm, >53µm and <53µm of the wet sieving before or after particle dispersion) was calculated as follow

(Eq 4.2). The biochar-C percentage in a soil sample of the different soil fraction size (>250µm, >53µm and <53µm of the wet sieving before or after particle dispersion) was estimated by multiplying the biochar-C fraction (Eq 4.2) by the total soil organic carbon of its respective soil sample (TOC).

Eq 4.2 Biochar-C fraction

$$\text{Biochar} - \text{C fraction} = \frac{(\delta^{13}\text{C of soil fraction} - \delta^{13}\text{C of control})}{(\delta^{13}\text{C of biochar} - \delta^{13}\text{C of control})}$$

Being the δ^{13}C of control soil (S) -26.74‰, and that of the pine and corn cob biochar (PB and ZB) -28.03‰ and -13.12‰, respectively.

4.2.6 Mild oxidation of carbon by potassium dichromate

Easy oxidisable organic carbon was determined using 0,1M potassium dichromate in acid media and reported as mild oxidation (mO) carbon method (see more deatails in Chapter 1). The resultant oxidised carbon was denominated oxidisable-C (OXC) in this paper.

4.2.7 Mean weight diameter (MWD) of aggregates, water-stable aggregates and organic carbon fraction

4.2.7.1 Mean weight diameter (MWD)

Mean weight diameter was calculated as follow:

Eq 4.3 Mean weight diameter (MWD)

$$MWD = \sum_{i=1}^{n} \bar{x}_i w_i$$

Where x_i is the mean mesh-size between two consecutive sieves (mm) and w_i is the proportion of total weight of a sample retained on each sieve.

4.2.7.2 Mass balance

For estimate weight, total OC (TOC), oxidisable OC (OXC), resistant OC (ROC), OC attributed to native soil (SOC) and OC attributed to biochar (BOC) in each soil fraction (occluded and free) and size (5000-250µm, 250-23µm and <53µm) through both methods (mO and isotope partitioning) (Table 4.1) a mass balance was conducted. The respective equations used could be seen in the appendix C section.

Table 4.1 List of acronyms used in this paper

Soil fraction	
Whole	Weight or OC content of soil samples
Macroaggregates	Relative to water-stable aggregates fraction (5000-250µm)
Microaggregates	Relative to water-stable aggregates fraction (250-53µm)
Occluded	OC content of water-stable aggregates fraction
Free	Weight or OC located outside of water-stable aggregates fraction. It is equivalent to the particles or soil fraction retained on a mesh after hexametaphosphate wet sieving (particulate fraction).
WSF	Water sieved soil fraction. Weight or OC content of a soil fraction remaining on the mesh after wet sieving with distilled water. WSF include occluded in aggregates and free particles of biochar or organic matter in a soil fraction.
Variable	
OXC	Oxidisable OC by mild dichromate oxidation
TOC	Total OC of a soil fraction
ROC	Resistant OC of a soil fraction (=TOC-OXC)
SOC	OC attributed to native organic matter of soil
BOC	OC attributed to biochar
Fraction	**Size mesh interval**
$X_{5000-250}$	5000-250µm
$X_{5000-53}$	5000-53µm
X_{250-53}	250-53µm
X_{53-0}	<53µm

4.2.8 Data analysis

Before statistical analysis, data were tested for normality using the Shapiro-Wilk test. Global significance tests of effects of biochar amendment (S, S+PB, S+ZB) were performed using ANOVA tests. Significant differences between biochar treatments were assessed by using Tukey test at a probability level of 0.05. Significant differences between aggregates fraction size were assessed by using t-test at a probability level of 0.05. All the tests were carried out using R software (R Core Team, 2016).

4.3 Results

4.3.1 Distribution and mean weight diameter of water stable aggregates

Regarding aggregate size, very similar values of MWD (mm, Eq 4.3) of <5 mm soil fraction were found in all treatments ($p=0.9$) -being 1.49±0.19, 1.52±0.08 and 1.51±0.12

for control soil (S), pine-biochar amended (S+PB) and corn cob biochar amended (S+ZB) soil, respectively-, as can be seen in figure 4.1, no significant differences of the water-stable aggregates weight in macroaggregates (Eq C.1) and microaggregates (Eq C.2) were found between treatments. However, significant differences were found between the weight of water-stable aggregates size fraction (p-value=0.04) being higher in microaggregates than macroaggregates (Figure 4.1).

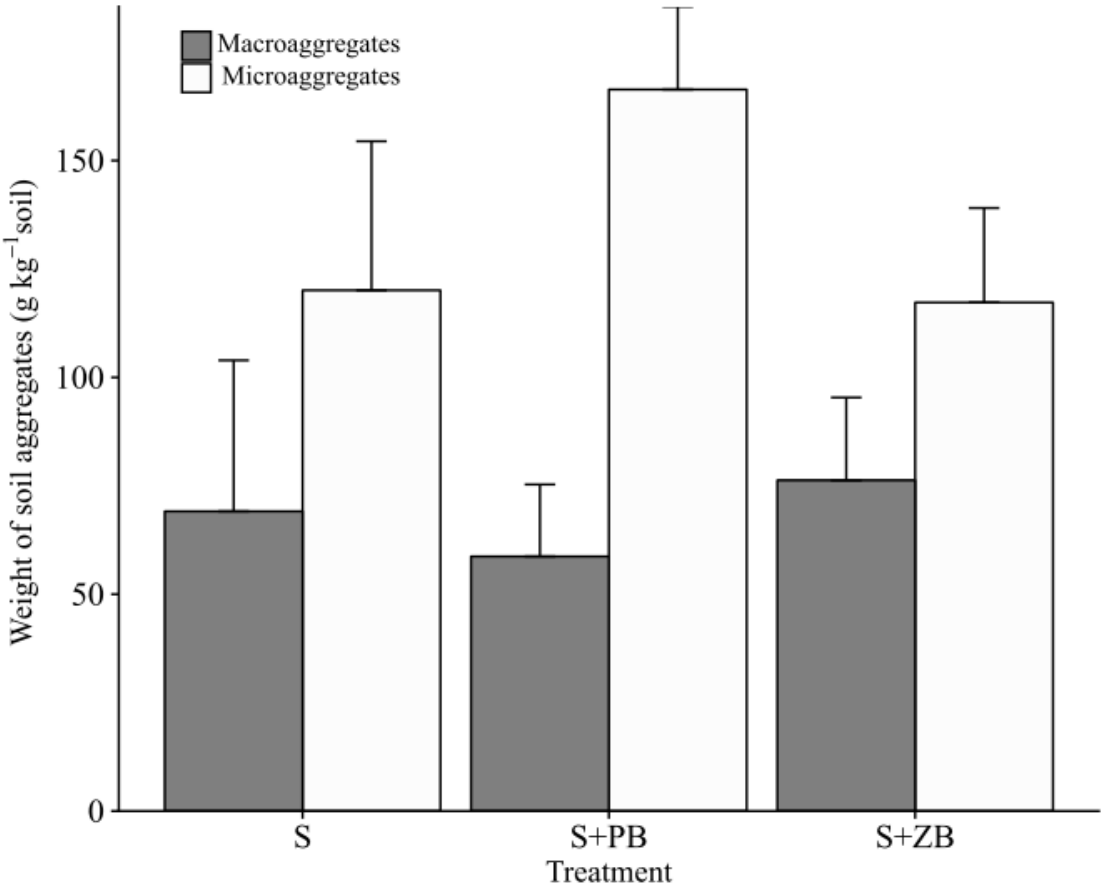

Figure 4.1. Weight of macro (5000-250µm) and micro (250-53µm) water-stable aggregates (g kg^{-1} soil) of a control soil (S) and soil treated with pine (S+PB) or corn cob (S+ZB) biochar, 31 months after biochar application.

4.3.2 Organic carbon occluded into aggregates

4.3.2.1 Total organic carbon

Significant differences in total organic carbon (TOC) of whole soil samples of S (11.68±0.75 g kg^{-1}) compared to S+PB (16.60±0.23 g kg^{-1}) and S+ZB (18.78 ±2.53 g kg^{-1}) were found (being p-value 0.02 and <0.01, respectively). Conversely, no significant

differences between treatments were found in occluded-$TOC_{5000-250}$ (Eq C.3) and occluded-TOC_{250-53} (Eq C.7) (Figure 4.2a), even though a tendency of increasing occluded-TOC in macro and micro water-stable aggregates was observed in biochar-amended soils compared to S. In any case, microaggregates contains more occluded C than macroaggregates, with significant differences between occluded-$TOC_{5000-250}$ and occluded-TOC_{250-53} (p=0.049) (Figure 4.2a).

4.3.2.1 Oxidisable carbon (mild dichromate oxidation)

Oxidisable organic carbon content (OXC) of the whole soil was very similar in all treatments (5.06 ± 0.46 g kg^{-1}) (*p= 0.578*). Likewise, no significant differences of occluded oxidisable C (occluded-OXC) between treatments in macro (Eq C.4) and microaggregates (Eq C.8) were found (Figure 4.2b). Moreover, no significant differences in occluded-OXC were observed between macro and microaggregates (Figure 4.2b). However, significantly higher amount of occluded-$OXC_{5000-53}$ (Eq C.11) –meaning the sum of occluded-OXC in macro and microaggregates- in S+ZB soil (1.15±0.22 g kg^{-1} soil) compared to S (0.49±0.23 g kg^{-1} soil) and S+PB (0.59±0.30 g kg^{-1} soil) (p=0.04) was found.

