BELOWGROUND BIOTA RESPONSES TO MAIZE BIOCHAR ADDITION TO THE SOIL OF A MEDITERRANEAN VINEYARD

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Abstract

Biochar is a high carbon material resulting from biomass pyrolysis that, when applied to croplands, can increase soil carbon and soil water retention. Both effects are of critical importance in semi-arid regions, where carbon decline and desertification are the main drivers of soil degradation. Since most environmental services provided by soil are mediated by belowground biota, effects of biochar on soil microbial and invertebrate communities must be evaluated under field conditions before its agricultural application can be recommended. We tested maize biochar for its mid-term effect on soil microbes and micro-arthropods of a Mediterranean vineyard. We applied biochar to three field plots with neutral sandy loam soils at a dose of 5 Mg ha^{-1}. During two years, we monitored the abundance of functional groups of soil micro-arthropods and estimated the biomass of soil microbial groups. We also analyzed the $\delta^{13}C$ value of microbial PLFA biomarkers to determine biochar-C utilization by each microbial group taking advantage of the $\delta^{13}C$ natural abundance differences between the applied biochar and the soil. Biochar addition significantly reduced soil microbial biomass but did not alter the functional microbial diversity nor the abundance or biodiversity of soil micro-arthropods. The contribution of biochar-C to the diet of most microbial groups was very low through the monitoring period. However, two gram-negative bacterial groups increased their biochar-derived carbon uptake under extreme soil dryness, which suggests that biochar-C might help soil microbes to overcome the food shortage caused by drought. The decrease in microbial biomass observed in our experiment and the concomitant decrease of SOM mineralization could contribute to the carbon sequestration potential of Mediterranean soils after biochar addition.

Keywords: biochar, Mediterranean soils, soil biota, soil microbial biomass, microbial biochar utilization, PLFA.

1. Introduction

Counteracting soil carbon decline is a key priority for sustainable soil management in the arid and semiarid areas of Europe, since there is evidence that soil degradation will further progress as climate variability increases and extreme weather events become more frequent (Montanarella, 2007). Biochar production and application to soil is promoted as a way to increase the recalcitrant soil carbon pool while improving soil water-holding capacity (Atkinson et al., 2010). Biochar is a by-product of the pyrolysis of biomass at temperatures ranging from 350ºC to more than 800ºC in the absence (or at very low concentration) of oxygen (Sohi et al., 2009). Agricultural lands produce high quantities of organic residues that, when naturally returned to soil, are efficiently processed by the underground food web. After 5 to 10 years, 80 to 90% of the C-biomass will have been released back to the atmosphere as CO_2. Pyrolysis of these residues leads to sequestration of about 50% of their carbon into recalcitrant biochar-C with an estimated residence time in soil of hundreds to thousands of years (Lehmann et al., 2006). For this reason, biochar production and application to agricultural soils has been suggested as a potential strategy to develop more sustainable agricultural systems while mitigating greenhouse gas emissions (Roberts et al., 2009; Woolf et al., 2010).

Biochar is claimed to enhance crop yields (Atkinson et al., 2010; Spokas et al., 2012). However, increases in crop production have only been proven for nutrient-poor acidic and coarse and medium textured soils, while moderately fertile arable soils in temperate regions rarely show significant yield increases after biochar application (Sorrenti et al., 2016; Agegnehu et al., 2017; Jeffery et al., 2017). Together with improvement in soil physical conditions and nutrient status (Biederman and Harpole, 2013), the liming effect of biochar is thought to be the main mechanism underlying yield increase in acidic soils (Jeffery et al., 2011). In neutral to basic and light-textured soils under temperate and dry climates, the agricultural benefits of biochar are more often attributable to the improvement of soil water-holding capacity (Olmo et al., 2014; Baronti et al., 2014; Genesio et al., 2015).

Most environmental services provided by soil, including agricultural fertility, carbon sequestration and water cycle regulation are substantially mediated by the activity of a highly diverse soil community of microbes and invertebrate animals (Lavelle et al., 2006). In the surface horizon of temperate agricultural soils, microbial biomass is in the range of

400 to 880 mg C kg soil^{-1} (Dalal, 1998) and animals are present at densities of 10^6 m^{-2} for nematodes, 10^5 m^{-2} for micro-arthropods and 10^4 m^{-2} for other invertebrates (Altieri et al., 1999). Soil multifunctionality and even plant biodiversity are closely dependent on soil communities (Wagg et al., 2014) and the interaction between plants and belowground organisms regulates primary production and plant health (Wardle et al., 2004). Under the current scenario of climatic uncertainty, sustainable agricultural management must aim to increase soil ecosystem resilience that is closely dependent on soil biodiversity and belowground food web structure (Andrés et al., 2017).

Biochar can influence belowground communities through changes in soil albedo (Meyer et al., 2012), soil chemistry and physical structure, moisture and aeration (Atkinson et al., 2010), nutrient availability, pH (McCormack et al., 2013) and toxicity (Hilber et al., 2017), and by providing a carbon source to the soil biota (Soong et al., 2017). Soil porosity highly determines microbial abundance because a large proportion of the soil bacteria live in micropores inside and around soil microaggregates that offer favorable conditions of water and substrate availability and protection against predators (Rabbi et al., 2016; Sessitsch et al., 2001). Thanks to its highly porous structure, biochar is considered a good soil conditioner but its efficiency in improving soil porosity depends on pore size distribution of the biochar particles that varies with pyrolysis conditions and biomass feedstock (Downie et al., 2009). Both the type of pyrolysis and the chemistry of the feedstock will also determine the recalcitrance of biochar to mineralization and the amount of nutrients available to microbes that ultimately determines biochar stability and the overall biochar-C sequestration capacity of the soil (Schlesinger and Andrews, 2000; Thies and Rillig, 2009).

It is widely recognized that biochar degradation is both biotically and abiotically mediated (Jones et al., 2011) and that its application to soil may have significant effects on belowground processes. Therefore, consequences of biochar on the soil microbial community must be evaluated before agricultural application of biochar can be recommended (Pressler et al., 2017). However, understanding biochar degradation by microbes is far from being complete, and the impact of biochar on soil eukaryotes remains poorly known. Data on the effects of biochar on soil invertebrates are constrained to lab ecotoxicological tests on selected or model animals (Marks et al., 2014; Domene et al., 2015) and to a few short-term field experiments with earthworms (Weyers and Spokas, 2011; Tammeorg et al., 2014), protists and nematodes (Eo et al., 2018) and epigeous macroinvertebrates (Castracani et al., 2015) as indicators.

Biochar chemical and physical properties, including its resistance to microbial utilization, change with aging (Mukherjee et al., 2014) which has profound implications for the estimation of the long-term capacity of biochar-amended soils to sequester carbon (Spokas, 2013). Biochar mineralization may be described following an exponential model (Lehmann et al., 2009) with an initial phase of fast decomposition of the labile fraction followed by a second phase of slow decomposition of the recalcitrant aromatic condensed carbon components (Wang et al., 2016). But this model is continuously reshaped by soil-biochar-soil biota interactions that also change over time (Ameloot et al., 2014). However, only very recently, multiyear experiments have begun to provide data on the mid-term evolution of soil microbial communities in biochar-amended agricultural soils under field conditions (Nielsen et al., 2014; Watzinger et al., 2014; Mackie et al., 2015, Jones et al. 2012; Yao et al., 2017; Mitchell et al., 2016). Regrettably, data about mid-term effects of biochar on higher levels of the soil community are even scarcer and, to our knowledge, restricted to the works of Domene et al. (2014) on soil invertebrate feeding activity and Pressler et al. (2017) on several groups of the soil food web.

Based on biochar's proven ability to alter soil chemistry and physical structure and to improve soil water retention capacity, we hypothesized that biochar addition to our soils will: (a) increase soil microbial biomass, (b) lead to greater abundance of soil micro-arthropods and particularly of the water-dependent forms, and (c) alter the composition of the soil microbial and micro-arthropods' communities in the short- and mid-term. We also posited that (d) soil microbes would feed on biochar-derived carbon at least in the initial period after biochar application to soil.

To test these hypotheses, a maize-derived biochar was applied to the soil of a vineyard and soil microbes and soil micro-arthropods were monitored during the following two years in biochar-amended and control plots. Maize is a C_4 plant and our experimental vineyard soils historically developed under C_3 vegetation. The difference between the ^{13}C isotopic signature of C_4 and C_3 plant-derived soil organic matter (SOM) was used to monitor the inclusion of biochar-C in the diet of different soil microbial groups over time.

The sustainability of the biochar strategy will depend on to what extent biochar is degraded and on its medium and long-term effects on the native soil biota, which are ultimately responsible for soil environmental services. With this in mind, our work aimed to assess the effects of biochar on the soil biota of a vineyard under semi-arid Mediterranean conditions.

