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Influence of select bioenergy by-products on soil carbon and microbial activity: A laboratory study

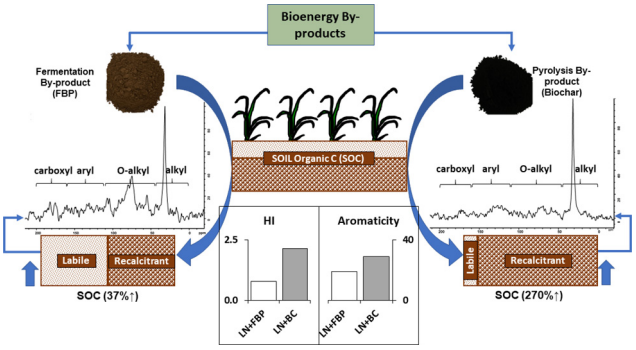
T. Bera [a], L. Vardanyan [a], K.S. Inglett [a,*], K.R. Reddy [a], G.A. O'Connor [a], J.E. Erickson [b], A.C. Wilkie [a]

[a] Soil and Water Sciences Department, University of Florida, Gainesville, FL 32611, USA
[b] Agronomy Department, University of Florida, Gainesville, FL 32611, USA

HIGHLIGHTS

- Bioenergy by-products can be used to replenish the soil organic C (SOC).
- Fermentation by-product (FBP) and pyrolysis by-product (biochar) increased SOC.
- Biochar increased soil recalcitrant C whereas FBP increased soil labile C.
- FBP amendment can be used to stimulate microbial response in soils.
- Biochar could be used to facilitate C sequestration over time.

GRAPHICAL ABSTRACT



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ABSTRACT

Concerns about the negative impacts of crop biomass removal on soil ecological functions have led to questioning the long-term sustainability of bioenergy production. To offset this potential negative impact, use of organic C rich by-products from the bioenergy industries have been proposed as a means to replenish soil C in degraded soils. However, the impact of these by-products application on soil carbon dynamics is not fully understood. We measured biogeochemical changes in soil organic C following a three-year field application of two by-products, biochar (BC) and fermentation-by product (FBP), of bioenergy industry processes in an elephant grass [*Pennisetum purpureum* (L.) Schum.] field. There was a significant increase in overall soil organic C (SOC) observed in BC (270%) treated plots, however the higher labile SOC (51%) content was present in FBP treated plots. Solid-state ^{13}C NMR spectroscopy further revealed increased aromatic and alkyl groups in BC amended soils which lend to its significantly higher hydrophobicity index, HI (2.13) compared with FBP amended soils (HI = 0.8). Initial biogeochemical responses of amended soils to drought conditions were also investigated during a short-term experiment with drying and rewetting of soils. Increased concentrations of extractable C and higher stimulation of microbial activities (respiration and enzyme activities) in FBP amended soils were measured. Overall, our results reveal different impacts of the two soil amendments, where FBP soil application can affect the labile SOC availability, and stimulate rapid microbial response in drought affected soils, and biochar soil application lowers the labile SOC and microbial stimulation facilitating C sequestration over time.

* Corresponding author at: Wetland Biogeochemistry Laboratory, Soil and Water Sciences Department, University of Florida, 3161 McCarty Hall A, P.O. Box 110290, Gainesville, FL 32611, United States.
E-mail address: kanika@ufl.edu (K.S. Inglett).

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1. Introduction

Improvements in enzyme technology and thermochemical conversion facilitate the use of non-grain crop biomass for bioenergy production. To meet the growing demand of either bioethanol or pyrolysis oil, crop biomass is removed from the field which has been shown to seriously impact soil quality in general, and soil organic carbon (SOC) in particular (Lal, 2004). Several studies have reported negative impacts of crop biomass removal including loss of SOC stock, increase soil erosion and decrease soil microbial activities (Lal, 2005; Liska et al., 2014; Wegner et al., 2018). Meanwhile studies have also shown the soil C and nutrients in crop biomass to be energy sources for soil biota and impact the SOC (Lal, 2005). Concerns about the negative impacts of crop biomass removal on soil ecological functions have led to questioning the long-term sustainability of bioenergy production (Lal, 2005; Wegner et al., 2018). To offset the potential negative impact of removing crop biomass for bioenergy production, by-products of bioenergy industries rich in organic C need to be returned to soil (Patzek, 2007). Such action would provide safe and economical waste disposal strategies, while improving soil quality (Pinto and Ilileji, 2009).