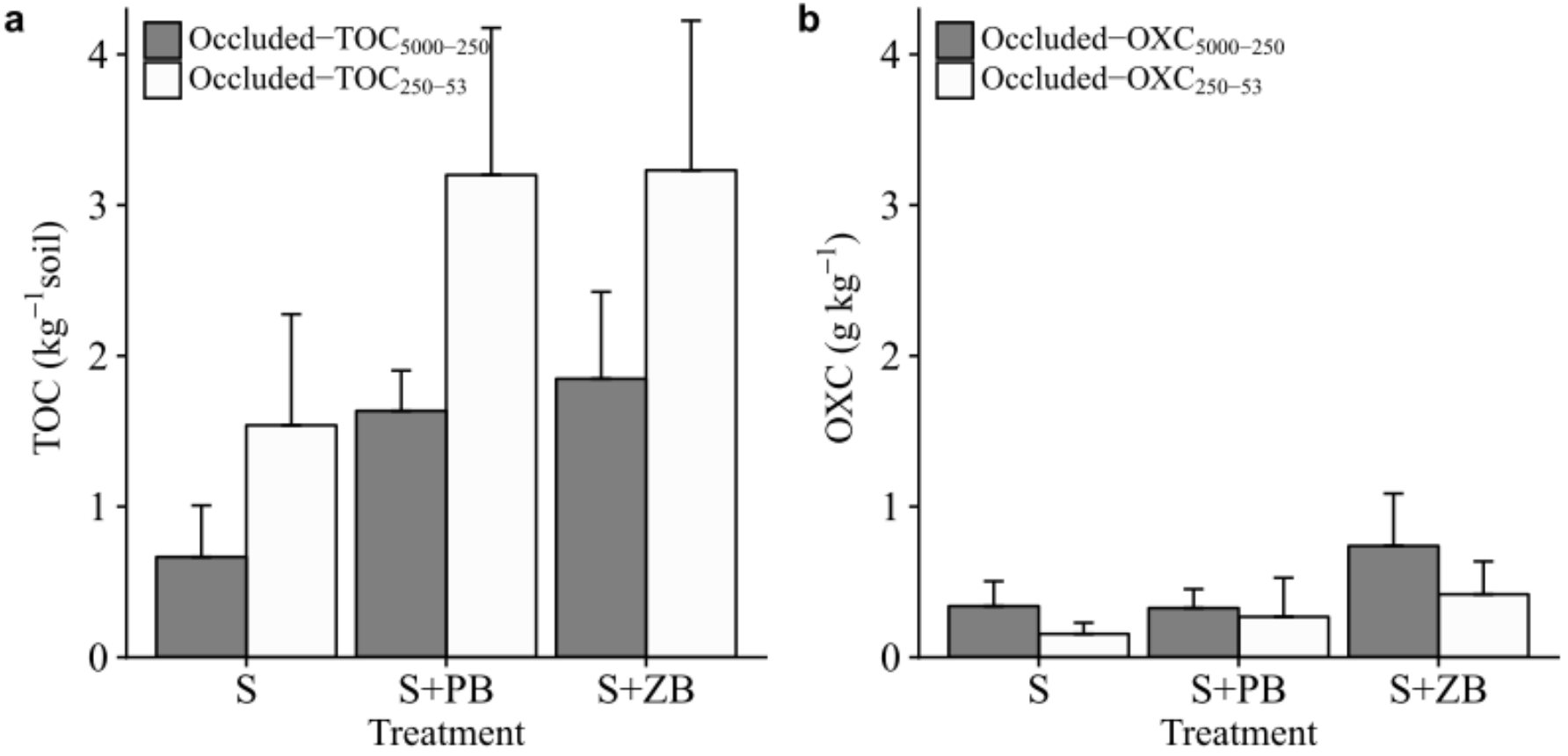

Figure 4.2 a) Total organic carbon (TOC) and b) Oxidisable organic carbon (OXC) (g kg^{-1} soil) of macro ($occluded_{5000-250}$) and micro ($occluded_{250-53}$) water-stable aggregates of a control soil (S) and soil treated with pine (S+PB) or corn cob (S+ZB) biochar, 31 months after biochar application.

4.3.3 Oxidisable vs. resistant soil organic carbon distribution in free particles and aggregate fractions

No-significant differences were observed in free and occluded OXC and ROC in the 5000-250µm (Eq C.4, Eq C.6) and 250-53µm (Eq C.8, Eq C.10) soil fractions (Figure 4.3). Similarly, no significant differences were found between treatments in OXC_{53-0} (appendix C.4.a) (1.21±0.35, 1.19±0.19 and 1.31±0.25 g kg^{-1} in S, S+PB and S+ZB, respectively) and ROC_{53-0} (appendix c.4.b) (1.99±0.36, 3.47±0.86 and 3.19±0.88 g kg^{-1} in S, S+PB and S+ZB, respectively) in <53µm soil fraction. However, significantly higher free-$ROC_{5000-53}$ amount (Eq C.14) –meaning the sum of free-ROC soil in 5000-250µm (Eq C.5) and 250-53µm (Eq C. 10) fractions- in S+ZB compared to S was found (*p=0.01*). Moreover, similar values of free-$OXC_{5000-53}$ (Eq C.12) were found in all the treatments (Figure 4.3). Also, higher values of occluded-$ROC_{5000-53}$ (Eq C.13)and ROC_{53-0} (appendix c.4.b) were observed in biochar-amended soils (especially in S+PB) compared to S, although, no-significant differences between treatments were detected.

In addition, similar ROC attributed to biochar organic carbon (BOC-ROC, Eq C.15) amount in both amended soil (6.61±2.69 and 7.7±3.13 g kg^{-1} soil in S+PB and S+ZB, respectively) was observed.

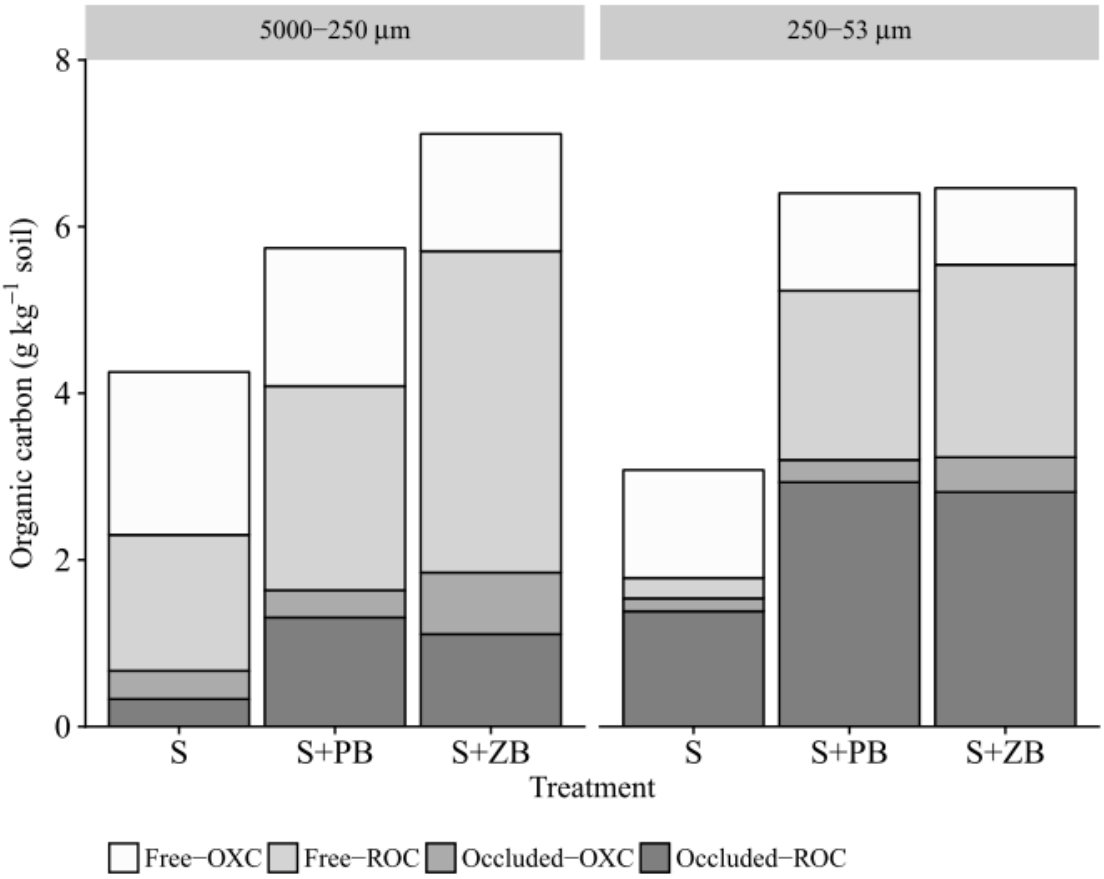

Figure 4.3 Resistant (ROC) and oxidisable (OXC) organic carbon (g kg^{-1} soil) of three fraction size of water-stable aggregates (5000-250 µm; 250-53 µm and <53 µm) and its distribution inside (occluded) and outside (free) aggregates of a control soil (S) and soil treated with pine (S+PB) or corn cob (S+ZB) biochar, 31 months after biochar application.

4.3.4 Biochar and soil organic carbon contribution to soil aggregation

Significant differences between treatments were observed in free-$SOC_{5000-53}$ (Eq C.23) (*p=0.04*) (Table 4.2). However, the amount of native soil organic carbon (SOC) in water-stable aggregates estimated by isotope partitioning method (occluded-$SOC_{5000-53}$, Eq C.22) in S+ZB was 1.07 g kg^{-1} higher than control, although no-significant differences were detected between these treatments (Table 4.2). In addition, estimated organic carbon attributed to corn cob biochar in whole soil (BOC, Eq C.26) was 6.32 g kg^{-1} (Table 4.2).

Table 4.2 Organic carbon attributed to native soil (SOC) and biochar (BOC) (g kg^{-1}) estimated using $\delta^{13}C$ signature and TOC: 1) occluded in water-stable aggregates of two fraction size ($occluded_{5000-250}$, $occluded_{250-53}$); 2) particulate carbon outside of aggregates of two fraction size ($free_{5000-250}$ and $free_{250-53}$); and 3) $fraction_{53-0}$ of the control soil (S) and soil amended with corn cob biochar (S+ZB) 31 months after biochar application. Data correspond to the mean and sd of three replicates (n=3).

Treatment	Origin	$Occluded_{5000-250}$	$Occluded_{250-53}$	$Free_{5000-250}$	$Free_{250-53}$	f_{53-0}
S	SOC	0.67±0.58	2.16±1.21	2.74±0.17	0.5±0.85	3.20±0.35
S+ZB	BOC	0.70±0.60	0.86±0.80	1.80±0.23	1.98±0.79	0.98±0.45
	SOC	1.20±0.42	2.70±0.37	2.13±0.22	3.28±0.35	3.51±0.68

4.4 Discussion

4.4.1 Aggregates stability and OC in biochar-amended soils

Our results showed that the two biochars did not have noticeable effects in the aggregates stability as no significant differences were found with control in the weight of water-stable aggregates (Figure 4.1) and MWD. The lack of significant effects can be explained probably by the relatively low biochar dose applied to the field experiment. Nonetheless, it is important highlight that the biochar dosage used was low because it was intended to represent a realistic agricultural scenario (Jeffery et al. 2011). These results are consistent with Zhang et al. (2017) who do not observe changes in soil aggregation with biochar similar dose (8t ha^{-1}) whereas it does at higher one (16 t ha^{-1}). However, a tendency of increasing occluded-TOC in biochar amended soil was observed (Figure 4.2a), suggesting that BOC of corn cob and pine biochars was incorporated into aggregates. Moreover, Grunwald et al. (2017) -in an experiment related to climate change where soil temperature is increased 2.5^{0}C and 30 t ha^{-1} of *Miscanthus* biochar is applied- observe that biochar is incorporated into aggregates, increasing 10% of total OC. Moreover, Zhang et al (2017) find higher amount of OC in macroaggregates while Kelly (2017) observe an increase of OC in microaggregates. These diverse results could be attributed to different physical and physicochemical characteristics of the biochars and soils studied. However, as we

explained later, the increasing TOC tendency observed was the combination of SOC and BOC occlusion, similarly to the findings described in a 6-year study by Du et al. (2017).