2. Material and methods

2.1. Work area

The experimental plots were located in a vineyard located in Vimbodí i Poblet (Tarragona, Spain; 41º 22' 43.8" N; 01º 04' 30.3" E, 527 m.a.s.l.). Local topography is gentle (8% slope) and soils are deep and well drained *Fluventic Haploxerept* (Soil Survey Staff, 2014) evolved from Quaternary detrital materials. Surface and sub-surface horizons

(0-40 cm) contain great amounts of coarse elements (55% to 70%). The surface horizon (0-20 cm) is sandy loam and has a neutral pH, poor cation exchange capacity (7.1 $cmol_c$ kg^{-1}) and low content of carbonates. Soil organic matter content (about 1.7%) is within the normal range for agricultural soils (see other soil properties in Table 2). Climate is dry continental Mediterranean, with 550 mm of total annual rainfall and 14.6°C of mean annual temperature. Daily temperature and rainfall during the working period are shown in Fig. S1.

Vines (*Vitis vinifera* ssp. *vinifera*) were planted in 1992 at a density of 4000 plants ha^{-1} with a planting pattern of 2.20 m x 1 m. The vine plants are trellised and managed with double Royat pruning. Pests are controlled with copper hydroxide (50%), wettable sulphur (80%), sulphur in powder (95.5%) and Spinosad (SPINTOR 480, Dow Agrosciences LLC, USA), a natural insecticide obtained from *Saccharopolyspora spinose*, commonly used in organic farming. Weeds are mechanically removed by ploughing the interrow spaces of the plantation to a depth of 15 cm three to six times per year. In 1990, the vineyard was fertilized with compost made of cow manure, after which no other fertilizer has been applied.

2.2. Biochar production

In order to trace the fate of biochar-C in the vineyard soil, biochar from maize corn cob rachis was used. Maize (*Zea mais*) was chosen because, being a C_4 plant, its ^{13}C isotopic signature significantly differs from that of Mediterranean soils historically cultivated with C_3 plants ($\delta^{13}C$ value ranges from -24 to -32 ‰ for C_3 plants and from −7 to −17‰ for C_4 plants; Boutton,1996). This difference allows the flux of the biochar-derived carbon to be followed through the belowground food web (Fry et al., 1978) by isotope analysis of carbon resources and consumers. Corn cob biomass contained 30% water and was pyrolyzed in the furnace of the Environmental North Valorization Center of the Touro mine (A Coruña, Spain). The slow pyrolysis started at ambient temperature and reached a final temperature of 450 to 500 ºC. The residence time of the biomass at final temperature was two hours. 50 to 65% of the initial biomass-C (equivalent to 25% to 32% of the initial biomass) was recovered as biochar. Biochar chemical-physical properties were analyzed as described in Raya-Moreno et al., (2017) and are reported in Table 1.

2.3. Experimental design and sampling plan

In May 2013, six contiguous 90 m^2 field plots (Fig. S2) were demarcated in a two-hectare vineyard and set up as a field experiment following a random design with three plots assigned to biochar application (Bc) and three more plots assigned to non-amended controls (Co). Biochar was homogeneously spread on the soil of the Bc plots with a fertilizer spreader at a dose of 5 Mg C ha^{-1} (equivalent to 6.5 g kg^{-1}) and incorporated into the soil by ploughing at 15 cm depth. The control plots were ploughed the same way. A week after biochar application and ploughing, top soil (0-10 cm) was sampled from each biochar-amended and control plot and analyzed for basic properties. All plots were analyzed two, fourteen and twenty-four months after biochar application for total, inorganic and organic soil carbon (Table 2).

From July 2013 to April 2015, we conducted two different soil sampling campaigns. To evaluate the effects of biochar on soil microbial communities and on biochar-C exploitation by soil microbes, the field plots were sampled seasonally for a total of eight times. At each sampling date, six soil samples per plot were extracted as described below and combined in pairs to produce three composite samples per plot. To measure effects of biochar on soil micro-arthropod communities, we sampled the plots the first day of February, May, August and November 2014 and took eight soil samples per plot each time. At all times, soil samples were extracted with 5 x 5 x 15 cm soil borers from random points situated 1 m away from each other in the central line of the four interrows between vines of each plot.

2.4. Microbial phospholipid fatty acid (PLFA) extraction and isotopic ratio determination

PLFAs were microwave-extracted from freeze-dried soils with a 0.1 M phosphate buffer:choloform:methanol solution at a 0.8:1:2 ratio. For the quantification and identification of PLFAs, 20 µl of 19:0 phosphatidylcholine (Avanti Polar Lipids Inc., Alabaster, AL) were added as internal standard. Lipids were extracted and partitioned into glycolipids, phospholipid and neutral lipids and phospholipids were transesterificated to obtain fatty acid methyl esters (FAMEs). FAMEs were analyzed with capillary gas chromatography with flame ionization detector (GC-FID 7820A, Agilent Technologies, Palo Alto, USA) with a HP1-MS capillary column (60 m x 0.25 mm x 0.25 µm film thickness). The program started at 80ºC, followed by a heating rate of 10ºC minute^{-1} to 170 ºC, 2 ºC minute^{-1} to 230 ºC, 5 ºC minute^{-1} to 310 ºC, with a final hold of 10 minutes. FAMEs were identified and quantified from mass spectral and retention time matches to the NIST 2008 mass spectral library. The isolated PLFAs were grouped into biomarkers of microbial groups as shown in Table 3.

Effects of biochar on the diet of soil microbial groups were explored by comparing the $\delta^{13}C$ signature of their specific PLFA biomarkers in the biochar-amended and control soils. The $\delta^{13}C$ unit was used to report ^{13}C isotope data as in Craig (1953):

$$\delta^{13}\mathrm{C} = \frac{R_{sample} - R_{PDB}}{R_{PDB}}\ 1000\ ‰$$

The $\delta^{13}C$ signature and carbon content of the most significant FAMEs were analyzed (only microbial markers present in samples in sufficient concentration over time were taken into account) by capillary gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) (Trace GC Ultra, GC-C Combustion III and DeltaV IRMS, Thermo Scientific, Bremen, Germany). FAME separation was performed with a capillary GC column type DB-5 (length 60 m, i.d. 0.25 mm, film thickness 0.25 µm; Agilent Technologies, Santa Clara, CA). The GC temperature programme started at 80 ºC with a 1-minute pause, followed by a heating rate of 10 ºC minute^{-1} to 170 ºC, 2 ºC minute^{-1} to 230 ºC and 5 ºC minute^{-1} to 310 ºC, with a final pause of 10 minutes. The $\delta^{13}C$ values were corrected by using working standards (18:0 and 24:0) calibrated on an elemental analyser-IRMS (Flash 1112, Thermo Scientific, Bremen, Germany) coupled to a DeltaV IRMS continuous flow IRMS (Thermo Scientific, Bremen, Germany). The final $\delta^{13}C$ values of the PLFAs, were obtained after correcting the measured $\delta^{13}C$ FAME values for the addition of the methyl group during transesterification by simple mass balance (after Denef et al., 2007):

$$\delta^{13}\mathrm{C}_{\mathrm{PLFA}} = \frac{N\ x\ \delta^{13}\mathrm{C}_{\mathrm{PLFA-Me}} - \delta^{13}\mathrm{C}_{\mathrm{MeOH}}}{(N-1)}$$

where the isotope value of the PLFA ($\delta^{13}C_{PLFA}$) was calculated from the isotope ratio of the PLFA methyl ester ($\delta^{13}C_{PLFA\text{-}Me}$), the isotope ratio of methanol used for methylation ($\delta^{13}C_{Me\text{-}OH}$), and the number of C atoms of the methylated PLFA (N).

To evaluate a possible effect of biochar on the efficiency of PLFA extraction from soil samples (Gomez et al., 2014), C19:0 PLFA was added in a known dose to three soil samples taken from the non-amended control plots and to three more samples taken from the biochar-amended plots. All samples were processed for PLFA extraction as explained above. Results were corrected for extraction efficiency (EE), calculated as the percentage of C19:0 recovered relative to the dose added. EE was 72.7% in the non-amended control soils and 71.7% in the biochar-amended soils.

2.5. Determination of soil micro-arthropod community size and composition

Soil micro-arthropods were heat extracted from soil samples with 70% ethyl alcohol using Tullgren funnels (Moore et al., 2000) during eight days. The extractors were operated in the dark during the first two days to prevent mortality due to fast soil drying. The animals collected where classified under the microscope to different taxonomic levels, counted and classified into functional groups based on common food preferences and life traits (Table S1).

2.6. Data analysis

The sum of all biomarkers (in nMol PLFA g^{-1} soil) was used as a proxy for total microbial biomass. The fungal to bacterial biomass ratio was calculated by dividing the biomass of the fungal PLFA 18:2ω6,9c by the sum of PLFAs a15:0, i16:0, i17:0, a17:0, 16:1ω7t, 16:1ω7c, 17:1ω7c, 17:0cy, 19:0cy, 18:1ω5c, 14:0, 15:0, 17:0 and 18:0.

Effects of biochar amendment and time after soil amendment on microbial biomass, abundance of micro-arthropods, fungal-to-bacterial biomass ratio and PLFA isotopic signature were tested according to a mixed model design, with “treatment”, “time” and their interaction as fixed factors and “plot” as a random factor. The analyses were performed with the *lmer* function of the *lme4* package (Bates et al., 2015) in R (R Development Core Team, 2016). Tests for fixed effects were done with the *lmerTest* package (Kuznetsova et al., 2016) with the Kenward-Roger's approximation for denominator degrees of freedom for F (Kenward and Roger, 1997). Tests for differences between treatment levels after fitting the linear models were evaluated from predicted marginal means using the *lsmeans* package (Lenth, 2016).