Fermentation by-product (residual of enzymatic degradation) and biochar (residual of thermochemical conversion) are common organic C rich substances produced from converting biomass to bioenergy (Demirbas, 2001; Saini et al., 2015). The production processes are different; hence, the C characteristics and impacts in soil are expected to be different. During bioethanol production, cellulose and hemicellulose of lignocellulosic biomass residues are metabolized to produce sugar through enzymatic hydrolysis leaving lignin, waxes and nutrients to concentrate in fermentation by-product (Kim et al., 2008). Fermentation by-product, deprived of cellulose and hemicellulose, is the remains of plant and microbial biomass used for fermentation, along with pretreatment solution added in the process, e.g., phosphoric acid, ammonium hydroxide, etc. (Gubicza et al., 2016). In the enzymatic hydrolysis process, there is no conversion of cellulose or hemicellulose to aromatic structure. Further, no graphene nucleation or carbonization occurs in fermentation by-product generation, as there is during the pyrolysis process. Biochar is produced by pyrolysis through condensation of aliphatic (cellulose and hemicellulose) C to polymeric aromatic C, graphene nucleation and carbonization (Amonette and Joseph, 2009; Bera et al., 2017). Volatilization loss of hydrogen (H) and oxygen (O) leaves biochar with highly condensed and graphite-like material, enriched in C. Overall, the lignocellulosic biomass residues convert to carbonized recalcitrant biochar.

The portion of SOC that decays rapidly is defined as labile SOC and the portion that does not is considered as recalcitrant soil C (Parton et al., 1987). Increased labile C has been related to increased energy source in the soil food web (De Vries and Caruso, 2016), increasing soil quality (Ghani et al., 2003) and soil organic matter quality (Debusk and Reddy, 2005). Long-term storing and C sequestration have been credited to increase in recalcitrant C (Lal, 2004). The labile SOC is characterized by rapid turnover time and quantified through various chemical means (Gregorich et al., 2006). Among chemical means, different extractants including solvent (like water) and oxidizing solutions (mineral acids) were used to quantify labile SOC in grass, forest or crop land ecosystems (Rovira and Vallejo, 2002; Ghani et al., 2003). Besides extraction, C functional group was also determined by spectroscopic analysis such as Fourier-transform infrared spectroscopy (FTIR) or ^{13}C nuclear magnetic resonance (NMR) spectroscopy and linked to labile SOC content (Baldock et al., 1997). The turnover time of SOC is a function of soil microbial activity, which is often measured as respiration, microbial biomass or extracellular enzyme activity. Labile SOC has been frequently positively correlated with soil microbial biomass, respiration, and enzyme activity (Rovira and Vallejo, 2002). Thus, characterizing labile SOC by chemical extractant and ^{13}C NMR spectroscopy complemented with soil microbial activity measurement would provide comprehensive information on labile SOC.

Labile SOC responds quickly to any change in either soil or agronomic management practices (Lal, 2004). Therefore, soil application of organic C-rich amendments, e.g. fermentation by-product or biochar, would influence SOC. Several laboratory and field studies described the use of biochar as a soil amendment to increase C sequestration (Lehmann et al., 2006; Zhang et al., 2010; Zimmerman et al., 2011; Zhang et al., 2012; Liu et al., 2014; Bera et al., 2016), but the impact of biochar on SOC, specifically labile and recalcitrant, is not fully understood yet. For example, Rutigliano et al. (2014) showed no significant difference in soil SOC even after applying 60 Mg ha^{-1} under a wheat crop. Similar observation has also been reported by Quilliam et al. (2012) who applied 25–50 Mg ha^{-1} but failed to record an increase in soil SOC under maize wheat cropping. The differential impact of biochar on soil SOC in the field was attributed to biochar and soil characteristics (Lehmann et al., 2006). From a meta-analysis, Wang et al. (2016) reported that 97% of biochar C was recalcitrant in nature with mean residence time (MRT) of 556 years. The 556 years MRT of biochar C was estimated based on information derived mostly from laboratory incubation studies. Under field conditions, biochar-C decomposition can be altered due to the suboptimal nutrient, moisture and temperature, boosting effect of bioturbation and photodegradation, and biochar-C loss due to surface runoff or leaching (Zimmerman, 2010). Thus, to quantify the effect of applying biochar on SOC specifically labile and recalcitrant SOC, through further field experiments are necessary. Besides soil C amendment, biochar has also been used for reducing contaminant leaching in polluted land (O'Connor et al., 2018; Rens et al., 2018). Compared to biochar, fewer studies have emphasized the application of fermentation by-products to increase soil C (Cayuela et al., 2010, 2014). Cayuela et al. (2010) found 16–19% of fermentation by-product C to be mineralized within 60 days of incubation. Easy decomposability of fermentation by-product contributes to greater soil respiration and soil labile C content, whereas the role of fermentation by-product in C sequestration is unconvincing. The reported nature of C in fermentation by-product and biochar leads to the hypothesis that fermentation by-product contributes to the soil labile C pool, whereas biochar increases soil recalcitrant C. Hence, the objectives of this study were to investigate: (1) the impacts of biochar and fermentation by-product on soil labile and recalcitrant C and (2) soil microbial responses to biochar and fermentation by-product application in amended soil.