4.4.2 SOM and BOC distribution in soil aggregates

Contrasting results of the distribution of OC from native soil (SOC) and biochar (BOC) inside and outside of aggregates were found in both biochar-amended soils. Firstly, higher SOC (OC attributed to native soil) was detected in S+ZB aggregates (Figure 4.2b and Table 4.2) compared to S+PB and S (Figure 4.2b). The divergent results between the two biochar amended soils could be attributed to biochar properties resulting of different feedstocks and pyrolysis processes. These results are in agreement with Sun and Lu (2014) study who find an increment of macro-aggregates in soils amended with straw biochar, whereas do not observe effects in soils amended with woodchip biochar. The underlying process in S+ZB treatment that explain the increment of SOM inside of aggregates was the promotion of microbial activity after biochar application. In our previous studies, a short term positive priming effect after ZB application has been observed while the opposite has been found in PB. The enhancement or suppression of microbial activity seems to be biochar type dependent (Zimmerman et al., 2011) that agrees with the factors of soil aggregate promotion discussed before. Therefore, the increment of SOC inside and outside the small aggregate fractions of S+ZB (Table 4.2) could be justified by the microbial biomass grown. In addition, microbial decomposition processes can increase C=O groups in biochar surface promoting links with soil particles (mineral and organic matter) (Grunwald et al., 2017). Moreover, the combination of the increasing linkage opportunities, microbial grown and the products from microbial activity can explain the increment of SOC found inside and outside of soil aggregates on S+ZB treatment (Table 4.2) and can enhance soil aggregation (Grunwald et al., 2017). It is in agreement with Sarker et al (2018) who observe large aggregation stability when decomposable OC is applied, although, those aggregates have less persistence than those promoted by cellulose-rich material because the last is more resistant to decomposition. Otherwise, native $\delta^{13}C$ (Table 4.2) and free-ROC (Figure 4.3) of S+ZB treatment indicated that corn cob BOC was found mainly in the free fraction as the relatively big particle size of ZB limits biochar occlusion. It is in agreement with other studies where most of biochar remained in non-occluded fraction (Grunwald et al., 2017). Nevertheless, an opposite tendency of increasing occluded-ROC in S+PB was observed. There are several factors that can contribute to the preferential occlusion of PB BOC such as

hydrophobicity, small particle size and sorption properties. Mainly, PB biochar is hydrophobic as a consequence of the high temperatures during pyrolysis process resulting in a high biochar surface sorption and relative large particles surface area (Zheng et al., 2018). Also, Li et al (2017) concluded that the sorptive surface of biochar facilitate the formation of cationic bridges. Moreover, the small BP particle size increased the possibility of being occluded inside of preformed aggregates. At the same time, the high surface could enables elevated content of exchangeable cations (e.g. Ca^{2+}, Mg^{2+}) and the formation of cationic bridges between SOM and biochar (Zheng et al., 2018). As well, biochar can be flocculated via van der Vaals forces and interparticles strength (Ajayi and Horn, 2017). Moreover, according to Li et al (2017), biochar enhance inter-granular porosity in microenvironments increasing soil organic matter accumulation and improving long term soil aggregation. Therefore, aromatic C fraction could be protected inside of aggregates due to water repellency and internal binding (Sarker et al., 2018). Our results agree with this findings, as a tendency of increasing occluded PB BOC inside aggregates was observed as a result of the high hydrophobicity and sorption capacity of PB. Nevertheless, according to Kelly et al., (2017) biochar might sorb the microbial products, although the biological factor is discarded when PB was applied because negative priming effect was observed in previous studies.

In addition, a great portion of biochar was recovered in <53 μm fraction. It is plausible that both biochars had been breaked down in small pieces as a result of microbial activity and environmental agents. The environmental exposition could change biochar particles surface, oxidising aromatic components (e.g. wetting and drying cycles, microbial and rhizosphere activity…) breaking biochar in small pieces that can be loosed to deeper soil layers. However, oxidation process could increase carboxyl and other functional groups density in biochar surface (Archanjo et al., 2015; Zheng et al., 2018) resulting in a increment of links between minerals, organic matter and biochar (Zheng et al., 2018) that promote soil aggregation. In the same way, it has been observed that black carbon from Amazonian dark earth (*terra preta*), formed as a result of ancient anthropic activity (mainly pyrogenic waste from cooking), is basically as a carbon skeleton, with spread O and Fe, Al, Si, Ca and P located in the shell (Archanjo et al., 2015). The element enrichment found in the surface is principally the result of aging processes (Archanjo et al., 2015). At the same time, other environmental agents could affect soil aggregation process. For example, according to Rahman et al (2018) soil wetting-drying cycles improve C stabilization in microaggregates of a Vertisol amended with straw biochar

amended. In our study area, summer drought is an environmental climate characteristic, therefore, it was expected that biochar increased soil aggregates stabilization, however no effects were observed. Another factor is soil pH that changes the availability of bivalent particles (mainly metals) in soil solution which can act as binding agents. For example, in acid soils biochar carboxyl groups might be associated to Al promoting soil structure (Li et al., 2017). Moreover, other authors suggest that interaction between biochar and organic matter could be pH- dependent (Kelly et al., 2017; Yu et al., 2016). This factor did not affect to our soil as has a neutral pH, however cannot be discarded the Ca^{++} cation as a binding (Moreno-Barriga et al., 2017).

4.4.3 Dosage and effects of biochar in soil aggregation

The estimated BOC recovered in the different soil fractions, calculated trough a mass balance, was close to the biochar amount applied to amended soils (6.5g kg^{-1}). However, an overestimation was detected in ZB treatment estimated with mild oxidation method (7.7 g kg^{-1}) while this was not observed in BOC estimated by isotope method (6.3 g kg^{-1}). As it is said above, it seems that ZB promote SOC incorporation into aggregates. Then, the overestimation could be explained by the protection of resistant SOC occluded in S+ZB aggregates compared to S and PB treatments and, consequently, increases attributed ROC BOC estimated with this mild oxidation method. Therefore, the lack of significance found in this study cannot be explained by biochar losses. Otherwise, the lack of biochar effect was attributed mainly to the low dosage used. Other studies suggest that biochar effects might be dependent on the rate of biochar applied (Zhang et al., 2017), e.g. Huang et al. (2017) attributed the lack of response of biochar applied at 10 t ha^{-1} while Li et al. (2017) observe that the optimal dosage of biochar to obtain great results in soil aggregation stability was 40 t ha^{-1}. The dosage used in this experiment was 5 t C ha^{-1}. It seems that biochar beneficial effects will be greately detected when optimal biochar dose is applied (which depend on soil and biochar type), too low or high biochar amount application could result in no significant (our study) or negative aggregation effects (Ajayi and Horn, 2017). Nevertheless, our study highlights that the benefits to improve soil physical and chemical properties which have been reported in incubation studies may not be achieved by using a relatively low but realistic biochar application rate of 5 t C ha^{-1} in field conditions, at least at the medium term (2 years) and with single application.

4.5 Conclusion

In conclusion, no effects of pine and corn cob biochar were observed in soil aggregation on a vineyard soil after 31 months of biochar application. The lack of effect was attributed to the small biochar dose applied. However, contrasting OC distribution was observed depending of biochar type (feedstock and pyrolysis). Corn cob biochar significantly promoted the occlusion of native soil organic carbon into aggregates being the more probable reason the enhancement of microbial activity. In contrast, pine biochar due to its small particle size, hydrophobicity and surface sorption properties tends to increase occluded BOC in soil aggregates suggesting that the preferential location of this biochar was inside of aggregates.

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General Discussion

The main aim of this thesis was to assess the effects of pine (PB) and corn cob (ZB) biochars on soil organic carbon persistence in a Mediterranean agricultural soil using chemical (Chapters 1, 2), biological (Chapter 3), and physical (Chapter 4) methodological approaches. Biochar has been proposed as a global change mitigation solution, as part of a C-negative strategy, as its application on soil could immobilize large amounts of soil organic C at a millennia time scale (Cheng et al., 2006; Qin et al., 2016). However, contrasting results have been described in different biochar amended soils as the effects of biochar on soil carbon content and dynamics are both biochar (Fang et al., 2014; Singh et al., 2012) and soil dependent (Bird et al., 1999; Lützow et al., 2006). Additionally, benefits of biochar application in temperate regions are usually less evident than in acidic unfertile tropical soils. Moreover, most biochar-amended soils studies have been carried out in short-term laboratory experiments with high rates of biochar addition, with only a few studies conducted in field conditions with realistic doses and assessment in the medium-term. In this thesis we have used chemical, physical and biological methods to study in detail the properties and impacts of two very different biochars in carbon persistence, applied at realistic agricultural doses in a field experiment conducted on a Mediterranean soil over two years.