Effects of treatment and time on the communities of soil microbes and micro-arthropods were studied by permutational analyses of variance (PERMANOVA) and were graphically represented using distance-based redundancy analyses (dbRDA). The contribution of each group of microbes or micro-arthropods to dissimilarity between samples was evaluated by SIMPER analyses. PERMANOVAs and dbRDAs were performed with PERMANOVA+ for PRIMER (Anderson et al., 2008), and SIMPER analyses with PRIMER v.7 (Clarke and Gorley, 2015).

3. Results

3.1 Physical and chemical characteristics of the experimental soils

Biochar addition doubled the carbon content of the experimental soils (from 10.7 g C kg soil^{-1} in the control plots to 21.3 g C kg soil^{-1} in the biochar amended plots). This increase particularly affected the non-soluble and non-oxidizable fractions of the soil carbon pool that amounted to 9.4% of total carbon in the control soils and to 26.7% in the amended soils (Table 2). The soil pH increased from 7.3 to 7.7 and the C/N ratio from 15.3 to 26.6. Electrical conductivity almost doubled after biochar addition although the new value (0.14 dS m^{-1}) remained below the adequate salinity threshold for agricultural soils. There was no change in total soil carbon in the control plots over time. In contrast, during the two-year monitoring period, there was a 22% reduction of the total soil carbon content in the biochar amended soils, due to the decline of both the organic and the inorganic fractions of the carbon pool (22.6% and 14.3% respectively) (Table 2).

3.2. Effects of biochar on soil microbial biomass

Biochar had either no effect or a negative effect on total soil microbial biomass depending on time (Treatment x Time: p = 0.0005). Microbial PLFA content was significantly lower in the biochar-amended soils than in the control soils on most sampling dates (Fig. 1a). The greatest difference between control and biochar-amended soils (85.3 ± 1.2 and 32.2 ± 1.2 nMol PLFAs g^{-1} soil respectively) occurred in April 2014. Only on two dates (November 2013 and April 2015), when microbial biomass was very low in the control soils (2.6 ± 1.2 and 7.5 ± 1.2 nMol PLFAs g^{-1} soil respectively), was the effect of biochar insignificant.

3.3. Effects of biochar on the structure of the microbial community

The effect of biochar on the fungal-to-bacterial biomass ratio depended on time (Treatment x Time: $p = 0.037$). The ratio was affected by the treatment only in January 2014 and was significantly lower in the biochar-amended soils (0.04 ± 0.01) than in controls (0.11 ± 0.01) (Fig. 1b).

The composition of the soil microbial community, as indicated by the proportion of individual PLFA microbial biomarkers, significantly depended on time (PERMANOVA: $p = 0.0001$) but was not affected by the addition of biochar (Table S2 and Fig. 2a). In the dbRDA, 63.1% of the variance was explained by Axis I along which the samples were ranked by sampling dates. Sampling T4 and T5, located towards the right side of the axis, were done immediately after rainy periods while sampling T2, in the opposite end of the axis, was done after an extended dry period (Fig. S1). A regression analysis showed that the scores of the samples on Axis 1 were positive and significantly related with total microbial biomass ($R^2 = 0.4834$; $p < 0.0001$) and with the fungi-to-bacteria ratio ($R^2 = 0.505$; $p < 0.0001$). The SIMPER analysis showed that the main contributors to the formation of Axis I were the universal microbial marker 16:0, the fungal marker 18:2ω6,9c and the gram-negative marker 16:1ω7c.

3.4. Soil micro-arthropod community abundance and composition

Biochar did not alter the total abundance of soil micro-arthropods. Their abundance only depended on time ($p = 0.0007$): they were significantly ($p < 0.05$) more abundant (24,121 individuals m^{-2}) in the spring sampling (May 2014) than in any other sampling date (10,686 in winter -February 2014-; 13,467 in summer -August 2014-; 12,915 in fall –November 2014).

Biochar had no effect on the composition of the micro-arthropod community (Table S2). The community composition only significantly changed over time (PERMANOVA: p = 0.0001). The dbRDA graph showed the samples grouped by sampling date along Axis 1, with the spring and winter samples located in opposite sides along the axis (Fig. 2b). A SIMPER analysis showed that differences between samples were mainly due to endeostigmatic mites and immature oribatids that were more abundant in spring and summer than in winter or fall.

3.5. Isotopic signature of the PLFA microbial markers

$\delta^{13}C$ was -13.12 ± 0.01 for the maize biochar and -26.84 ± 0.05 for soil. The signature of the non-amended soil was measured three times (in 2013, 2014 and 2015) and changes over time were not significant.

Twelve microbial PLFAs were extracted from the soil samples in sufficient quantity to allow the measurement of their isotopic signature (Table S3) although some of them were not present in all samples. There were significant ($p < 0.05$) differences in mean annual isotopic values between PLFA types: 18:1ω5c ($\delta^{13}C$ = -19.9 ± 1.7) was the most ^{13}C enriched PLFA, followed by PLFAs a15:0, 15:0 and 16:1ω7c ($\delta^{13}C$ = -24.9 ± 0.3) and by the remainder PLFAs, with $\delta^{13}C$ values between -27.1 and -31.2.

The signature of all PLFAs varied significantly over time (Fig. 3). Moreover, biochar modified significantly the isotopic signature of four PLFAs (15:0, 16:1ω7c, 16:0, and 18:0). In the four cases PLFAs were enriched in ^{13}C in the biochar-amended soils relative to controls (Fig. 4 and Table 4). Two more PLFAs (a15:0 and i16:0) were sensitive to the interaction between time and treatment in such a way that they were ^{13}C enriched by the addition of biochar compared to control only in sampling T2 (Fig. 5 and Table 4).

4. Discussion

4.1. Effects of biochar on soil microbial biomass

We had hypothesized that amendment with biochar will increase soil microbial biomass, but this did not happen. In the control plots, microbial biomass evolved according to the phenology of the vine plants, with a spring peak from March to September when vines are active and labile carbon is provided to soil microbes by roots (Amendola et al., 2017), low values from November to March, during the plant dormancy period in the region (Camps et al., 2012) and minima occurring during drought. Biochar had no effect on the winter basal soil microbial biomass but suppressed its spring peak. Given the great sorption capacity of biochar (Wang et al., 2010), the suppressive effect could be attributable to a reduction of available resources due to sorption of the spring labile rhizodeposition carbon onto biochar surfaces (Foster et al., 2016). Positive effects on soil microbial biomass have been previously reported after alkaline biochar application to acidic soils (Pragoyo et al., 2010; Paz-Ferreiro et al., 2011; Ameloot et al., 2013; Mackie et al., 2015; Jiang et al., 2016; Zheng et al., 2016). However, our results are in line with those of a number of studies that show no effect (Castaldi et al., 2011) or reduced microbial biomass when biochar is applied to neutral or alkaline soils (Warnock et al., 2010; Luo et al., 2011; Dempster et al., 2012; Lu et al., 2014; Ameloot et al., 2014).

4.2. Effects of biochar on the soil microbial community

We expected that biochar application would cause changes in the relative abundance of soil microbial functional groups but, surprisingly, neither the fungi-to-bacteria ratio nor the overall microbial community composition were significantly affected. A number of previous studies have shown that biochar selectively stimulates microbes involved in nitrogen cycling and phosphate solubilization (Ducey et al., 2013), gram-positive and gram-negative bacteria (Gomez et al., 2014), actinomycetes (Prayogo et al., 2014) and fungi (Steinbeiss et al., 2009). These effects are often explained by changes in quantity and quality of available nutriments: the labile biochar fraction might benefit copiotrophic against oligotrophic bacteria (Xu et al., 2016; Jenkins et al., 2017) while the recalcitrant fraction might favor oligotrophic and gram-positive bacteria (Ding et al., 2013). The lack of response of our microbial groups to biochar application may be due to the lower agronomic dose at which biochar was applied compared to most experiments (Ameloot et al., 2013, Abujabhah et al., 2018, Watzinger et al., 2014). In the same sense, this moderate dosage resulted in minor changes in soil pH, which is the main driver of changes in soil microbial community composition (Fernández-Calviño et al., 2010; Rousk et al., 2010; Anders et al., 2013; Farrell et al., 2013).

4.3. Effect of biochar on soil micro-arthropods

We had also postulated that biochar would increase the abundance of micro-arthropods at different trophic levels of the soil food web, based on an expected increase of basal resources and microbial preys (Abujabhah et al., 2016). But instead, the abundance of the targeted fauna was not affected by biochar. Our results are in line with those of Domene et al. (2014) and Pressler et al. (2017) who did not find effects of biochar on the feeding activity of the soil fauna nor on the abundance of any functional group of the soil food web, respectively. Despite the paucity of data for comparison, it seems that the response of soil micro-arthropods to biochar application follows the same trend as soil microbial biomass: a positive response in acidic soils (as in Abujabhah et al., 2016) and no response in neutral to alkaline and hard-textured soils (as in Domene et al., 2014, Pressler et al., 2017). In the same sense, we had anticipated biochar addition to favor soil water-demanding invertebrates (in particular collembolans) given the biochar ability to improve soil water retention under arid and semi-arid conditions (Novak et al., 2012; Obia et al., 2016), but this effect was not observed. Again, this could be due to the moderate rate at which biochar was applied.