2. Materials and methods

2.1. Study site description and amendments

The study site soil was categorized as sandy soil (98.4% sand, 1.2% silt and 0.4% clay) based on USDA soil textural classification with a bulk density of 1.61 g cm^{-3}, a pH of 6.8 and SOC of 5.67 g kg^{-1}. Mehlich-1 extractable P, K, Mg, and Ca content were 64.7, 12.2, 20.9, and 925.2 mg kg^{-1}, respectively in surface soil (0–20 cm soil depth). Sugarcane fermentation by-product was used as the by-product of bioethanol produced through enzymatic hydrolysis. Among the various feedstocks available, sugarcane bagasse is the most common source for second-generation bioethanol production (Saini et al., 2015). Contrarily, agricultural residue specifically crop biomass are limited for burning (Zhao et al., 2018). In the pyrolysis industry, wood biomass is preferred because of its easy availability at commercial scale and greater lignin content and lower ash content than crop biomass (Demirbas, 2001; Cacho et al., 2018). Pine chip biochar was used in this study as the by-product of bioenergy production through pyrolysis. Both amendments were applied at the same C loading rate of 4.37 Mg ha^{-1} yr^{-1} which approximates the mass of C obtained from elephant grass yields for each of the respective conversion technologies. Fermentation by-product used in the experiment was obtained from the University of Florida Stan Mayfield Biorefinery in Perry, Florida, following bioethanol production

from milled sugarcane bagasse (Gubicza et al., 2016). Biochar, obtained from Standard Purification, Dunnellon, Florida, was produced by pyrolysis of reclaimed pine bark for about 30 min at 760 °C via indirect-fired rotary kilns. Low temperature biochar was not included in the present study, because low temperature biochar also known as 'agrichar' is not the byproduct of bioenergy production through pyrolysis (Lehmann, 2007). Characteristics of the fermentation by-product and biochar used are presented in Table 1.

The details of the experimental design are given in Reyes-Cabreraa et al. (2017). Briefly, experiment was conducted for three growing seasons (2013–2015) at the University of Florida Plant Science Research and Education Unit (29°24′N and 82°9′W). Eight rows of elephant grass [*Pennisetum purpureum* (L.) Schum.] breeding line 'UF-1' spaced at 1 m were planted in 90 m^2 (10 × 9 m) plot. The experiment was arranged in a randomized complete block design with three treatments and one control. These naturally N-limited soils had been fertilized with base N fertilizer (Ammonium nitrate, 50 kg N ha^{-1} yr^{-1}) in all plots to maintain minimal plant productivity (Knoll et al., 2012) and to minimize the weed pressure. Therefore, the control plots were identified as (low fertilizer N: LN). The three treatments were: (1) LN plus 200 kg N ha^{-1} yr^{-1} (intensive fertilizer N: LN + N); (2) LN plus 9 Mg dry fermentation by-product ha^{-1} yr^{-1} (low fertilizer N with fermentation by-product: LN + FBP); (3) LN with 7 Mg biochar ha^{-1} yr^{-1} (low fertilizer N with biochar: LN + BC). After fertilization, amendments were hand applied and mixed with the surface soil of respective treatment plots in mid-May each year.