Key properties of biochars to understand its effects on a Mediterranean agricultural soil

Biochar properties depend on the feedstock and pyrolysis process conditions (temperature, pressure, heating rate, reaction and residence time). However, in our study even though the two biochars came from very different feedstocks and pyrolysis processes, no noticeable differences were observed in total organic carbon (789.4 and 783.1 g kg^{-1} in PB and ZB, respectively), O/C ratio (0.10) and ash content (91 g kg^{-1}) (Table 1). This similarity could be explained by the usage of "pure" biomass which consisted in pine wood without tree bark and corn cob without kernels to produce PB and ZB, respectively. However, differences in H/C ratio indicate differences in aromaticity, with PB being richer in aromatic-C content. According to Downie et al. (2009), C-aromaticity increases with increasing pyrolysis temperatures because the fundamental physical changes (i.e. the release of volatiles, the formation of intermediate melts and the volatilization of the intermediate melts) and molecular rearrangement are both

temperature dependent. This agrees with our results as higher temperatures were applied in the gasification process used to obtain PB (600-900°C), which has lower H/C values and therefore higher C-aromaticity than in the slow pyrolysis used to obtain ZB (450-500°C) (0.19 and 0.29 in PB and ZB, respectively) (Table 1). In any case, H/C of both biochars was less than 0.3 therefore indicating high aromatic-C content (Krull et al., 2009). Other contrasting properties were observed between biochars, for example, particle size analysis showed that the fractions >0.2 mm in PB and ZB were 42.7% and 93.8% respectively (Chapter 4). The small particle size observed in PB was the consequence of high temperatures which cause shrinkage stresses followed by biochar cracking as a result of microstructural rearrangement and the differential decomposition rate between the biochar surface and matrix (Downie et al., 2009). In contrast, the large particle size observed in ZB was attributed to a lower pyrolysis temperature and long reaction time (2 hours). As explained by Downie et al. (2009), the low temperatures could cause a low amount of continuity of pores due to synthesis of tars from thermal decomposition of biomass particles. In contrast, high temperatures achieved during PB pyrolysis allowed the volatilization of tar components increasing pore accessibility. Also, the incorporation of mineral ash (e. g. Na, K, Ca, Mg, P, S, Si) into the biochar matrix increases with pyrolysis temperatures, however they were more accessible (located in more superficial places) in biochars produced at low pyrolysis temperatures. The availability of mineral ash increases electrical conductivity with higher values measured in ZB (2.54 dS m^{-1} 25°C) compared to PB (0.69 dS m^{-1} 25°C). Moreover, biochar surface might be oxidized by the aging process, increasing ionizable functional groups, and therefore charge density, on the biochar surface (Sorrenti and Toselli, 2016). Also, higher functional group content on the biochar surface increases the hydrophilicity of biochar. In our study PB showed hydrophobic properties while ZB was more hydrophilic (Chapter 1).

In addition, a higher amount of dissolved organic carbon was measured in corn cob biochar (11.15 g kg^{-1}) compared to PB (1.13 g kg^{-1}) (Chapter 3). The labile carbon available was more abundant in corn cob than in pine biochar, and could be explained mainly by the different temperatures used in pyrolysis process, although this represented a very small biochar carbon fraction. However, this minor biochar carbon fraction was a key difference to understanding the contrasting effect on native soil organic matter of both biochars and in the promotion of microbial activity and soil aggregation. In addition, a very small but similar proportion of easily oxidisable organic carbon (dichromate mild

oxidation) was found in both biochars (44 g kg^{-1}) corroborating biochar resistance to chemical reagents (Chapter 1).

Therefore, to understand the effects on native soil organic carbon the key (sometimes contrasting) properties of both biochars are summarized in table 1.

Table 1 Elemental analysis, molar ratios and chemical properties of biochar from pine wood (PB) and corn cob (ZB). Electrical conductivity (EC), total carbon (TC), hydrogen (H), total organic oxygen (O), ash, O/C and H/C molar ratios, dissolved organic carbon with hot water (dissolved-C), organic carbon destroyed by mild potassium dichromate oxidation (mO, 0.1M $K_2Cr_2O_7$ / 2M H_2SO_4 and oxidised at 60°C for 8 h) and percentage of particle size higher >0.2 mm.

Biochar feedstock	**Pine (PB)**	**Corn cob (ZB)**
EC (dS m^{-1} 25°C)	0.69	2.54
TC (g kg^{-1})	793.4	785.8
H (g kg^{-1})	12.2	19.1
O (g kg^{-1})	90.15	89.36
Ash (g kg^{-1})	91.9	91.1
H/C	0.19	0.29
O/C	0.11	0.11
Dissolved-C (g kg^{-1})	1.13	11.15
mO (g kg^{-1})	46.82	43.69
Particles >0.2 mm (%)	42.7	93.8

Contribution of biochar to soil carbon resistance, microbial availability and protection

Biochar application greatly increases resistant organic carbon (ROC) amount in soils as a result of its high innate chemical stability (Chapter 1). Our results showed that while application of both biochars had a significant positive effect on ROC compared to the control (Chapter 1), there was no difference between the two types of biochar, with similar amounts of ROC, estimated by chemical methods (AH, LPO and mO), for PB and ZB (Chapter 1). Moreover, some clues about the cause of this result can be deduced from other biochar properties, such as the H/C ratio that was lower than <0.3 for both PB and ZB, and which is associated with a high aromatic-C content (Krull et al., 2009). Innate

biochar resistance to chemical agents (e. g. $K_2Cr_2O_7$, HCl and H_2O_2) in the medium-term was corroborated as no significant loss of ROC in biochar amended soil over two years was observed (Chapter 1). Moreover, biochar carbon estimated using the different thermo-chemical methods through a mass balance method (using data from biochar-amended and control soils of TOC, LOI, mO and AH methods) was similar to the real amount of biochar applied (6.5 g Kg^{-1}). These results corroborate the innate biochar resistance to thermo-chemical methods after two years of its soil application (Chapter 1). However, our results also indicate that biochar could be partially oxidised as it was slightly underestimated by LPO methods (Chapter 1). This agrees with other authors who have suggested that biochar could suffer an aging process (mainly surface oxidation) after its application, due to environmental exposure (by weathering and drying-wetting cycles) or by partial microbial degradation (Sorrenti and Toselli, 2016). In addition, a decrease in biochar carbon concentration over two years was detected when corn cob biochar presence was estimated by isotope partitioning using total carbon and $\delta^{13}C$ of treated soil (Chapter 2). Some possible hypotheses about biochar diminution were: i) partial decomposition of the labile biochar fraction by microbes; ii) biochar translocation to deeper soil layers by physical processes; iii) changes in soil $\delta^{13}C$ due to some external biomass input, and iv) biochar dilution into bulk soil. Biochar decomposition was discarded as a main explanation for biochar losses. In fact, during the microbial decomposition process, soil microorganisms preferentially use the carbon that needs less energy to be degraded. Thus, materials made of more easily metabolizable molecules (e.g. cellulose) and rich in ^{12}C will be used before compounds with complex molecules (e.g. lignin) and rich in ^{13}C. Following this natural process, soils tend to be ^{12}C depleted (Werth and Kuzyakov, 2010). In our experiment ^{12}C enriched soil was obtained in corn cob biochar amended soil after two years of biochar application, therefore we concluded that biological decomposition was not the main explanation of biochar losses (Chapter 2). Some authors have described the eluviation of biochar particles into soil as a result of a biochar transformation due to an aging process. The surface of biochar particles can be oxidised and it might be broken into small pieces, which can be transported to deeper soil layers (Bird and Ascough, 2012). However, this hypothesis seems less plausible in our study as biochar particles have a very low density and there were few rain events which makes the transport of biochar to deeper layers less probable, although this hypothesis cannot be completely discarded (Chapter 2). Moreover, biochar estimation was carried out using a simple two components model (soil and biochar) without taking into account

possible external biomass inputs (e.g. from rhizosphere activity and weeds), which could modify $\delta^{13}C$ of amended-soil and be a source of error (Chapter 2). Finally, biochar dilution into a thicker layer of soil as a result of the periodic tillage seems to be the main explanation (Chapter 2).

A positive short term priming effect after 2 months of biochar application was observed in soils amended with ZB whereas negative priming was found with PB (Chapter 3). Moreover, in both treatments, slightly negative priming was observed after two years (Chapter 3). The contrasting short term priming effects can be explained by the difference in the properties of the biochars such as: i) water-soluble organic carbon content; ii) sorption capacity, and iii) hydrophobic/hydrophilic properties. The amount of CO_2 released in short-term incubation (30 days) for corn cob biochar amended soil was double that of the control and S+PB soils (Chapter 3). It seems that even the little input of dissolved organic carbon of the ZB biochar (11.15 g kg^{-1}) could have promoted microbial activity. As a result, it would have increased microbial biomass and metabolic products. Moreover, these microbial products could act as glue, bonding mineral, native soil organic matter and biochar, resulting in promotion of soil aggregation. This expected increment in soil aggregation allows the occlusion of organic matter, and therefore increases carbon protection against microbial decomposition (Chapter 4). Previously it has been suggested that biochar also acts as a bonding agent to promote soil aggregation, but in this case low ZB content was found inside of aggregates, due to its relatively big particle size that limited its occlusion into aggregates (Chapter 4). However, ZB application had an indirect effect to increase soil aggregation via promotion of microbial activity, which consequently, increased the occlusion of native soil organic carbon (Chapter 4). Therefore, organic carbon content and protection increased as a result of microbial activity promotion due to ZB application (Chapter 3 and Chapter 4).

Interestingly, a very different process occurred in S+PB. Dissolved soil organic carbon input from PB was not observed due to its high hydrophobicity (Chapter 3). It seems that pine biochar sorbed dissolved soil organic carbon limiting its availability for microorganisms and causing a short term negative priming effect (Chapter 3). Moreover, some degree of toxicity that could affect microbial activity cannot be discarded, although it was not tested in this study. However, a tendency of PB organic carbon occlusion into aggregates was detected. This might be explained by three properties of this biochar: i) small particle size; ii) sorptive capacity; iii) hydrophobicity. Biochar hydrophobicity could lead to biochar particles grouping together by themselves. Then, as they are close

to each other, H-bonding and Van der Wall forces between biochar particles could help to keep them grouped (Solomon et al., 2012). Also, over time, the biochar surface could be modified by aging processes, which could increase links with minerals and result in soil organic matter becoming occluded into aggregates. Moreover, as pine biochar has a small particle size it could penetrate directly inside soil aggregates and pores, and be retained. Thus, pine biochar carbon will tend to be protected inside of soil aggregates increasing occluded organic carbon (Chapter 4).

A decrease of soil organic carbon degradation after two years of biochar application to soil was explained by the depletion of labile organic carbon and organic carbon protection in aggregates. The reduced labile organic carbon availability resulting from the low carbon inputs during the two years of the field test (due to suppression of organic fertilization during the experiment and the limited inputs from grapevine plant leaves, branches and roots), led to a strong reduction in CO_2-C released over time (Chapter 3). Moreover, protection against degradation of soil organic matter might be explained by its occlusion into aggregates (Chapter 4). Also, the high level of aromaticity and chemical resistance of biochar means that it is not generally suitable as a C-source for microorganisms (Chapter 1).

In conclusion, the main effects of pine and corn cob biochar were the increment of stable organic carbon content in a Mediterranean agricultural soil by: i) increasing its resistance to biotic and abiotic decomposition due to its high content of aromatic-C and ii) increasing soil organic carbon protection by promotion of soil aggregation as a result of microbial biomass activity and biochar interaction with soil minerals and organic matter. Therefore, biochar application increases soil organic carbon content, resistance and protection over time and may be a possible global change mitigation solution, using a C-negative strategy, in Mediterranean areas. Therefore, it can be an effective way to sequester organic carbon in soils.