4.4. Microbial utilization of biochar and other soil resources

We assumed that SOM and biochar were the only two providers of carbon to the soil food web and that the isotopic signature of the microbial PLFAs would lay between the $\delta^{13}C$ values of soil (-26.84 ‰) and biochar (-13.12 ‰). Unexpectedly, most PLFAs showed $\delta^{13}C$ values far below those of the bulk SOM. Such negative values may be due to two main causes: ^{13}C fractionation by microbes and selective preferences of microbial groups towards diverse carbon sources. There is growing evidence that microbial isotopic fractionation may be significant (Henn et al., 2002) and that relative deviation of $\delta^{13}C$ values of individual PLFAs compared to the $\delta^{13}C$ value of bulk SOM may be remarkable (Glasser, 2005). Several experiments have shown that microbial PLFAs can be strongly depleted in $\delta^{13}C$ (to up to 17 ‰) compared to the exploited substrate depending on microbial metabolism and environmental conditions (Abraham et al., 1998; Burke et al., 2003; Ruess et al., 2005). For example, isotopic fractionation between bulk SOM and PLFA 16:0 has been shown under anaerobic conditions (Cifuentes and Salata, 2001). Our very negative PLFA $\delta^{13}C$ values might reveal the existence of carbon sources other than SOM and biochar (Williams et al., 2006) as well as microbial preferences for specific fractions of the SOM (Schweizer et al., 1999; Ehleringer et al., 2000) and in particular for those rich in lignin and lipids that are ^{13}C depleted relative to sugar, starch and cellulose (Bowling et al., 2008). Comparable results have been provided by Kramer and Gleixner (2008) who found soil fungal PLFA biomarkers ^{13}C depleted compared to bulk SOM, probably due to preferential exploitation of lignin (Glaser, 2005).

In our vineyard, the diet of all soil microbial groups changed over time, most likely following shifts in resource availability. In this sense, the lowest $\delta^{13}C$ values were found for all groups in the 2015 winter sampling that was carried out a few days after the pruning of the vine trees, when lignified (and therefore ^{13}C depleted) vine cuttings fell to the ground. The only exception to this trend was provided by the 18:1ω5c biomarker that indicates a gram-negative group that clearly prefers more ^{13}C enriched resources.

We found evidence that biochar alters the diet of several soil bacteria. In four cases (a gram-negative marker and three general bacterial markers), the biomarkers were ^{13}C enriched in the biochar amended soils relative to controls throughout the whole monitoring period. Although being significant, the ^{13}C enrichment was constant and always low ($\Delta \approx 1‰ - 2‰$) which indicates little importance of biochar in the diet of the soil bacteria (and negligible use by fungi) during the two years following soil amendment.

It has been reported that the incorporation of biochar-derived carbon in microbial biomass starts immediately after biochar application to soil. The initial and very active decomposition phase last from two days to two months (Kuzyakov et al., 2009; Smith et al., 2010; Santos et al., 2012), after which the mineralization rate stabilizes at low levels and microbes continue to consume small doses of biochar carbon for many years (Kuzyakov et al., 2014). Since in this work the first sampling was carried out two months after biochar application, a potential short period of fast mineralization might have been missed, but another explanation for the low incorporation of biochar in microbial biomass might be the quality of the biochar. Some authors have found maize biochar more recalcitrant than biochar made of other feedstocks due to the presence of strong structural surface functional groups (Purakayastha et al., 2015). Biochar lability is directly related to oxygen content, and the very low O/C ratio of our biochar (0.1) might have contributed to its recalcitrance, since biochars with $O/C < 0.2$ have half-lives in the range of thousands of years (Spokas et al., 2010).

A very interesting finding is that the ^{13}C signature of gram-positive bacteria markers suggested that this group of bacteria only included biochar in its diet in November 2013, when microbial biomass was at its lowest after an extremely dry summer. For two biomarkers (a15:0 and i16:0), the measured ^{13}C enrichment (2.5‰ and 3.4‰ respectively), i.e. the proportion of biochar-derived carbon in their diet, was higher than the observed for any other bacterial group at any other sampling date. This suggests that biochar-C might be a food resource when no other option is available, at least for microbes able to decompose recalcitrant aromatic soil carbon as is the case of gram-positive bacteria (Kramer and Gleixner, 2008). This is in accordance with Jiang et al. (2016) who found that soil microorganisms prefer to use SOC sources more labile than biochar, when available. However, this interpretation must be used with caution, because summer drought stimulates the production of ^{13}C depleted biomass by plant roots (Bowling et al., 2008) and the observed shift in the two bacterial PLFAs might be the consequence of microbial consumption of rhizodeposition products more than of biochar.

5. Conclusion

This study demonstrated that corn cob biochar applied at agronomic doses to Mediterranean neutral to alkaline soils can reduce soil microbial biomass for at least two years after application. At this application rate, biochar had no significant effects on the community composition of soil microbes or micro-arthropods. Microbial utilization of biochar was very low which is promising in order to increase the residence time of biochar-derived carbon in soil. The isotopic signature of PLFA biomarkers indicated that in our soil, microbes feed preferably on organic matter fractions more ^{13}C depleted than the supplied biochar. However, under the severe conditions of Mediterranean summers, biochar might constitute an emergency resource for soil microbes to overcome food shortage during drought.

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Figure captions

Figure 1. Total microbial biomass (a) and fungi to bacteria ratio (b) in control (Co) and biochar-amended (Bc) plots throughout the sampling period. Asterisks indicate significant differences between control and biochar-amended soils at each sampling date after ANOVA on log transformed data. ***: p = 0.001; **: p = 0.01; *: p = 0.05. Vertical bars denote standard errors of the mean (n=3).

Figure 2. dbRDAs on effect of biochar addition on microbial PLFA biomarkers (a) and on micro-arthropod groups (b) over time. In (a) T1: July 2013, T2: November 2013, T3: January 2014, T4: April 2014, T5: July 2014, T6: October 2014, T7: January 2015, T8: April 2015. In (b), T1: January 2014, T2: May 2014, T3: August 2014, and T4: November 2014.

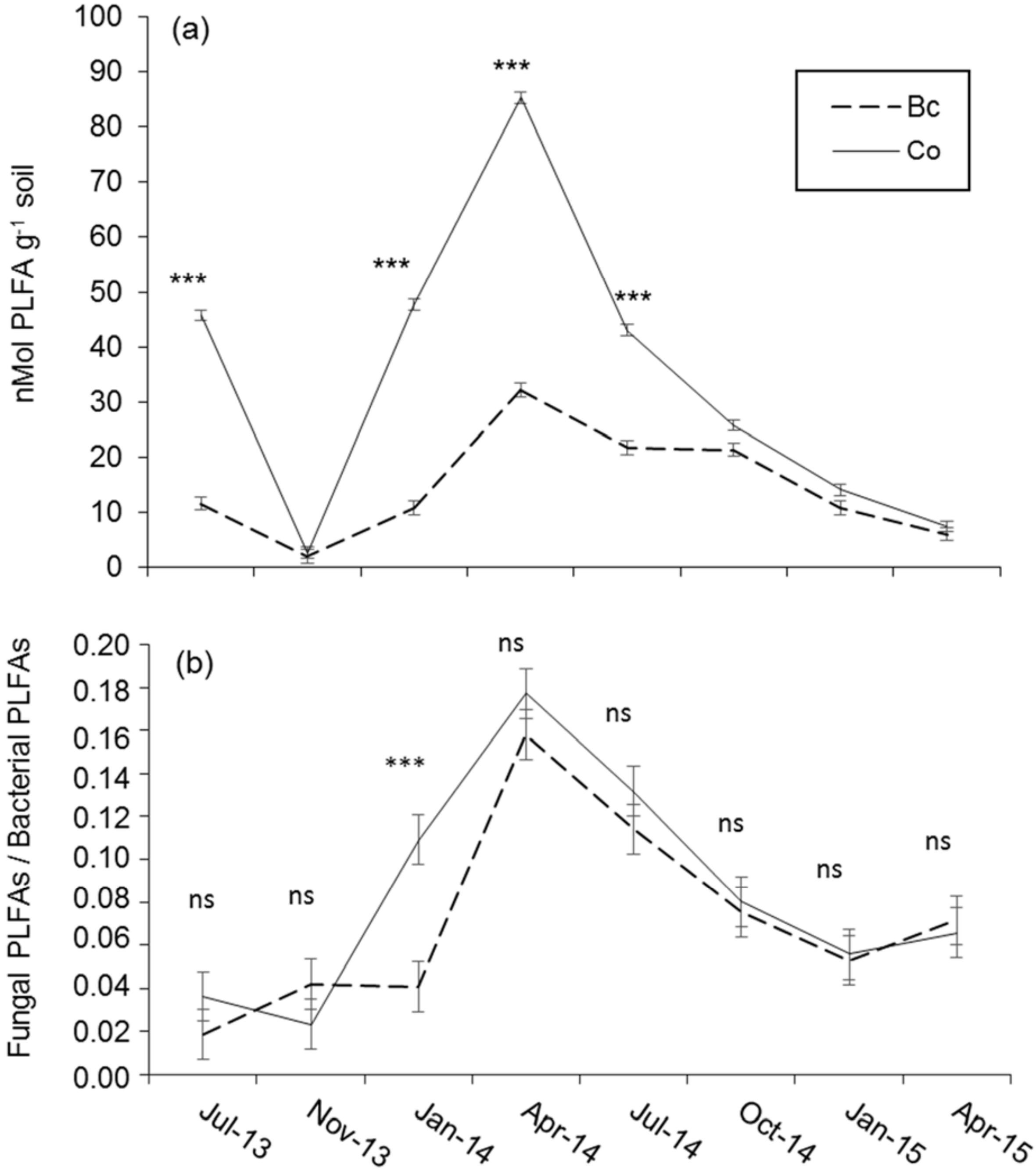
Figure 3. Mean $\delta^{13}C$ value of the soil microbial PLFA biomarkers over time. For each sampling date, the mean was calculated from all samples (including all biochar-amended and non-amended plots). Vertical bars denote standard errors of the mean (n=3).