2.2. Physicochemical characterization of amended soils

After harvest of aboveground biomass on November 10, 2015, soil samples were collected from the 0–10 cm soil layer on December 10, 2015. Replicate composite samples were prepared with six soil cores (0–10 cm depth, 3.8-cm diameter). Samples were air dried and sieved to pass a 2 mm screen. Air-dried samples were analyzed for pH in soil water suspension (1:2, w:v), loss on ignition (LOI) at 550 °C, and soil organic C and N by dry combustion in a Thermo Flash EA 1112 elemental analyzer (CE Elantech Inc., Lakewood, NJ, USA). As a measure of soil labile C, a sequential extraction process was performed to estimate cold water extractable C (CWC), hot water extractable C (HWC) and acid water extractable C (AEC) following procedures of Sihi et al. (2016) and Silveira et al. (2008). Briefly, the CWC and HWC were determined by extracting with double distilled (DDI) water using a 1:10 soil:water ratio at 23 °C for 30 min and at 80 °C for 16 h, respectively. Suspensions were shaken on an end-over-end shaker at 30 rpm, followed by centrifugation for 20 min at 4000 rpm, and filtration (0.45 μm filter). AEC was measured by treating residual soil from the hot water extraction step with 6 N HCl, while maintaining similar soil:water ratios, extraction times, shaking periods and filtration processes. Extracts were analyzed in a 5050A TOC auto-analyzer (Shimadzu Corp., Columbia, MD; EPA method 415.1). The combined CWC, HWC and AEC extracts represent labile soil C. Residual C (RSC) content, considered as recalcitrant C, was determined by subtracting labile soil C from the total C.

Table 1
Initial physico-chemical characteristics of soil and bioenergy by-products (biochar and fermentation by-product).

Properties	Biochar	Fermentation by-product
pH (H_2O)	9.4	5.1
Bulk density (g cm^3)	0.4–0.6	–
Total C (g kg^{-1})	625	486
Total N (g kg^{-1})	5.3	40.2
C:N	118	12
Mehlich-1 P (mg kg^{-1})	1800	360
Moisture content (%)	5.6	1.5
Volatile (g kg^{-1})	264	888
Fixed C (g kg^{-1})	618	88
Ash (g kg^{-1})	118	24

2.3. Solid-state ^{13}C nuclear magnetic resonance NMR analysis of amended soils

Solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy was used to characterize the C functional groups in SOC. Replicate samples from each treatment were composited into one sample for NMR spectroscopy analysis. ^{13}C NMR spectra were acquired using an Avance III spectrometer manufactured by Bruker Bio-Spin operating at a field strength of 14 T (600 MHz) with a 51 mm bore. Spectroscopy data were collected using TopSpin software (version 3.2 pl5), and imaging data were collected with ParaVision 6. Solid State NMR studies utilized Magic Angle Spinning (MAS) in which samples were spun at the magic angle (θm = ~54.74°) at speeds from 5 to 15 kHz that drastically decreases line broadening (Knicker, 2011). Finely ground and sieved (250 μm) samples were packed into 3.2 mm NMR zirconium rotors with Kel-F drive caps (Wang et al., 2011). The rotors then were placed in the NMR probe tuned to ^{13}C and ^{1}H nuclei frequencies and spun to 7 kHz at 233 K. Depending on C content, 10,000–18,000 transients were collected for each experiment with a recycle delay of 3 s. MAS ^{13}C Solid State NMR spectra were collected utilizing a Cross Polarization with Total Sideband Suppression (CPTOSS) pulse sequence. A 4.5 μs 1H π/2 pulse followed by a 1.5 ms ramped CP pulse were applied at 100 kHz (H) and 55 kHz (C). The MAS ^{13}C Solid State NMR spectral regions were referenced using an external standard (adamantine) and were integrated to determine the relative contribution of each C functional group in the sample based on referenced assignments: *O*-alkyl C into methoxyl C (45–60 ppm), carbohydrate C (60–90 ppm) and di-*O*-alkyl C (90–110 ppm); and aromatic C into aryl C (110–140 ppm) and phenolic C (140–160 ppm) (Baldock et al., 1997).