Future research

Some important effects of biochar on soil organic carbon resistance, availability and protection have been identified in this thesis and although many mechanisms have been suggested to explain these processes, some supported by ample evidence collected in the elaboration of the thesis, for the most part they remain as hypotheses. Moreover, some experimental limitations have been found during this research, of which the most important one was the low biochar dose applied which, at

times, was not enough to detect significant effects on soil organic carbon. Nevertheless, some tendencies were observed in most of the cases giving important clues to the related underling processes. However, future lines of research are required to corroborate the hypothesized mechanisms and to improve the detection of biochar effects. As a summary of recommendations:

- Higher application rates are required to improve the detection of biochar effects in Mediterranean soils however, this must be done very carefully to avoid overdose of application, which can result in negative effects.
- As biochar characteristics are feedstock and pyrolysis dependent, it would be recommendable to choose a pyrolysis condition and apply it to different feedstocks, and vice versa, to evaluate the precedence of each biochar property (feedstock or pyrolysis conditions). However, this could be limited by the availability of pyrolysis facilities in a region.
- If biochar obtained at high temperature, such as pine biochar, will be used in the future, a better knowledge of its possible toxicity is strongly desirable.
- Longer field experiment periods are necessary to determine the long term biochar persistence and effects in soils. Therefore, the maintenance of experimental field stations with different soils and biochar treatments is recommendable.
- Analysis of soil samples from deeper layers than topsoil should be carried out to discard biochar rearrangement by eluviation or other processes.
- Biochars should be tested in different soils with contrasting climatic conditions to determine the success of biochar in each soil type.
- Calculation of the net carbon balance during biochar production, transport and application study (Life Cycle Analysis) is essential to corroborate its positive effects in a C-negative strategy to contribute to global climate change mitigation solutions.

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Appendix A

A.1. Mass balance calculations

A mass balance of different organic fractions was performed to compare the different methods used to estimate resistant soil and biochar organic matter (ROM and BOM), and organic carbon (ROC and BOC) as follows.

A. 1.1 Resistant organic carbon (ROC) to chemical agents

The resistant organic carbon (ROC) was quantified by three methods corresponding to three different chemical attacks:

i) From the peroxide oxidation residue (ROC_{PO}) by elemental analysis.

ii) From the carbon remaining after mild dichromate oxidation (ROC_{mO})

$TOC\text{-}LSOC_{mO} = ROC_{mO}$ Eq (A.1)

TOC: Total organic carbon values estimated by TOC

$LSOC_{mO}$: Labile organic carbon values estimated by mO

ROC_{mO}: Resistant organic carbon values to mO

iii) From the residue of acid hydrolysis (ROC_{AH}) by elemental analysis.

A.1.2 Biochar quantification based on TOC

Differences in TOC between biochar treatments and control soil made it possible to determine the biochar C abundance in soil.

$BOC_{TOC} = TOC_{S+B} - TOC_{S}$ Eq (A.2)

BOC_{TOC}: Biochar of pine or corn cob organic carbon values estimated by TOC

TOC_{S+B}: Total organic carbon values of biochar-amended soil estimated by TOC

TOC_{S}: Total organic carbon values of control soil estimated by TOC

A.1.3 Biochar quantification based on LOI

LOI $_{CFS+B}$ minus LOI $_{CFS}$ were used to determine biochar organic matter (BOM_{LOI}) in the samples. Then biochar organic carbon (BOC_{LOI}) was calculated as follows:

$$BOC_{LOI} = BOM_{LOI} * F \qquad \text{Eq (A.3)}$$

BOC_{LOI}: Biochar of pine or corn cob organic carbon values estimated by LOI
BOM_{LOI}: Biochar pine or corn cob organic matter values of biochar-amended soil estimated by LOI 550°C.
F: Ratio OC/OM of biochar (F=0.89 in pine biochar and F=0.87 in corn cob biochar)

A.1.4 Biochar quantification based on PO method

Biochar organic carbon (BOC_{PO}) was calculated subtracting resistant native soil organic C from peroxide oxidation of control soil ($RSOC_{PO-S}$) of those samples amended with biochars ($RSOC_{PO-S+B}$), Eq (A.4), as follows:

$$BOC_{PO} = RSOC_{PO-S+B} - RSOC_{PO-S} \qquad \text{Eq (A.4)}$$

BOC_{PO}: Biochar of pine or corn cob organic carbon values estimated by PO
$RSOC_{PO-S+B}$: Resistant soil organic carbon values of biochar-amended to PO
$RSOC_{PO-S}$: Resistant soil organic carbon values of control soil to PO

A.1.5 Biochar quantification based on mild dichromate oxidation method

Resistant soil organic carbon ($RSOC_{mO-S}$) fraction was calculated as the difference between TOC and labile soil organic carbon estimated by mO values ($LSOC_{mO-S}$) of control soil samples, Eq (A.5). Secondly, the pool of resistant organic carbon of control soil which was added to labile soil organic carbon of biochar-amended soil ($LSOC_{mO-S+B}$) values, as this part of resistant organic carbon of soil was not quantified with mO analyses, Eq (A.6). Finally, soil organic carbon (SOC_{mO}) estimation was subtracted from total organic carbon values (TOC_{S+B}) to estimate biochar organic carbon (BOC_{mO}) Eq (A.7) as follows:

$$RSOC_{mO-S} = TOC_{S} - LSOC_{mO-S} \qquad \text{Eq (A.5)}$$

$SOC_{mO}=LSOC_{mO-S+B}+RSOC_{mO-S}$ Eq (A.6)

$BOC_{mO} = TOC_{S+B} - SOC_{mO}$ Eq (A.7)

$RSOC_{mO-S}$: Resistant soil organic carbon values of control soil estimated by mO
TOC_S: Total organic carbon values of control soils estimated by TOC
$LSOC_{mO-S}$: Labile soil organic carbon values of control soil estimated by mO
SOC_{mO}: Soil organic carbon values estimated by mO
$LSOC_{mO-S+B}$: Labile soil organic carbon values of biochar-amended samples estimated by mO
BOC_{mO}: Biochar pine or corn cob organic carbon values estimated by mO
TOC_{S+B}: Total organic carbon values of the biochar-amended soil by TOC

A.1.6 Resistant biochar fraction estimation based on acid hydrolysis (AH)

Biochar organic carbon (BOC_{AH}) was calculated subtracting resistant soil organic C from acid hydrolysis ($RSOC_{AH-S}$) of those samples amended with biochars ($RSOC_{AH-S+B}$), Eq (A.8), as follows:

$BOC_{AH}= RSOC_{AH-S+B}-RSOC_{AH-S}$ Eq (A.8)

BOC_{AH}: Biochar of pine or corn cob organic carbon values estimated by AH
$RSOC_{AH-S+B}$: Resistant soil organic carbon values of biochar-amended to AH
$RSOC_{AH-S}$: Resistant soil organic carbon values of control soil to AH

A.2 Statistics

A.2.1 Loss-on-ignition (LOI)

ANOVA RM: LOI 375

	numDF	denDF	F-value	p-value
(Intercept)	1	16	4679.525	0.000
Time	2	16	0.767	0.481
Treatment	2	6	22.748	0.002

Bonferroni test: LOI 375

contrast	estimate	SE	df	t.ratio	p.value
S,2months - S,14months	0.493	0.788	20.250	0.626	1.000
S,2months - S,26months	1.019	0.788	20.250	1.293	1.000
S,2months - S+PB,2months	-7.030	1.289	13.500	-5.454	0.003
S,2months - S+ZB,2months	-6.567	1.289	13.500	-5.095	0.007
S,14months - S,26months	0.526	0.788	20.250	0.668	1.000
S,14months - S+PB,14months	-7.030	1.289	13.500	-5.454	0.003
S,14months - S+ZB,14months	-6.567	1.289	13.500	-5.095	0.007
S,26months - S+PB,26months	-7.030	1.289	13.500	-5.454	0.003
S,26months - S+ZB,26months	-6.567	1.289	13.500	-5.095	0.007
S+PB,2months - S+PB,14months	0.493	0.788	20.250	0.626	1.000
S+PB,2months - S+PB,26months	1.019	0.788	20.250	1.293	1.000
S+PB,2months - S+ZB,2months	0.463	1.289	13.500	0.359	1.000
S+PB,14months - S+PB,26months	0.526	0.788	20.250	0.668	1.000
S+PB,14months - S+ZB,2months	-0.030	1.511	25.996	-0.020	1.000
S+PB,14months - S+ZB,14months	0.463	1.289	13.500	0.359	1.000
S+PB,26months - S+ZB,26months	0.463	1.289	13.500	0.359	1.000
S+ZB,2months - S+ZB,14months	0.493	0.788	20.250	0.626	1.000
S+ZB,2months - S+ZB,26months	1.019	0.788	20.250	1.293	1.000
S+ZB,14months - S+ZB,26months	0.526	0.788	20.250	0.668	1.000

ANOVA RM: LOI 550

	numDF	denDF	F-value	p-value
(Intercept)	1	16	7863.007	0.000
Time	2	16	1.865	0.187
Treatment	2	6	23.835	0.001

Bonferroni test: LOI 550

contrast	estimate	SE	df	t.ratio	p.value
S,2months - S,14months	-0.506	0.773	20.250	-0.655	1.000
S,2months - S,26months	1.023	0.773	20.250	1.325	1.000
S,2months - S+PB,2months	-7.744	1.459	13.500	-5.308	0.004
S,2months - S+ZB,2months	-8.029	1.459	13.500	-5.504	0.003
S,14months - S,26months	1.530	0.773	20.250	1.980	1.000
S,14months - S+PB,14months	-7.744	1.459	13.500	-5.308	0.004
S,14months - S+ZB,14months	-8.029	1.459	13.500	-5.504	0.003
S,26months - S+PB,26months	-7.744	1.459	13.500	-5.308	0.004
S,26months - S+ZB,26months	-8.029	1.459	13.500	-5.504	0.003
S+PB,2months - S+PB,14months	-0.506	0.773	20.250	-0.655	1.000
S+PB,2months - S+PB,26months	1.023	0.773	20.250	1.325	1.000
S+PB,2months - S+ZB,2months	-0.286	1.459	13.500	-0.196	1.000
S+PB,14months - S+PB,26months	1.530	0.773	20.250	1.980	1.000
S+PB,14months - S+ZB,14months	-0.286	1.459	13.500	-0.196	1.000
S+PB,26months - S+ZB,26months	-0.286	1.459	13.500	-0.196	1.000
S+ZB,2months - S+ZB,14months	-0.506	0.773	20.250	-0.655	1.000
S+ZB,2months - S+ZB,26months	1.023	0.773	20.250	1.325	1.000
S+ZB,14months - S+ZB,26months	1.530	0.773	20.250	1.980	1.000