Figure 4. Effect of biochar on the isotopic signature of four microbial PLFAs. Significance of differences between control soils (Co) and biochar-amended soils (Bc) after ANOVA on transformed data (after Ln -$\delta^{13}C$). ***: p = 0.001; **: p = 0.01; *: p = 0.05. Vertical bars denote standard errors of the mean (n=3).

Figure 5. Isotopic signature of PLFAs a15:0 (a) and i16:0 (b) in control soils (Co) and in biochar amended soils (Bc) over time (i16:0 concentration in samplings T3, T4 and T5 was too low for isotopic analysis). Significance of the difference between control and biochar-amended soils after ANOVA on transformed data (after Ln -$\delta^{13}C$). ***: p = 0.001; **: p = 0.01; ns = no significant difference. Vertical bars denote standard errors of the mean (n=3). In (a) T1: July 2013, T2: November 2013, T3: January 2014, T4: April

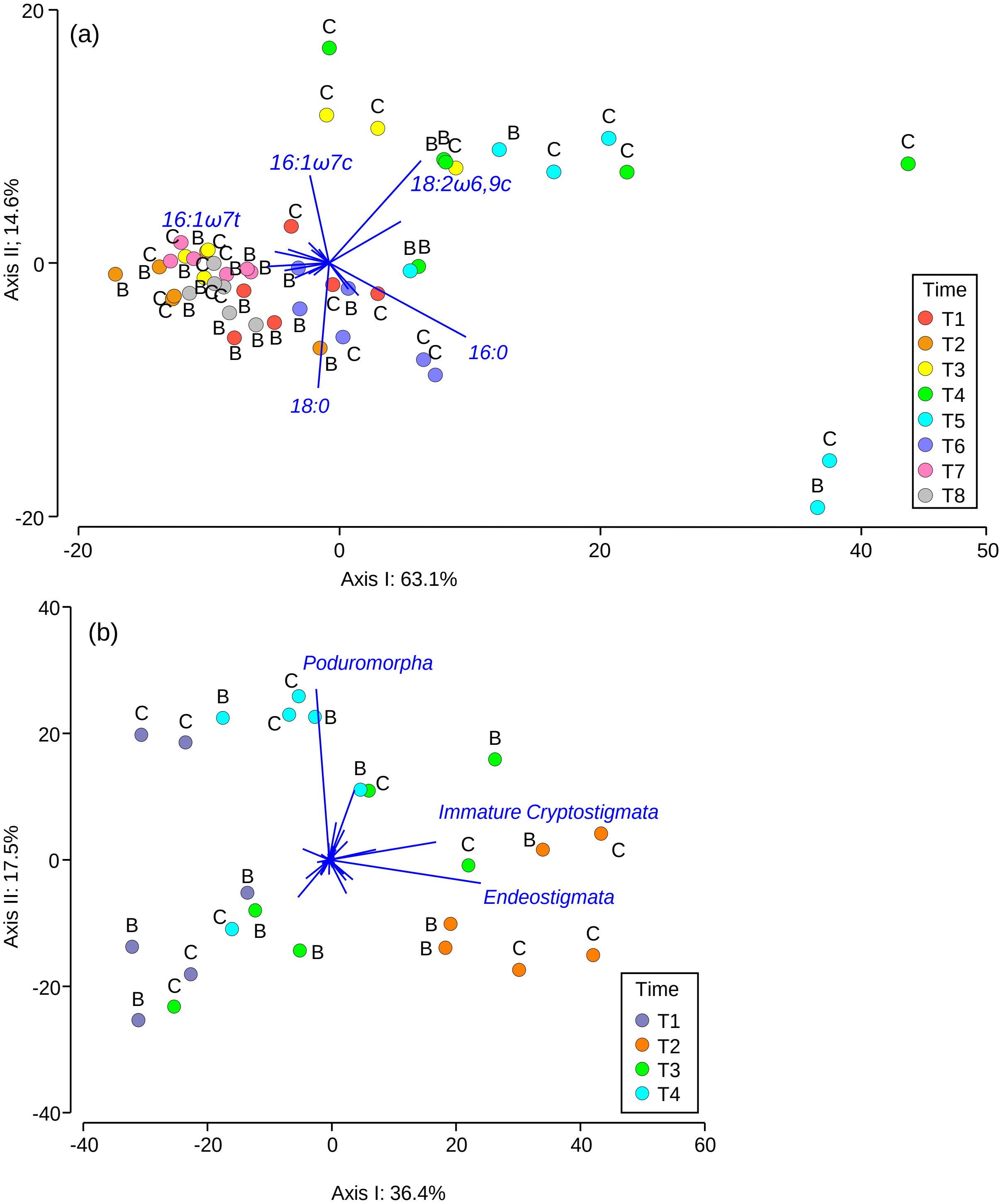
2014, T5: July 2014, T6: October 2014, T7: January 2015, T8: April 2015. In (b), T1: January 2014, T2: May 2014, T3: August 2014, and T4: November 2014.

Fig. 1



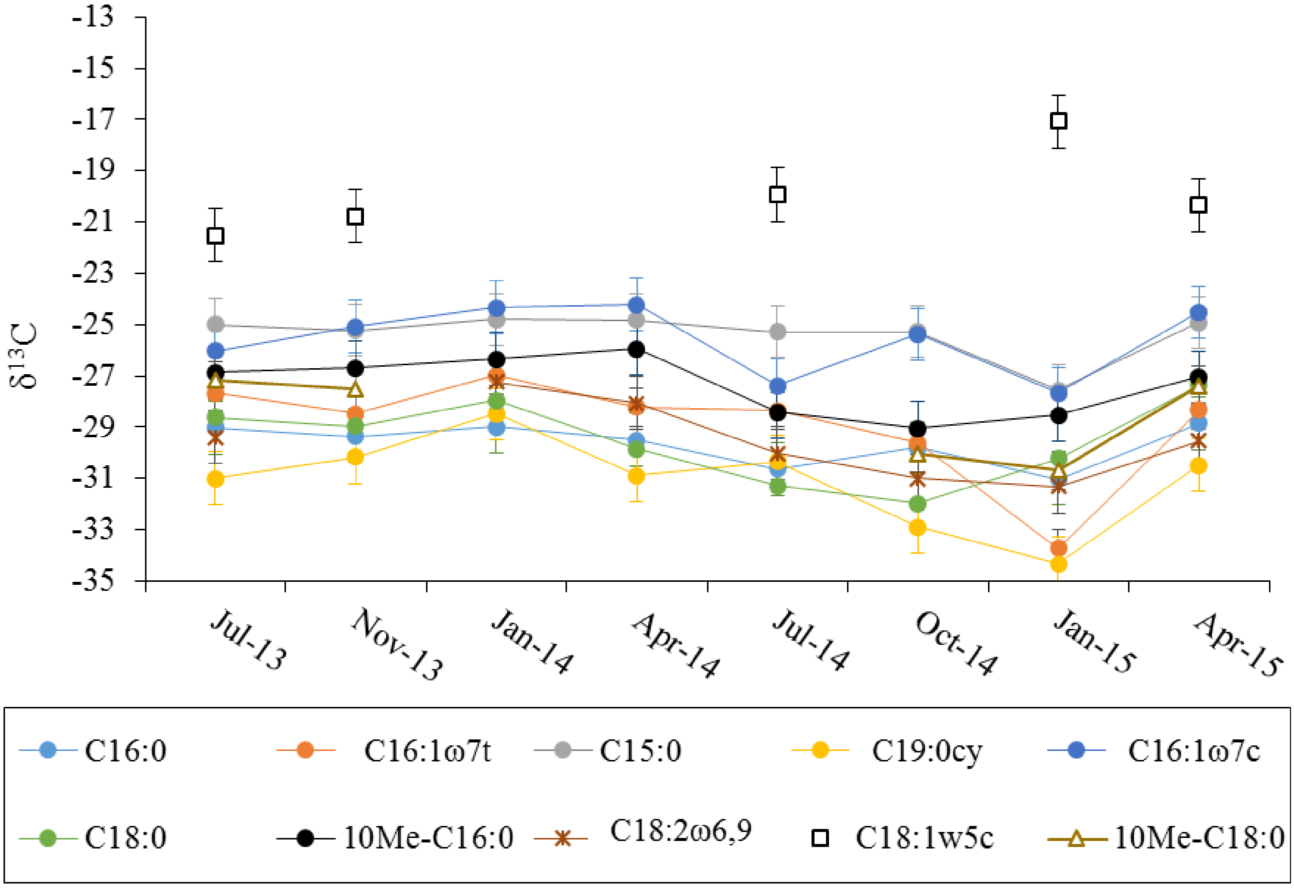
(a)
(b)
Bc
Co
nMol PLFA g-1 soil
Fungal PLFAs / Bacterial PLFAs
***
ns
Jul-13
Nov-13
Jan-14
Apr-14
Jul-14
Oct-14
Jan-15
Apr-15