The C stability of the amendments and the treated soil was estimated from the aromaticity and hydrophobicity of SOC (Baldock et al., 1997, Spaccini et al., 2002). Based on the integrated areas of C types from the ^{13}C NMR spectra, the following ratios among the organic groups present were determined which is a measure of stability of SOC:

$$Hydrophobicity = \frac{Aromatic + alkyls}{Carboxyl + O{-}alkyl}$$

$$Aromaticity = \frac{Aromatics}{Alkyl + O{-}alkyl + aromatic} \times 100\%$$

2.4. Laboratory incubation study

Florida being a semi-arid tropic region also has an extended dry period from October to late May, resulting in quick dry soil conditions (Sun et al., 2015). This followed by rainfall or irrigation creates frequent dry (similar to drought conditions) and wet cycles in the field. To study the effect of amendments on biogeochemistry of drought impacted soils, a laboratory incubation study was established with the air-dried soil from experimental plots. Fifty grams of soils in a mason jar (~200 mL) was initially moistened at 60% (by weight) of maximum water holding capacity (MWHC) and incubated aerobically at room temperature (23 °C) in the dark. Gas exchange was permitted for 72 h to obviate the "Birch" effect: the sudden increase in soil C and N mineralization caused by re-wetting air-dry soils (Birch, 1958). Additional DDI water was then added to increase the moisture content to 70% of MWHC and soils were stirred thoroughly to mix the added water. Mason jars, covered with parafilm, were arranged randomly in a water tub and incubated for 14 days at room temperature (23 °C) in the dark. Incubation units were checked regularly for moisture loss at 3–4 day interval. Moisture deficits were corrected by adding the same amount of DDI, uniformly sprinkled with a micropipette. Rates of

microbial respiration were measured periodically during the incubation at predetermined intervals. Before each sampling, the incubation units were flushed with CO_2-free air and fitted with a lid containing a septum to make the incubation unit a closed system. Periodic gas sampling of head space was conducted, and gas was analyzed with a gas chromatograph equipped with a thermal conductivity detector (120 °C injection, 50 °C detector temperature) (Shimadzu Scientific Instruments, Columbia, MD). Hourly CO_2-C production rates were calculated by linear regression of the CO_2 peaks over the sampling time using the ideal gas law. The rate of CO_2-C production was measured at predetermined sampling times, i.e. 0.25, 0.5, 1, 2, 5, 8, 11 and 14 days after starting the incubation. Cumulative CO_2-C productions (CCC) were estimated by linear interpolation of the hourly CO_2-C production rate at each sampling event.

2.5. Soil microbial biomass and enzyme analysis

Following incubation, soil microbial biomass C (MBC) was determined using a chloroform fumigation extraction procedure (Vance et al., 1987). Fumigated and non-fumigated extracts were filtered, followed by analyzing total dissolved C in a 5050A TOC auto-analyzer (Shimadzu Corp., Columbia, MD; EPA method 415.1) similarly as for water extractable C. Salt extractable C (SEC) in non-fumigated soil was considered labile C which is immediately available for microbial use (Sihi et al., 2016). MBC was estimated as the difference in extractable C between fumigated and non-fumigated soils using a correction factor of 0.45 (Vance et al., 1987). Extracellular enzymes (β-1,4-glucosidase (BGA), involved in catalyzing cellulose degradation; Leucine Aminopeptidase (LAP), involved in protein degradation; *N*-acetyl-β-D-glucosaminidase (NAG), involved in degrading chitin; and acid phosphatase (APA), involved in degrading organic phosphate) were measured using fluorescent-tagged substrates, as previously described (Inglett et al., 2011). Enzyme substrates (methyl-umbelliferone (MUF)-phosphate, MUF-glucoside and MUF-glucosaminide) were used to measure APA, BGA and NAG, respectively. L-Leucine-7-amido-4-methylcoumarin (AMC) hydrochloride was used to measure the LAP activity. Soil slurries were added with respective substrates and incubated 1 h. At the beginning and end of incubation, formation of the fluorescent product MUF or AMC was measured at excitation/emission wavelength of 360/460 in a fluorometer (Biotek, Winooski, VT). Quenching curves were prepared for each set of soil samples to account for any quenching of the fluorescent product by the soil matrix. Enzyme activity was calculated by using a standard curve (MUF and AMC) and a quenching curve. Specific soil enzyme activities were measured as the ratio of enzyme activity to the microbial biomass C and reported as kg MUF released kg^{-1} microbial biomass C h^{-1}. Enzyme activity ratio (AR) was calculated as the ratio of either C to N or C to P enzyme activity and reported as a unit-less number.