ANOVA RM: LOI 950

	numDF	denDF	F-value	p-value
(Intercept)	1	16	8864.376	0.000
Time	2	16	0.598	0.562
Treatment	2	6	22.092	0.002

Bonferroni test: LOI 950

contrast	estimate	SE	df	t.ratio	p.value
S,2months - S,14months	-0.613	0.748	20.250	-0.820	1.000
S,2months - S,26months	0.208	0.748	20.250	0.279	1.000
S,2months - S+PB,2months	-8.023	1.640	13.500	-4.892	0.010
S,2months - S+ZB,2months	-8.976	1.640	13.500	-5.473	0.003
S,14months - S,26months	0.821	0.748	20.250	1.099	1.000
S,14months - S+PB,14months	-8.023	1.640	13.500	-4.892	0.010
S,14months - S+ZB,14months	-8.976	1.640	13.500	-5.473	0.003
S,26months - S+PB,26months	-8.023	1.640	13.500	-4.892	0.010
S,26months - S+ZB,26months	-8.976	1.640	13.500	-5.473	0.003
S+PB,2months - S+PB,14months	-0.613	0.748	20.250	-0.820	1.000
S+PB,2months - S+PB,26months	0.208	0.748	20.250	0.279	1.000
S+PB,2months - S+ZB,2months	-0.953	1.640	13.500	-0.581	1.000
S+PB,14months - S+PB,26months	0.821	0.748	20.250	1.099	1.000
S+PB,14months - S+ZB,14months	-0.953	1.640	13.500	-0.581	1.000
S+PB,26months - S+ZB,26months	-0.953	1.640	13.500	-0.581	1.000
S+ZB,2months - S+ZB,14months	-0.613	0.748	20.250	-0.820	1.000
S+ZB,2months - S+ZB,26months	0.208	0.748	20.250	0.279	1.000
S+ZB,14months - S+ZB,26months	0.821	0.748	20.250	1.099	1.000

A.2.2 Loss-on-peroxide oxidation (LPO)

ANOVA RM: LPO

	numDF	denDF	F-value	p-value
(Intercept)	1	16	2191.744	0.000
Time	2	16	24.453	0.000
Treatment	2	6	2.738	0.143

Bonferroni test: LPO

contrast	estimate	SE	df	t.ratio	p.value
S,2months - S,14months	0.224	0.413	20.250	0.542	1.000
S,2months - S,26months	-2.494	0.413	20.250	-6.037	0.000
S,2months - S+PB,2months	-1.671	0.796	13.500	-2.098	1.000
S,2months - S+ZB,2months	-1.029	0.796	13.500	-1.292	1.000
S,14months - S,26months	-2.718	0.413	20.250	-6.579	0.000
S,14months - S+PB,14months	-1.671	0.796	13.500	-2.098	1.000
S,14months - S+ZB,14months	-1.029	0.796	13.500	-1.292	1.000
S,26months - S+PB,26months	-1.671	0.796	13.500	-2.098	1.000
S,26months - S+ZB,26months	-1.029	0.796	13.500	-1.292	1.000
S+PB,2months - S+PB,14months	0.224	0.413	20.250	0.542	1.000
S+PB,2months - S+PB,26months	-2.494	0.413	20.250	-6.037	0.000
S+PB,2months - S+ZB,2months	0.641	0.796	13.500	0.805	1.000
S+PB,14months - S+PB,26months	-2.718	0.413	20.250	-6.579	0.000
S+PB,14months - S+ZB,14months	0.641	0.796	13.500	0.805	1.000
S+PB,26months - S+ZB,26months	0.641	0.796	13.500	0.805	1.000
S+ZB,2months - S+ZB,14months	0.224	0.413	20.250	0.542	1.000
S+ZB,2months - S+ZB,26months	-2.494	0.413	20.250	-6.037	0.000
S+ZB,14months - S+ZB,26months	-2.718	0.413	20.250	-6.579	0.000

A.2.3 Total organic carbon (TOC)

ANOVA RM: TOC

	numDF	denDF	F-value	p-value
(Intercept)	1	16	2308.292	0.000
Time	2	16	3.877	0.042
Treatment	2	6	58.964	0.000

Bonferroni test: TOC

contrast	estimate	SE	df	t.ratio	p.value
S,2months - S,14months	0.379	0.799	23.014	0.475	1.000
S,2months - S,26months	2.110	0.799	23.014	2.642	0.524
S,2months - S+PB,2months	-7.115	0.832	10.168	-8.550	0.000
S,2months - S+ZB,2months	-8.005	0.832	10.168	-9.619	0.000
S,14months - S,26months	1.731	0.799	23.014	2.167	1.000
S,14months - S+PB,14months	-7.115	0.832	10.168	-8.550	0.000
S,14months - S+ZB,14months	-8.005	0.832	10.168	-9.619	0.000
S,26months - S+PB,26months	-7.115	0.832	10.168	-8.550	0.000
S,26months - S+ZB,26months	-8.005	0.832	10.168	-9.619	0.000
S+PB,2months - S+PB,14months	0.379	0.799	23.014	0.475	1.000
S+PB,2months - S+PB,26months	2.110	0.799	23.014	2.642	0.524
S+PB,2months - S+ZB,2months	-0.889	0.832	10.168	-1.069	1.000
S+PB,14months - S+PB,26months	1.731	0.799	23.014	2.167	1.000
S+PB,14months - S+ZB,14months	-0.889	0.832	10.168	-1.069	1.000
S+PB,26months - S+ZB,26months	-0.889	0.832	10.168	-1.069	1.000
S+ZB,2months - S+ZB,14months	0.379	0.799	23.014	0.475	1.000
S+ZB,2months - S+ZB,26months	2.110	0.799	23.014	2.642	0.524
S+ZB,14months - S+ZB,26months	1.731	0.799	23.014	2.167	1.000

A.2.4 Strong dichromate oxidation (sO)

ANOVA RM: sO

	numDF	denDF	F-value	p-value
(Intercept)	1	12	1076.426	0.000
Time	2	12	9.497	0.003
Treatment	2	6	20.288	0.002
Time:Treatment	4	12	3.788	0.032

Bonferroni test: sO

contrast	estimate	SE	df	t.ratio	p.value
S,2months - S,14months	-2.269	0.841	27.000	-2.699	0.427
S,2months - S,26months	1.771	0.841	27.000	2.107	1.000
S,2months - S+PB,2months	-6.055	1.193	26.876	-5.074	0.001
S,2months - S+ZB,2months	-6.107	1.193	26.876	-5.118	0.001
S,14months - S,26months	4.041	0.841	27.000	4.805	0.002
S,14months - S+PB,14months	-4.483	1.193	26.876	-3.757	0.030
S,14months - S+ZB,14months	-2.571	1.193	26.876	-2.155	1.000
S,26months - S+PB,26months	-6.150	1.193	26.876	-5.153	0.001
S,26months - S+ZB,26months	-6.880	1.193	26.876	-5.765	0.000
S+PB,2months - S+PB,14months	-0.697	0.841	27.000	-0.829	1.000
S+PB,2months - S+PB,26months	1.676	0.841	27.000	1.994	1.000
S+PB,2months - S+ZB,2months	-0.053	1.193	26.876	-0.044	1.000
S+PB,14months - S+PB,26months	2.374	0.841	27.000	2.823	0.318
S+PB,14months - S+ZB,14months	1.912	1.193	26.876	1.602	1.000
S+PB,26months - S+ZB,26months	-0.730	1.193	26.876	-0.612	1.000
S+ZB,2months - S+ZB,14months	1.267	0.841	27.000	1.506	1.000
S+ZB,2months - S+ZB,26months	0.999	0.841	27.000	1.188	1.000
S+ZB,14months - S+ZB,26months	-0.268	0.841	27.000	-0.319	1.000

A.2.5 Mild Oxidation (mO)

ANOVA RM: mO

	numDF	denDF	F-value	p-value
(Intercept)	1	16	1442.364	0.000
Time	2	16	6.308	0.010
Treatment	2	6	0.234	0.798

Bonferroni test: mO

contrast	estimate	SE	df	t.ratio	p.value
S,2months - S,14months	0.833	0.235	20.25	3.539	0.073
S,2months - S,26months	0.643	0.235	20.25	2.733	0.458
S,2months - S+PB,2months	-0.234	0.386	13.50	-0.606	1.000
S,2months - S+ZB,2months	-0.074	0.386	13.50	-0.192	1.000
S,14months - S,26months	-0.190	0.235	20.25	-0.806	1.000
S,14months - S+PB,14months	-0.234	0.386	13.50	-0.606	1.000
S,14months - S+ZB,14months	-0.074	0.386	13.50	-0.192	1.000
S,26months - S+PB,26months	-0.234	0.386	13.50	-0.606	1.000
S,26months - S+ZB,26months	-0.074	0.386	13.50	-0.192	1.000
S+PB,2months - S+PB,14months	0.833	0.235	20.25	3.539	0.073
S+PB,2months - S+PB,26months	0.643	0.235	20.25	2.733	0.458
S+PB,2months - S+ZB,2months	0.160	0.386	13.50	0.414	1.000
S+PB,14months - S+PB,26months	-0.190	0.235	20.25	-0.806	1.000
S+PB,14months - S+ZB,14months	0.160	0.386	13.50	0.414	1.000
S+PB,26months - S+ZB,26months	0.160	0.386	13.50	0.414	1.000
S+ZB,2months - S+ZB,14months	0.833	0.235	20.25	3.539	0.073
S+ZB,2months - S+ZB,26months	0.643	0.235	20.25	2.733	0.458
S+ZB,14months - S+ZB,26months	-0.190	0.235	20.25	-0.806	1.000

A.2.6 Acid hydrolysis (AH)

ANOVA RM: AH

	numDF	denDF	F-value	p-value
(Intercept)	1	16	855.852	0.000
Time	2	16	4.385	0.030
Treatment	2	6	28.929	0.001