Fig. 2



(a)
16:1ω7c
18:2ω6,9c
16:1ω7t
16:0
18:0
Axis II; 14.6%
Axis I: 63.1%
Time
T1
T2
T3
T4
T5
T6
T7
T8
(b)
Poduromorpha
Immature Cryptostigmata
Endeostigmata
Axis II: 17.5%
Axis I: 36.4%
Time
T1
T2
T3
T4

Fig. 3



δ13C
-13
-15
-17
-19
-21
-23
-25
-27
-29
-31
-33
-35
Jul-13
Nov-13
Jan-14
Apr-14
Jul-14
Oct-14
Jan-15
Apr-15
C16:0
C16:1ω7t
C15:0
C19:0cy
C16:1ω7c
C18:0
10Me-C16:0
C18:2ω6,9
C18:1w5c
10Me-C18:0

Fig. 4

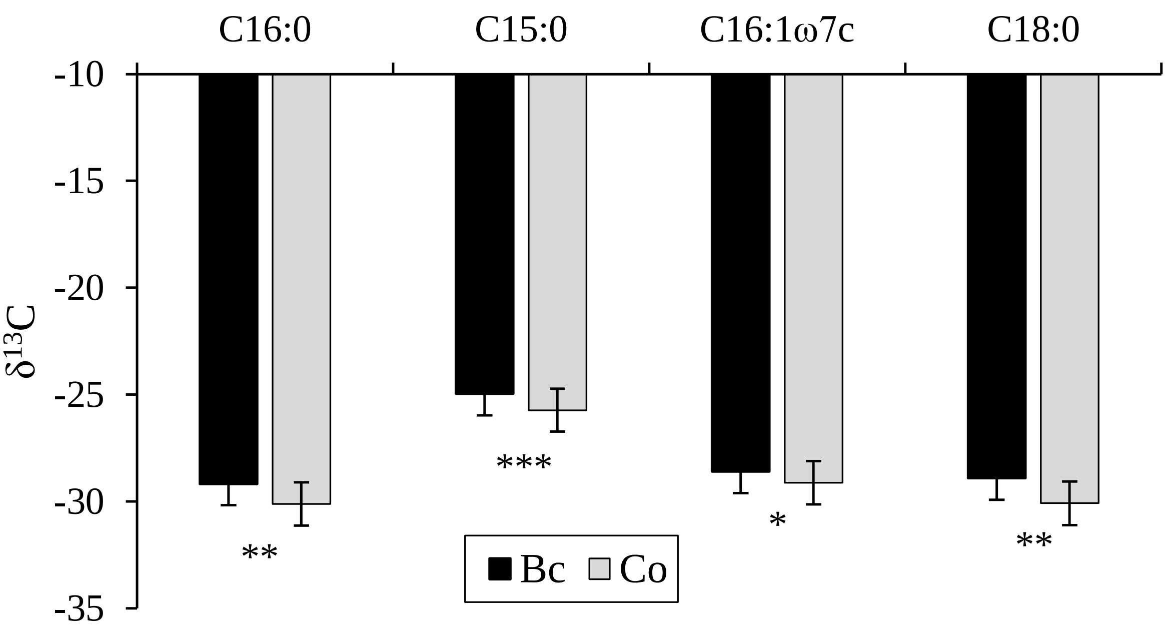
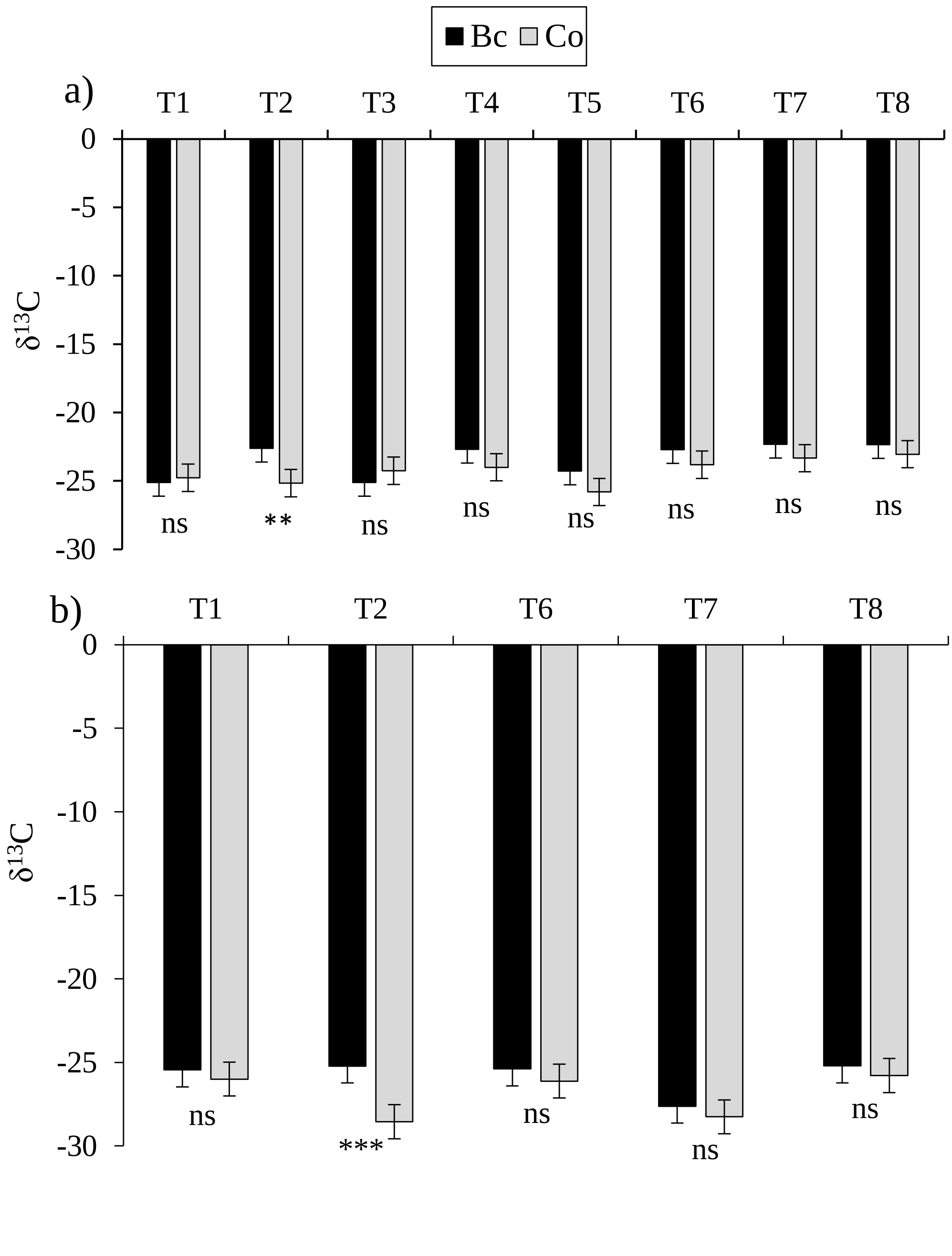


Fig. 5



Bc
Co
a)
T1
T2
T3
T4
T5
T6
T7
T8
0
-5
-10
-15
-20
-25
-30
δ13C
ns
**
ns
ns
ns
ns
ns
ns
b)
T1
T2
T6
T7
T8
0
-5
-10
-15
-20
-25
-30
δ13C
ns
***
ns
ns
ns

Table 1. Elemental analysis, molar ratios and chemical properties of biochar. LOI: weight loss-on-ignition, LPO: weight loss-on-peroxide oxidation, sO: organic carbon destroyed by strong potassium dichromate oxidation, mO: organic carbon destroyed by mild potassium dichromate oxidation, AH: organic carbon resistant to acid hydrolysis.