2.6. Statistical analysis

The laboratory experiment was laid out in a completely randomized design with three replications including treatments as the main factor. To determine the treatment effects, data were analyzed using analysis of variance in the PROC GLIM (SAS, 2013, SAS Institute Inc., Cary, NC, USA). The multiple mean separation with corresponding letter grouping method was performed using Tukey's honest significant difference (HSD) test in SAS. All statistical analyses were done at the 5% significance level ($\alpha = 0.05$). To correlate the measured soil properties and their relationship with the treatments imposed in the experiment, principal component analysis (PCA) was done using JMP version 5.1 (SAS Institute, Inc., Cary, NC, USA). A biplot of first and second component was performed. The PCA analysis was done based on directly measurable soil properties excluding the deduced properties like specific enzyme activity and enzyme activity ratios.

3. Results

3.1. Impact of 3 years of N fertilizer and bioenergy by-products applications on soil physicochemical properties

Application of amendments did not alter the soil pH except for in the case of FBP where the soil pH decreased (Table 2). As expected, the C-rich bioenergy by-products significantly influenced soil LOI, and organic C content compared to LN (Table 2). LOI and SOC followed a similar trend in the amended soils. A 246% increase in LOI in biochar-treated soil compared to LN also suggested the greatest SOC. Following the trend of LOI, LN + BC had a 270% increase in SOC over LN. Fermentation by-product increased both LOI and SOC in soil by 54% and 37%, respectively, compared to LN. Intensive N fertilizer (LN + N) also increased the SOC and LOI, but the increase was insignificant. In contrast to SOC, LN + BC resulted in the lowest soil nitrogen content among the treatments. Soil organic N (SON) decreased in the following order: LN + FBP ≥ LN ≥ LN + N ≥ LN + BC (Table 2). Consequently, the greatest soil C:N ratio was recorded for soil treated with LN + BC, followed by LN + N, LN + FBP and LN.

Characterization of labile organic carbon is essential to understand C cycling in the soil samples. To characterize the changes in the labile and recalcitrant C as impacted by the extra fertilizer N or bioenergy by-product treatments, a sequential C fractionation scheme was used. Fractions were defined operationally and quantified as the amount of the C extractable in either cold water, hot water, or acid water. Labile SOC (soil C fractions, Table 3) and recalcitrant C differed among the treatments. The LN + FBP samples had the greatest content of CWC (1.1% of SOC), HWC (4.4% of SOC) and AEC (45.6% of SOC) among the treatments (Table 3). Treatments involving the other bioenergy by-product, i.e. LN + BC had similar contents of CWC (0.06 ± 0.00 g kg^{-1}) and AEC (2.05 ± 0.13 g kg^{-1}), but lower HWC (0.07 ± 0.02 g kg^{-1}) compared to either LN or LN + N, and the greatest RSC (30.51 ± 0.93 g kg^{-1}). Both N fertilizer treatments had similar C content in all fractions (Table 3).

In addition to the chemical fractionation of SOC, molecular C functional groups of amended soils were characterized by ^{13}C NMR (Fig. 1). *The Alkyl C* (0–45 ppm) region of the C-NMR spectra was assigned to proteins, lipids, and aliphatic branched and short chained molecules. This region was mostly represented by the highest peak observed at 30 ppm (the methylene C in the long chains of aliphatic compounds) (Table 4). The NMR spectroscopy analysis clearly indicated that the alkyl functional group dominated the NMR spectra of LN (100% of total C) and LN + N (86%), as well as LN + BC (41%) soils (Table 4). In LN soil, no other groups were detected. In LN + N soil, 14% of total C was identified O-alkyl. The *O-alkyl C* (45–110 ppm) *region* was assigned primarily to O-substituted alkyl carbon in carbohydrates, but also included methoxyl carbon and N-substituted alkyl carbon in protein. C-NMR spectra showed that *O-alkyl* regions were dominant for soil amended with fermentation by-product (55%), followed by biochar (27%), but absent in LN and only 14% in LN + N soil treatments. *The aromatic region* (110–165 ppm) includes aromatic carbons linked to O or N and non-substituted and C-substituted aromatic carbons. The spectral regions between 140 and 165 ppm was assigned to lignin, phenols, aromatic ethers or amine moieties. The highest *aromatic*-C was identified in LN + BC soil (27%), followed by LN + FBP soil (19%) (Table 4). *The carboxylic region* (165–220 ppm) included carboxylic acids (—COOH), mainly organic acids that are free or involved in esters or amides, and carbonyl group (—C—O) present in aldehydes, ketones and organic acid. Similar to *aryl*-C, carboxyl-C was only detected in LN + FBP (1.4%) and LN + BC (5.2%) soils (Table 4). ^{13}C NMR spectroscopy also provided the relative stability of SOC in terms of HI and aromaticity (Fig. 2). Lower HI and aromaticity of LN + FBP (0.8 and 19, respectively) indicated short-term stability of fermentation by-product amended soil compared to biochar amended soil.