Bonferroni test: AH

contrast	estimate	SE	df	t.ratio	p.value
S,2months - S,14months	1.718	0.834	20.935	2.061	1.000
S,2months - S,26months	2.499	0.834	20.935	2.998	0.247
S,2months - S+PB,2months	-5.661	0.939	12.609	-6.032	0.002
S,2months - S+ZB,2months	-5.715	0.939	12.609	-6.089	0.002
S,14months - S,26months	0.781	0.834	20.935	0.937	1.000
S,14months - S+PB,14months	-5.661	0.939	12.609	-6.032	0.002
S,14months - S+ZB,14months	-5.715	0.939	12.609	-6.089	0.002
S,26months - S+PB,26months	-5.661	0.939	12.609	-6.032	0.002
S,26months - S+ZB,26months	-5.715	0.939	12.609	-6.089	0.002
S+PB,2months - S+PB,14months	1.718	0.834	20.935	2.061	1.000
S+PB,2months - S+PB,26months	2.499	0.834	20.935	2.998	0.247
S+PB,2months - S+ZB,2months	-0.054	0.939	12.609	-0.057	1.000
S+PB,14months - S+PB,26months	0.781	0.834	20.935	0.937	1.000
S+PB,14months - S+ZB,14months	-0.054	0.939	12.609	-0.057	1.000
S+PB,26months - S+ZB,26months	-0.054	0.939	12.609	-0.057	1.000
S+ZB,2months - S+ZB,14months	1.718	0.834	20.935	2.061	1.000
S+ZB,2months - S+ZB,26months	2.499	0.834	20.935	2.998	0.247
S+ZB,14months - S+ZB,26months	0.781	0.834	20.935	0.937	1.000

A.2.7 Resistant organic carbon (ROC)

ANOVA RM: ROC

	numDF	denDF	F-value	p-value
(Intercept)	1	69	3202	0
Methods	2	69	169	0
Treatment	2	6	30	0

Bonferroni test: ROC

contrast	estimate	SE	df	t.ratio	p.value
S,ROC_{mO}- S,ROC_{AH}	-8.577	2.285	78.513	-3.753	0.012
S,ROC_{mO}- S,ROC_{PO}	31.729	2.309	78.754	13.741	0.000
S,ROC_{mO}- $S+PB,ROC_{mO}$	-13.646	2.309	6.898	-5.910	0.023
S,ROC_{mO}- $S+ZB,ROC_{mO}$	-16.762	2.285	6.654	-7.334	0.007
S,ROC_{AH}- S,ROC_{PO}	40.306	2.309	78.754	17.455	0.000
S,ROC_{AH}- $S+PB,ROC_{AH}$	-13.646	2.309	6.898	-5.910	0.023
S,ROC_{AH}- $S+ZB,ROC_{AH}$	-16.762	2.285	6.654	-7.334	0.007
S,ROC_{PO} - $S+PB,ROC_{PO}$	-13.646	2.309	6.898	-5.910	0.023
S,ROC_{PO} - $S+ZB,ROC_{PO}$	-16.762	2.285	6.654	-7.334	0.007
$S+PB,ROC_{mO}$ - $S+PB,ROC_{AH}$	-8.577	2.285	78.513	-3.753	0.012
$S+PB,ROC_{mO}$ - $S+PB,ROC_{PO}$	31.729	2.309	78.754	13.741	0.000
$S+PB,ROC_{mO}$ - $S+ZB,ROC_{mO}$	-3.116	2.309	6.898	-1.349	1.000
$S+PB,ROC_{AH}$ - $S+PB,ROC_{PO}$	40.306	2.309	78.754	17.455	0.000
$S+PB,ROC_{AH}$ - $S+ZB,ROC_{AH}$	-3.116	2.309	6.898	-1.349	1.000
$S+PB,ROC_{PO}$ - $S+ZB,ROC_{PO}$	-3.116	2.309	6.898	-1.349	1.000
$S+ZB,ROC_{mO}$ - $S+ZB,ROC_{AH}$	-8.577	2.285	78.513	-3.753	0.012
$S+ZB,ROC_{mO}$ - $S+ZB,ROC_{PO}$	31.729	2.309	78.754	13.741	0.000
$S+ZB,ROC_{AH}$ - $S+ZB,ROC_{PO}$	40.306	2.309	78.754	17.455	0.000

A.2.8 Biochar organic carbon (BOC)

ANOVA RM: BOC

	numDF	denDF	F-value	p-value
(Intercept)	1	81	125.188972	0.0000000
Methods	4	81	4.848911	0.0014787
Treatment	1	81	3.649027	0.0596411

Bonferroni test: BOC

contrast	estimate	SE	df	t.ratio	p.value
S+PB,BOC_{mO}- S+PB,BOC_{AH}	1.200	0.557	91.303	2.154	1.000
S+PB,BOC_{mO}- S+PB,BOC_{LOI}	0.864	0.557	91.303	1.551	1.000
S+PB,BOC_{mO}- S+PB,BOC_{PO}	2.036	0.566	91.325	3.600	0.023
S+PB,BOC_{mO}- S+PB,BOC_{TOC}	-0.119	0.557	91.303	-0.213	1.000
S+PB,BOC_{mO}- S+ZB,BOC_{mO}	-0.681	0.355	91.312	-1.920	1.000
S+PB,BOC_{AH} - S+PB,BOC_{LOI}	-0.336	0.557	91.303	-0.603	1.000
S+PB,BOC_{AH} - S+PB,BOC_{PO}	0.836	0.566	91.325	1.478	1.000
S+PB,BOC_{AH} - S+PB,BOC_{TOC}	-1.319	0.557	91.303	-2.368	0.900
S+PB,BOC_{AH} - S+ZB,BOC_{AH}	-0.681	0.355	91.312	-1.920	1.000
S+PB,BOC_{LOI} - S+PB,BOC_{PO}	1.172	0.566	91.325	2.072	1.000
S+PB,BOC_{LOI} - S+PB,BOC_{TOC}	-0.983	0.557	91.303	-1.764	1.000
S+PB,BOC_{LOI} - S+ZB,BOC_{LOI}	-0.681	0.355	91.312	-1.920	1.000
S+PB,BOC_{PO} - S+PB,BOC_{TOC}	-2.155	0.566	91.325	-3.810	0.011
S+PB,BOC_{PO} - S+ZB,BOC_{PO}	-0.681	0.355	91.312	-1.920	1.000
S+PB,BOC_{TOC} - S+ZB,BOC_{TOC}	-0.681	0.355	91.312	-1.920	1.000
S+ZB,BOC_{mO} - S+ZB,BOC_{AH}	1.200	0.557	91.303	2.154	1.000
S+ZB,BOC_{mO} - S+ZB,BOC_{LOI}	0.864	0.557	91.303	1.551	1.000
S+ZB,BOC_{mO} - S+ZB,BOC_{PO}	2.036	0.566	91.325	3.600	0.023
S+ZB,BOC_{mO} - S+ZB,BOC_{TOC}	-0.119	0.557	91.303	-0.213	1.000
S+ZB,BOC_{AH} - S+ZB,BOC_{LOI}	-0.336	0.557	91.303	-0.603	1.000
S+ZB,BOC_{AH} - S+ZB,BOC_{PO}	0.836	0.566	91.325	1.478	1.000
S+ZB,BOC_{AH} - S+ZB,BOC_{TOC}	-1.319	0.557	91.303	-2.368	0.900
S+ZB,BOC_{LOI} - S+ZB,BOC_{PO}	1.172	0.566	91.325	2.072	1.000
S+ZB,BOC_{LOI} - S+ZB,BOC_{TOC}	-0.983	0.557	91.303	-1.764	1.000
S+ZB,BOC_{PO} - S+ZB,BOC_{TOC}	-2.155	0.566	91.325	-3.810	0.011

Appendix B

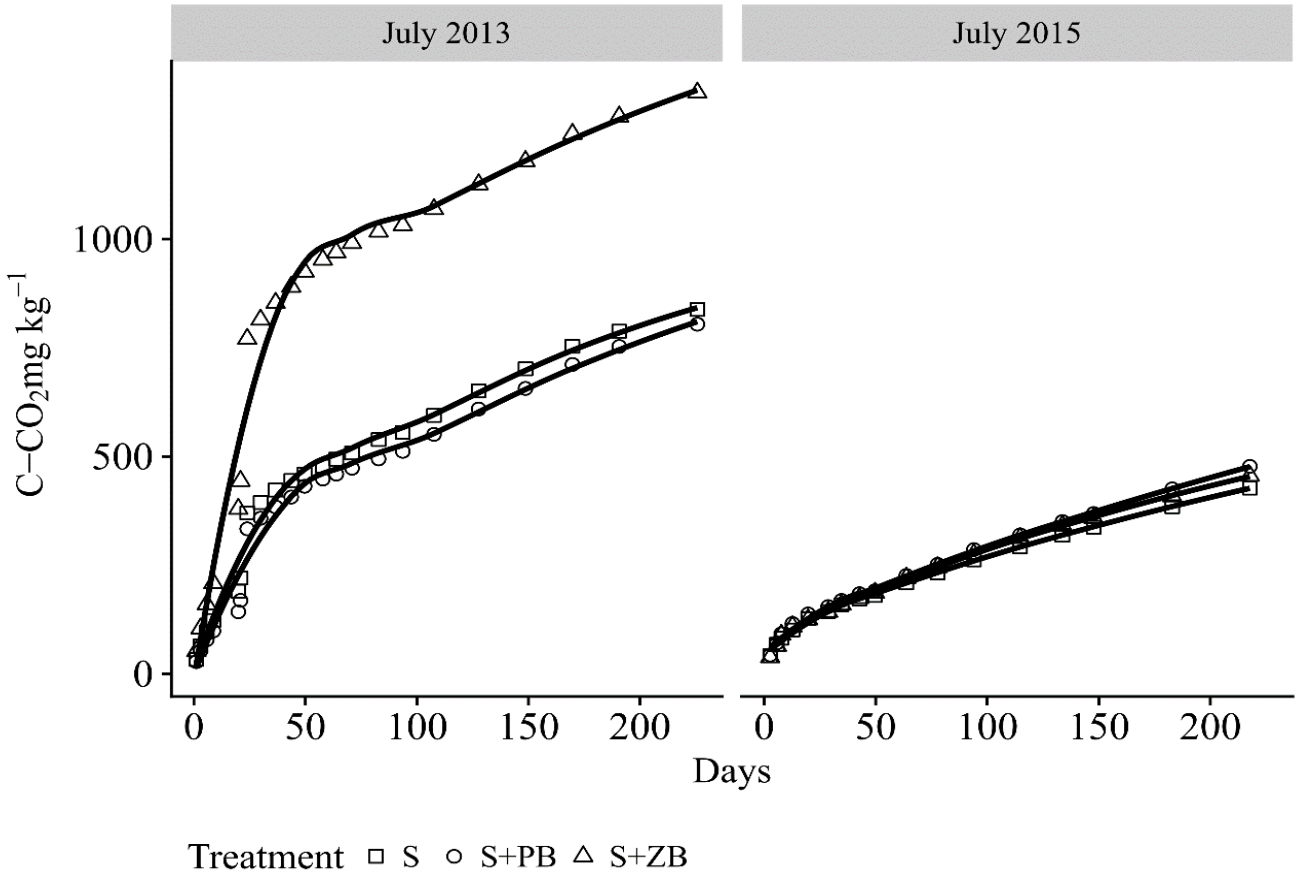

Figure B.1 Cumulative CO_2-C released along the 250 days of incubation from the control soil (S) and soil treated with pine (S+PB) or corn cob (S+ZB) biochar, 2 months (July 2013) and 26 months (July 2015) after biochar application.