BIOCHAR PROPERTIES				
pH (H_2O, 1:20) †		10.3 ± 0.04	$\delta^{13}C$ ‰	-13.12
EC dS m^{-1} (1:5, 25 ºC) †		2.5 ± 0.5	Exchangeable Ca (meq 100 g^{-1})	2.02
Total C (g kg^{-1}) †		785.8	Exchangeable Mg (meq 100 g^{-1})	1.1
Inorganic C (g kg^{-1}) †		2.7 ± 0.1	Exchangeable K (meq 100 g^{-1})	85.3
Total N (g kg^{-1}) †		6.8	Exchangeable Na (meq 100 g^{-1})	0.46
Total H (g kg^{-1}) †		19.1	Tot P (mg kg^{-1})	1,838
Total S (g kg^{-1}) †		0.64	Total Al (mg kg^{-1})	1,020
O (g kg^{-1}) †		89.4	Total Fe (mg kg^{-1})	7,810
Ash (g kg^{-1}) †		91.1	Total Na (mg kg^{-1})	200
H/C †		0.29	Total K (mg kg^{-1})	23,400
O/C †		0.11	Total Ca (mg kg^{-1})	2,550
LOI (g kg^{-1}) †			Total Mg (mg kg^{-1})	1,100
	375 ºC	891.7 ± 0.3	Total Cu (mg kg^{-1})	52
	550 ºC	897.9 ± 0.2	Total Co (mg kg^{-1})	10
	950 ºC	917.7 ± 0.2	Total Cr (mg kg^{-1})	17
LPO (g kg^{-1}) †		19.5 ± 3.8	Total Ni (mg kg^{-1})	38
sO (g kg^{-1}) †		235.3 ± 40.1	Total Pb (mg kg^{-1})	13
mO (g kg^{-1}) †		43.7 ± 3.6	Total V (mg kg^{-1})	10
AH (g kg^{-1}) †		65.7 ± 8.5	Total Zn (mg kg^{-1})	410
Particle sizes (% d. w.)			Total As (µg kg^{-1})	396
	5-2 mm	2.8	Total Cd (µg kg^{-1})	38
	2-1 mm	40.1	PAHs (16 US EPA, mg kg^{-1})	40
	1-0.5 mm	24.6		
	0.5-0.2 mm	27.3		
	<0.2 mm	5.2		

† Data from Raya-Moreno et al., 2017.

Table 2. Selected soil characteristics in control plots and in plots amended with biochar one week after biochar application. Data correspond to the top 10 cm of the soil and are reported as mass ratio in the < 2mm soil fraction (except stoniness). Mean ± Stdev (n=3).

	Control plots	Biochar-amended plots
One week after biochar application		
Stoniness (% of field sample)	61.7 ± 4.2	64.4 ± 3.9
Sand (%)	57.7 ± 2.6	60.6 ± 1.3
Loam (%)	26.9 ± 1.9	23.7 ± 2.0
Clay (%)	14.8 ± 0.6	15.3 ± 0.5
$\delta^{13}C$ ‰	-26.84	-19.81
pH (water 1:2.5)	7.3 ± 0.4	7.7 ± 0.3
EC µS cm^{-1} (1:5, 25ºC)	76.3 ± 15.4	140.1 ± 29.2
Total C (g kg^{-1})	10.7 ± 0.8	21.3 ± 1.5
Oxidizable C (g kg^{-1})	9.6 ± 0.6	15.5 ± 0.4
Soluble C (1:2.5 mg kg^{-1})	90.7 ± 33.3	115.4 ± 24.7
N (Kjeldahl, %)	0.07 ± 0.001	0.08 ± 0.006
P (Olsen, mg kg^{-1})	12.7 ± 2.1	18.3 ± 1.5
$CaCO_3$ (g kg^{-1})	0.95 ± 0.2	1.12 ± 0.9
CEC (meq 100 g^{-1})	7.1 ± 1.2	7.3 ± 0.4
Exchangeable Ca (meq 100 g^{-1})	6.1 ± 1.2	5.8 ± 0.5
Exchangeable Mg (meq 100 g^{-1})	0.5 ± 0.1	0.5 ± 0.1
Exchangeable K (meq 100 g^{-1})	0.4 ± 0.02	0.9 ± 0.1
Exchangeable Na (meq 100 g^{-1})	< 0.07	< 0.07
Two months after biochar application		
Total C (g kg^{-1}) †	10.72 ± 0.79	21.33 ± 1.50
Total inorganic C (g kg^{-1}) †	0.94 ± 0.25	1.12 ± 0.95
Total organic C (g kg^{-1}) †	9.77 ± 0.54	20.21 ± 2.37
14 months after biochar application		
Total C (g kg^{-1}) †	11.41 ± 0.86	18.53 ± 2.98
Total inorganic C (g kg^{-1}) †	1.43 ± 0.99	1.35 ± 0.37
Total organic C (g kg^{-1}) †	9.99 ± 1.03	17.15 ± 3.31
24 months after biochar application		
Total C (g kg^{-1}) †	10.29 ± 0.73	16.60 ± 1.03
Total inorganic C (g kg^{-1}) †	0.49 ± 0.29	0.96 ± 1.11
Total organic C (g kg^{-1}) †	9.80 ± 0.85	15.64 ± 2.03

† Data from Raya-Moreno et al., 2017.

Table 3. Microbial functional groups assigned to phospholipid fatty acid (PLFAs) biomarkers.

Functional group	PLFA markers	References
Gram-positive bacteria	a15:0, i16:0, i17:0, a17:0	Frostegård & Bååth (1996), Zelles (1997)
Gram-negative bacteria	16:1ω7t, 16:1ω7c, 17:1ω7c, 17:0cy, 19:0cy, 18:1ω5c	Frostegård & Bååth (1996), Zelles (1997)
Actinomycetes	10Me16:0, 10Me18:0	Ringelberg et al. (1997)
Saprophytic fungi	18:2ω6,9c	Frostegård & Bååth (1996), Bossio & Scow (1998)
Non-specific bacterial	14:0, 15:0, 17:0, 18:0	Bossio & Scow (1998)
Universal microbial	16:0	Bossio & Scow (1998)

Table 4. Two-way ANOVA (GLM procedure) for the ^{13}C isotopic signatures of all microbial PLFA markers depending on treatment (soil *vs* soil + biochar) and time (eight sampling dates over two years). Only significant interactions are shown.

		DF	F value	Pr (>F)
18:1ω5c				
	Treatment	1	13.049	0.2668
	Time	4	71.411	0.0009 ***
a15:0				
	Treatment	1	130.482	0.0012 **
	Time	7	81.336	0.0238 *
	Treat x Time	7	26.553	0.0313 *
15:00				
	Treatment	1	14.376	0.0005 ***
	Time	7	9.508	0.0012 **
16:1ω7c				
	Treatment	1	71.934	0.0109 *
	Time	7	56.563	0.0001 ***
16:1ω7t				
	Treatment	1	14.368	0.2383
	Time	7	97.889	0.0007 ***
i16:0				
	Treatment	1	32.637	0.0320 *
	Time	4	21.483	0.0027 **
	Treat x Time	4	5.948	0.0039 **
10Me-16:0				
	Treatment	1	28.796	0.0983
	Time	7	42.94	0.0015 **
10Me-18:0				
	Treatment	1	0.2915	0.5953
	Time	4	101.713	0.0001 ***
16:00				
	Treatment	1	121.828	0.0012 **
	Time	7	42.928	0.0014 **
18:00				
	Treatment	1	76.842	0.0087 **
	Time	7	71.71	0.0221 *
18:2ω6,9c				
	Treatment	1	18.901	0.1790
	Time	6	36.78	0.0070 **
19:0cy				
	Treatment	1	0.308	0.5824
	Time	7	75.498	0.0133 *

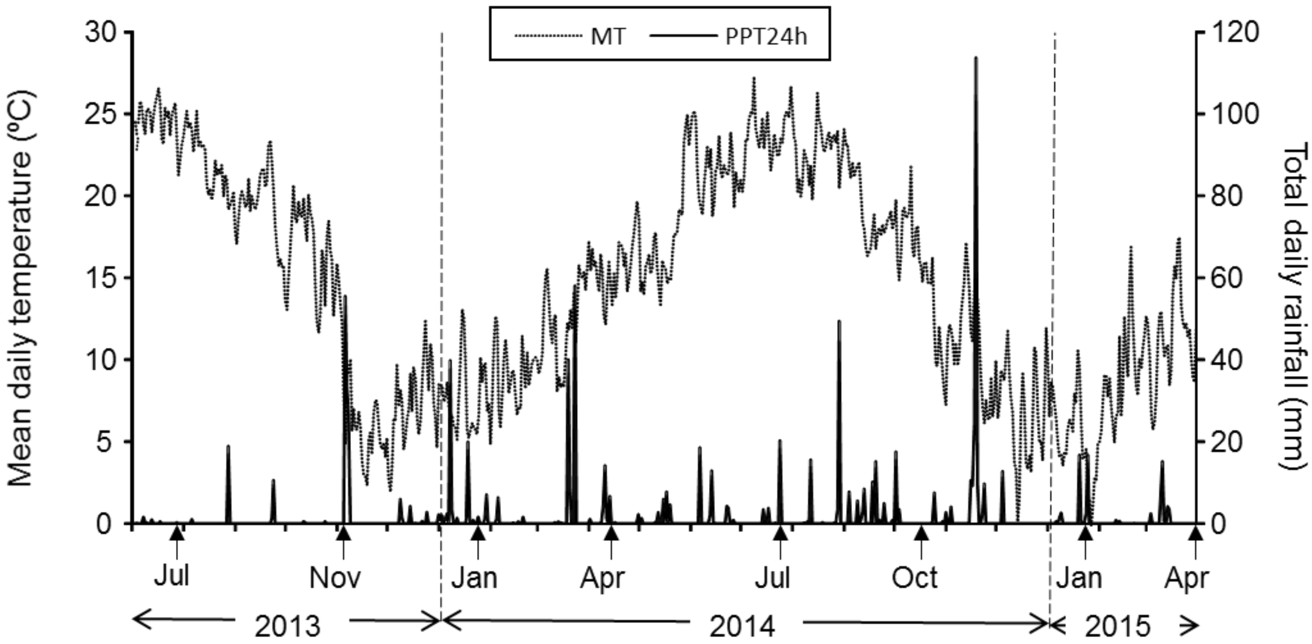


Figure S1. Mean daily temperature (MT) and total daily precipitation (PPT24h) during the study period. Data provided by the Montblanc automatic weather station (41º 22' 25" N; 1º 9' 48" E).