Table 2
Soil physico-chemical characteristics after three annual applications of N or bioenergy by-products.

Treatments	pH	LOI (g kg^{-1})	SOC (g kg^{-1})	SON (g kg^{-1})	C: N
LN	6.7 ± 0.15^{a}	12.7 ± 1.5^{c}	8.82 ± 0.25^{c}	0.63 ± 0.03^{ab}	14 ± 0.96^{b}
LN + N	6.9 ± 0.18^{a}	15.9 ± 1.5^{bc}	10.5 ± 0.13^{bc}	0.52 ± 0.02^{ab}	20 ± 0.70^{b}
LN + FBP	6.2 ± 0.16^{b}	19.9 ± 0.1^{b}	12.1 ± 0.25^{b}	0.68 ± 0.05^{a}	18 ± 1.16^{b}
LN + BC	7.0 ± 0.09^{a}	45.0 ± 2.2^{a}	32.6 ± 0.86^{a}	0.49 ± 0.06^{b}	68 ± 7.73^{a}

(LN: Low N; LN + N: LN with extra fertilizer N; LN + FBP: Low N with fermentation by-product; LN + BC: Low N with biochar; LOI: Loss on ignition. Values within column followed by same superscript letter are statistically similar based on Tukey honest significant difference test ($p < 0.05$)).

3.2. Soil respiration, microbial biomass, salt extractable carbon and enzyme activity

Soil treated with fertilizer N and bioenergy by-products showed significant difference in pattern of CO_2-C production rate (Fig. 3). Fermentation by-product amended soil always had the greatest CO_2 production rate except at the last sampling event among the treatments. CO_2-C production rate peaked sharply within a day of incubation in LN + FBP, and then decreased gradually until day 8 before becoming stable. In contrast, biochar amendment increased the CO_2-C production rate gradually during the initial 2 days of incubation and then decreased and continued to be stable from day 8 onwards. The gradual increase in CO_2-C production rate was also evident in LN and LN + N soil, but the rate peaked within 2 days of incubation and then decreased thereafter to become steady from the 5th day of incubation for LN and from the 8th day of incubation for LN + N. The significant difference in the CO_2-C production rate ensured the significant difference in cumulative CO_2-C production (CCC). Parallel to CO_2-C production rate, LN + FBP had the greatest CCC which was 85% greater than LN (Fig. 4). The biochar and LN + N had a similar CCC as that of LN. At the end of 14 days incubation, MBC was also greatest in LN + FBP but not significantly different from LN + BC. Both LN + FBP and LN + BC had 85 and 32% greater MBC than LN, respectively. LN + N did not affect the soil MBC compared to LN. Similarly, Fig. 4 shows significant differences in treatment effect on the SEC. Soil treated with fermentation by-product had the highest SEC while biochar amendment resulted in the lowest SEC. The SEC content in LN + FBP was 15% greater and in LN + BC was 21% lower than LN.

Soil amendment with bioenergy by-products and N fertilizer significantly influenced the soil extracellular enzyme activity, as presented in Fig. 5. Application of fermentation by-product had significantly greater enzyme activity (except for NAG) than biochar, LN + N and LN. For BGA, LAP and APA, the enzyme activity displayed a similar pattern, i.e. greatest in LN + FBP and then statistically similar activity in LN, LN + N and LN + BC amended soil. The enzyme assay showed that fermentation by-product amendment in soil increased BGA, LAP, NAG and APA activity by 1400, 250, 200 and 177%, respectively, compared to LN. The specific enzyme activity was used to compare the stimulation of enzyme activity in sols as triggered by the amendments. The specific activity of BGA was substantially greater in LN + FBP than in any other treatment (Table 5). Specific BGA activity in LN, LN + N and LN + BC was similar, like BGA activity. The specific LAP activity was greatest in LN + FBP (252 ± 34 mmol g^{-1} MBC h^{-1}) which was similar to LN + N and LN, and greater than LN + BC. Specific enzyme activity observed for NAG was greatest in LN + FBP (211 ± 29 mmol g^{-1} MBC h^{-1}), and lowest in LN + BC (28 ± 5 mmol g^{-1} MBC h^{-1}). Likewise, specific enzyme activity of phosphomonoesterase activity was greatest in LN + FBP (375 ± 37 mmol g^{-1} MBC h^{-1}) and lowest in LN + BC (99 ± 8 mmol g^{-1} MBC h^{-1}). Furthermore, the stoichiometries of enzyme activities were significantly influenced by the treatments (Table 5). The C:N enzyme activity ratio was similar in LN, LN + N and LN + BC. However, the LN + FBP samples had the greatest C:N enzyme activity ratio (1.80 ± 0.22) among the treatments. The trend of C:P enzyme activity ratio mirrored that of C:N enzyme activity ratio. The LN + FBP treatment had the greatest C:P enzyme activity ratio (2.20 ± 0.23). The LAP: NAG ratio was significantly greater (3.8 ± 0.5) in the LN + BC treatment, but comparable in the other three treatments.