Appendix C

C.1) Weight of water-stable aggregates and particulate fraction: macroaggregates, microaggregates and free fraction

The weight of water-stable aggregates in each fraction size (Table 4.1) was determined, as follow:

1) Weight of water-stable macroaggregates (Eq C.1) was calculated subtracting the weight of particulate fraction ($free_{5000-250}$) to the weight of soil fraction retained on the 250 μm mesh after wet sieving with distilled water ($WSF_{5000-250}$).

Eq C.1 Macroaggregates weight = $WSF_{5000-250}$ - $free_{5000-250}$

2) Weight of water-stable microaggregates (Eq C.2) was calculated subtracting the weight of particulate fraction (free-$W_{5000-250}$) and macroaggregates weight (Eq C.1) to the weight of soil fraction left on the mesh of 53 μm after wet sieving with distilled water (WSF_{250-53}).

Eq C.2 Microaggregates weight=WSF_{250-53} - $free_{250-53}$ – macroaggregates

C.2) Organic carbon distribution by mild oxidation method

Total organic carbon (TOC), mild oxidisable organic carbon (OXC) and mild resistant organic carbon (ROC) free (outside aggregates) and occluded in water-stable aggregates (Table 4.1) was carried out as follow:

1) Water-stable macroaggregates TOC (occluded-$TOC_{5000-250}$, Eq C.3) and OXC (occluded-$OXC_{5000-250}$, Eq C.4) was calculated subtracting the OC of particulate fraction higher than 250μm (free-$TOC_{5000-250}$ and free-$OXC_{5000-250}$) to the OC of soil fraction left on the mesh of 250 μm after wet sieving with distilled water (WSF-$TOC_{5000-250}$ and WSF-$OXC_{5000-250}$). Free mild oxidation resistant organic carbon (ROC) and occluded ROC in water-stable macroaggregates (free-$ROC_{5000-250}$ (Eq C.5) and occluded-$ROC_{5000-250}$ (Eq C.6), respectively) was estimated subtracting $OXC_{5000-250}$ to $TOC_{5000-250}$ of free and occluded soil fraction separately.

Eq C.3 Occluded-$TOC_{5000-250}$= WSF-$TOC_{5000-250}$ – free-$TOC_{5000-250}$

Eq C.4 Occluded-$OXC_{5000-250}$= WSF-$OXC_{5000-250}$ – free-$OXC_{5000-250}$

Eq C.5 Free-$ROC_{5000-250}$= free-$TOC_{5000-250}$ – free-$OXC_{5000-250}$

Eq C.6 Occluded-$ROC_{5000-250}$= occluded-$TOC_{5000-250}$ – occluded-$OXC_{5000-250}$

2) To calculated water-stable microagregates TOC (occluded-TOC_{250-53}, Eq C.7) or OXC (occluded-OXC_{250-53} ,Eq C.8), particulate fraction higher than 53µm TOC (free-$TOC_{5000-53}$) or OXC (free-$OXC_{5000-53}$) and occluded-$TOC_{5000-250}$ (Eq C.3) or occluded-$OXC_{5000-250}$ (Eq C.4) were subtracted to the TOC or OXC of soil fraction left on the mesh of 53 µm after wet sieving with distilled water (WSF-$TOC_{5000-53}$ or WSF-$OXC_{5000-53}$). Free ROC (free-ROC_{250-53}, Eq C.9) and occluded ROC in water-stable macroaggregates and (occluded-ROC_{250-53}, Eq C.10) was estimated subtracting OXC_{250-53} to TOC_{250-53} of free and occluded soil fraction separately.

Eq C.7 Occluded-TOC_{250-53}=WSF-$TOC_{5000-53}$ - free-$TOC_{5000-53}$ – Occluded-$TOC_{5000-250}$

Eq C.8 Occluded-OXC_{250-53}=WSF-$OXC_{5000-53}$ - free-$OXC_{5000-53}$ – Occluded-$OXC_{5000-250}$

Eq C.9 Free-ROC_{250-53}= free-TOC_{250-53} – free-OXC_{250-53}

Eq C.10 Occluded-ROC_{250-53}= occluded-TOC_{250-53} – occluded-OXC_{250-53}

3) Total occluded and free OXC (occluded-$OXC_{5000-53}$ (Eq C.11) and free-$OXC_{5000-53}$ (Eq C.12)) and ROC (occluded-$ROC_{5000-53}$ (Eq C.13) and free-$ROC_{5000-53}$ (Eq C.14)) was calculated as the sum of OC from OXC and ROC estimated in 5000-250 and 250-53 soil fraction (see above)

Eq C.11 Occluded-$OXC_{5000-53=}$ occluded-$OXC_{5000-250}$ (Eq C.4) + occluded-OXC_{250-53} (Eq C.8)

Eq C.12 Free-$OXC_{5000-53=}$ free-$OXC_{5000-250}$ + free-OXC_{250-53}

Eq C.13 Occluded-$ROC_{5000-53=}$ occluded-$ROC_{5000-250}$ (Eq C.6) + occluded-ROC_{250-53} (Eq C.9)

Eq C.14 Free-$ROC_{5000-53=}$ free-$ROC_{5000-250}$ (Eq C.5) + free-ROC_{250-53} (Eq C.10)

4) No aggregates were considered in <53µm soil fraction size. For this reason, only the global resistant and oxidisable OC (ROC_{53-0} and OXC_{53-0}) in this soil fraction size was determined:
    a) Mild oxidisable organic carbon quantified in the <53µm soil fraction size was considered OXC_{53-0}.
    b) OXC_{53-0} was subtracted to TOC of <53 soil fraction size to obtain ROC_{53-0}.

5) Organic carbon attributed to biochar contribution (BOC-ROC, Eq C.15) was determined subtracting the sum of ROC of S to the sum of ROC of amended soils.

Eq C.15 BOC-ROC= Σ S+PB or S+ZB-ROC (occluded-ROC + free-ROC + ROC_{53-0}) - Σ S-ROC (occluded-ROC + free-ROC + ROC_{53-0})

C.3) Organic carbon distribution by isotope partitioning methods

Organic carbon attributed to native sol (SOM) and biochar (ROC) free (outside aggregates) and occluded in water-stable aggregates (Table 4.1) was carried out as follow:

1) Organic carbon attributed to biochar (BOC) inside of water-stable aggregates (occluded-$BOC_{5000-250}$, Eq C.16) was calculated subtracting the BOC of particulate fraction higher than 250µm (free-$BOC_{5000-250}$) to the BOC of soil fraction left on the mesh of 250 µm after wet sieving with distilled water (WSF-$BOC_{5000-250}$). Native soil organic carbon (SOC) outside and inside of aggregates (free-$SOC_{5000-250}$ (Eq 17) and occluded-$SOC_{5000-250}$ (Eq C.18)) in water-stable macroaggregates was estimated subtracting $BOC_{5000-250}$ to $TOC_{5000-250}$ of free and occluded soil fraction separately.

Eq C.16 Occluded-$BOC_{5000-250}$= WSF-$BOC_{5000-250}$ − free-$BOC_{5000-250}$

Eq C.17 Free-$SOC_{5000-250}$= free-$TOC_{5000-250}$ − free-$BOC_{5000-250}$

Eq C.18 Occluded-$SOC_{5000-250}$= occluded-$TOC_{5000-250}$ − occluded-$BOC_{5000-250}$

2) To calculated organic carbon attributed to BOC in water-stable microagregates (occluded-BOC_{250-53}, Eq C.19), particulate fraction higher than 53µm BOC (free-$BOC_{5000-53}$) and occluded-$BOC_{5000-250}$ (Eq C.16) were subtracted to the BOC of soil fraction left on the mesh of 53 µm after wet sieving with distilled water (WSF-$BOC_{5000-53}$). Free SOC (free-SOC_{250-53}, Eq C.20) and occluded SOC in water-stable macroaggregates (occluded-SOC_{250-53}, Eq C.21) was estimated subtracting BOC_{250-53} to TOC_{250-53} of free and occluded soil fraction separately.

Eq C.19 Occluded-BOC_{250-53}=WSF-$BOC_{5000-53}$ - free-$BOC_{5000-53}$ – Occluded-$BOC_{5000-250}$

Eq C.20 Free-SOC_{250-53}= free-TOC_{250-53} – free-BOC_{250-53}

Eq C.21 Occluded-SOC_{250-53}= occluded-TOC_{250-53} – occluded-BOC_{250-53}

3) Total occluded and free SOC (occluded-$SOC_{5000-53}$ (Eq C.22) and free-$SOC_{5000-53}$ (Eq C. 23)) and BOC (occluded-$BOC_{5000-53}$ (Eq C.24) and free-$BOC_{5000-53}$ (Eq C.25)) was calculated as the sum of OC from SOC or BOC estimated in 5000-250 and 250-53 soil fraction (see above)

Eq C.22 Occluded-$SOC_{5000-53=}$ occluded-$SOC_{5000-250}$ (Eq C.18) + occluded-SOC_{250-53} (Eq C.21)

Eq C.23 Free-$SOC_{5000-53=}$ free-$SOC_{5000-250}$ (Eq C.17) + free-SOC_{250-53} (Eq C.20)

Eq C.24 Occluded-$BOC_{5000-53=}$ occluded-$BOC_{5000-250}$ (Eq C.16) + occluded-BOC_{250-53} (Eq C.19)

Eq C.25. Free-$BOC_{5000-53=}$ free-$BOC_{5000-250}$ (Eq C.5) + free-BOC_{250-53} (Eq C.10)

4) No aggregates were considered in <53µm soil fraction size. For this reason, only the global BOC and SOC (BOC_{53-0} and SOC_{53-0}) in this soil fraction size was determined:
    a) BOC_{53-0} was estimated as the product of biochar-C_{53-0} fraction (Eq 4.2) and TOC_{53-0}
    c) BOC_{53-0} was subtracted to TOC_{53-0} of <53 soil fraction size to obtain SOC_{53-0}.

5) Organic carbon attributed to biochar contribution was determined as the sum of BOC in all soil fractions.

Eq C.26 $BOC = \Sigma$ S+ZB-BOC (occluded-$BOC_{5000-53}$ (Eq C.24) + free-$BOC_{5000-53}$ (Eq C.25) + BOC_{53-0})