Figure 2. General view of the experimental field plots.

Table S1. Micro-arthropods found in the soil of the experimental plots and their main food preferences after Muraoka & Ishibashi (1976). Walter et al. (1986). Walter (1988). Behan-Pelletier (1999). Gerson et al. (2003). Krantz & Walter (2009). Walter & Proctor (2013). Castilho et al. (2015) and Van Leeuwen (2016).

ACARI	
Endeostigmata	
Nanorchestidae spp.	Predators
Alycidae	Fungivores
Sphaerolichida	
Sphaerolichidae	Predators
Oribatida	
Phthiracarus sp.1	Polyphages
Brachychthonius sp.1	Polyphages
Liochthonius sp.1	Polyphages
Cosmochthonius sp.1	Polyphages
Eohypochthonius sp.1	Polyphages
Epilohmannia sp.1	Polyphages
Papillacarus sp.1	Polyphages
Nothrus sp.1	Polyphages
Microzetes sp.1	Polyphages
Oppiidae spp	Polyphages
Suctobelbidae sp.1	Polyphages
Tectocepheus velatus	Polyphages
Oribatula tibialis	Polyphages
Ceratozetes sp.1	Polyphages
Liebstadia sp.1	Polyphages
Sheloribatidae sp.1	Polyphages
Sheloribatidae sp.2	Polyphages
Immature Oribatida	Polyphages
Astigmata	
Acaridae	Fungivores/Nematophages
Hipopus forms	Inactive
Prostigmata	
Eupodidae	Fungivores
Anystidae	Predators
Scutacaridae	Fungivores
Tydeidae	Fungivores/ Predators/Microphytophages
Paratydeidae	Predators

Tarsonemidae	Fungivores
Rhagidiidae	Predators on arthropods
Penthalodidae	Phytophages
Raphignathidae	Predators
Stigmaeidae	Predators on arthropods
Cunaxidae	Predators on nematodes
Erythraeidae	Predators
Trombididae	Predators
Mesostigmata	
Ascidae sp.1	Predators
Rhodacaridae spp.	Predators
Parasitidae spp.	Predators
Veigaiidae	Predators on arthropods
Uropodidae	Fungivores
Zerconidae	Fungivores
Immature Mesostigmata	Predators
MYRIAPODA	
Chilopoda (Geophilomorpha)	Predators
Symphyla	Root-feeders/saprophages
Pauropoda	Fungivores
INSECTA	
Protura	Fungivores
Diplura	
Diplura (Japygidae)	Polyphages (mainly predators)
Diplura (Campodeidae)	Polyphages
Collembola	
Poduromorpha	Fungivores/Nematophages
Entomobryomorpha	Fungivores
Symphypleona	Fungivores
Psocoptera	Polyphages

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Table S2. Effect of biochar and time after biochar application to soil on soil microbial and soil micro-arthropod communities after PERMANOVA. Tr: treatment; Ti: time, Pl: plot. (***, significant at *P* > 0.001)

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Microbial community						
Tr	1	570.32	570.32	2.3866	0.1975	10
Ti	7	8004.5	1143.5	8.5555	0.0001***	9924
Pl (Tr)	4	955.86	238.97	1.7879	0.0624	9929
Tr x Ti	7	890.98	127.28	0.95231	0.5235	9907
Res	28	3742.4	133.66			
Total	47	14164				
Micro-arthropod community						
Tr	1	897.59	897.59	1.0671	0.3985	10
Ti	3	16,875	5,625	5.1452	0.0001***	9923
Pl(Tr)	4	3364.7	841.16	0.76942	0.7783	9906
Tr x Ti	3	2317.4	772.45	0.70657	0.79	9913
Res	12	13119	1093.2			
Total	23	36574				

Table S3. Isotopic signatures ($\delta^{13}C$) of the PLFA microbial markers depending on treatment (Co: control soils; Bc: biochar amended soils) and sampling dates (T1 to T8 in Fig. 1). PLFAs in (A) and (B) were affected by time or by biochar amendment (treatment) of by both (treatment and time) independently; PLFAs in (C) were affected by the interaction "Treatment x Time". Mean ± Std. Error (n=3).

(A)	*16:1ω7t*	*19:10cy*	*10Me-16:0*	*18:1ω5c*	*18:2ω6.9c*	*10Me-18:0*	*15:0*	*16:0*	*16:1ω7c*	*18:0*
T1	-27.69 ± 1.02	-31.00 ± 1.02	-26.88 ± 1.02	-21.51 ± 1.04	-29.38 ± 1.04	-27.19 ± 1.02	-25.00 ± 1.01	-29.03 ± 1.01	-26.02 ± 1.01	-28.61 ± 1.02
T2	-28.49 ± 1.02	-30.18 ± 1.02	-26.69 ± 1.02	-20.78 ± 1.04		-27.54 ± 1.02	-25.22 ± 1.01	-29.37 ± 1.01	-25.08 ± 1.01	-28.96 ± 1.02
T3	-26.97 ± 1.02	-28.49 ± 1.02	-26.35 ± 1.02	-	-27.25 ± 1.04	-	-24.80 ± 1.01	-29.01 ± 1.01	-24.34 ± 1.01	-27.96 ± 1.02
T4	-28.22 ± 1.02	-30.89 ± 1.02	-25.96 ± 1.02	-	-28.07 ± 1.04	-	-24.83 ± 1.01	-29.50 ± 1.01	-24.23 ± 1.01	-29.85 ± 1.02
T5	-28.37 ± 1.02	-30.33 ± 1.02	-28.41 ± 1.02	-19.93 ± 1.04	-30.02 ± 1.04	-	-25.29 ± 1.01	-30.65 ± 1.01	-27.39 ± 1.01	-31.30 ± 1.02
T6	-29.61 ± 1.02	-32.89 ± 1.02	-29.05 ± 1.02	-	-31.00 ± 1.04	-30.02 ± 1.02	-25.28 ± 1.01	-29.81 ± 1.01	-25.38 ± 1.01	-31.98 ± 1.02
T7	-33.72 ± 1.02	-34.33 ± 1.02	-28.55 ± 1.02	-17.09 ± 1.04	-31.34 ± 1.04	-30.68 ± 1.02	-27.56 ± 1.01	-31.04 ± 1.01	-27.69 ± 1.01	-30.24 ± 1.02
T8	-28.34 ± 1.02	-30.49 ± 1.02	-27.05 ± 1.02	-20.36 ± 1.04	-29.56 ± 1.04	-27.38 ± 1.02	-24.92 ± 1.01	-28.86 ± 1.01	-24.52 ± 1.01	-27.36 ± 1.02

(B)	*15:0*	*16:0*	*16:1ω7c*	*18:0*
Bc	-24.97 ± 1.01	-29.18 ± 1.01	-28.60 ± 1.01	-28.91 ± 1.02
Co	-25.74 ± 1.01	-30.12 ± 1.01	-29.13 ± 1.01	-30.09 ± 1.02

(C)	Treatment	T1	T2	T3	T4	T5	T6	T7	T8
a15:0									
	Bc	-25.13 ± 1.02	-22.62 ± 1.02	-25.12 ± 1.02	-22.69 ± 1.02	-24.28 ± 1.02	-22.73 ± 1.02	-22.32 ± 1.02	-22.36 ± 1.02
	Co	-24.78 ± 1.02	-25.17 ± 1.02	-24.26 ± 1.02	-24.00 ± 1.02	-25.81 ± 1.02	-23.81 ± 1.02	-23.34 ± 1.02	-23.05 ± 1.02
i16:0									
	Bc	-25.45 ± 1.02	-25.22 ± 1.01	-	-	-	-25.39 ± 1.01	-27.63 ± 1.01	-25.21 ± 1.01
	Co	-26.00 ± 1.01	-28.55 ± 1.02	-	-	-	-26.12 ± 1.01	-28.25 ± 1.01	-25.79 ± 1.01