Table 3
Soil C fractions of a sequential extraction procedure including cold water extractable C (CWC), hot water extractable C (HWC), acid extractable C (AEC) and residual C (RSC) following three annual applications of N or bioenergy by-products.

Fractions of C (g kg^{-1})	CWC	HWC	AEC	RSC
LN	0.07 ± 0.01^{b} (0.8%)	0.26 ± 0.00^{b} (2.8%)	2.26 ± 0.07^{b} (24.7%)	6.57 ± 0.27^{c} (71.7%)
LN + N	0.07 ± 0.01^{b} (0.7%)	0.22 ± 0.02^{b} (2.0%)	2.13 ± 0.05^{b} (19.8%)	8.33 ± 0.16^{b} (77.5%)
LN + FBP	0.15 ± 0.01^{a} (1.2%)	0.56 ± 0.02^{a} (4.4%)	5.85 ± 0.08^{a} (45.6%)	6.26 ± 0.28^{c} (48.8%)
LN + BC	0.06 ± 0.00^{b} (0.2%)	0.07 ± 0.02^{c} (0.2%)	2.05 ± 0.13^{b} (6.3%)	30.51 ± 0.93^{a} (93.3%)

(Values in parenthesis indicate the individual fractions percentage of the total C. LN: Low N; LN + N: LN with extra fertilizer N; LN + FBP: Low N with fermentation by-product; LN + BC: Low N with biochar. Values within each column followed by same superscript letter are statistically similar based on Tukey honest significant difference test ($p < 0.05$).

4. Discussion

4.1. Changes in SOC due to bioenergy by-products amendment

Three years of C-rich bioenergy-byproduct amendments in field soil were expected to induce differences in SOC content. Despite adding 13.1 Mg ha^{-1}C over 3 years through both amendments (fermentation by-product and biochar), greater SOC content in biochar amended soil suggested the greater persistence and stability of biochar C than fermentation by-product confirming previously reported results (Lehmann et al., 2006; Zhang et al., 2010; Zimmerman et al., 2011; Zhang et al., 2012; Liu et al., 2014; Bera et al., 2016). Several investigators have indicated the recalcitrant and long-term stability of biochar C against microbial decomposition in soil (Zimmerman et al., 2011; Wang et al. 2016). The longer biochar-C MRT has always been associated with increased condensation of C through aromatic polymerization and graphene nucleation (Amonette and Joseph, 2009; Bera et al., 2017). In comparison to biochar, fermentation by-product C stability in soil has attracted less attention in the past decade. In a 60-day incubation study, Cayuela et al. (2010) estimated that only 16–19% of applied fermentation by-products remained at the end of 1 year with mean annual temperature of 10 °C in temperate soils. The greater decomposability of fermentation by-products compared to biochar could be ascribed to differences in C polymer structure and functional groups as detected by NMR spectroscopy (Fig. 1 & 2 and Table 4). Mostly, fermentation by-products go through pretreatment with acidic or alkaline solution at high temperature (Gubicza et al., 2016). The pretreatment breaks down the cell wall architecture of crop residue by disrupting the β-O-4 linkages in the lignin polymer (Kim et al., 2008). In this way, the remaining portion of cellulose and hemicellulose, which otherwise is generally protected in heavily cross-linked lignin moiety, is exposed to easy microbial attack when applied to soil (Kim et al., 2008). Even though fermentation by-product is predicted to have greater decomposability than biochar, a thoughtful interpretation is needed

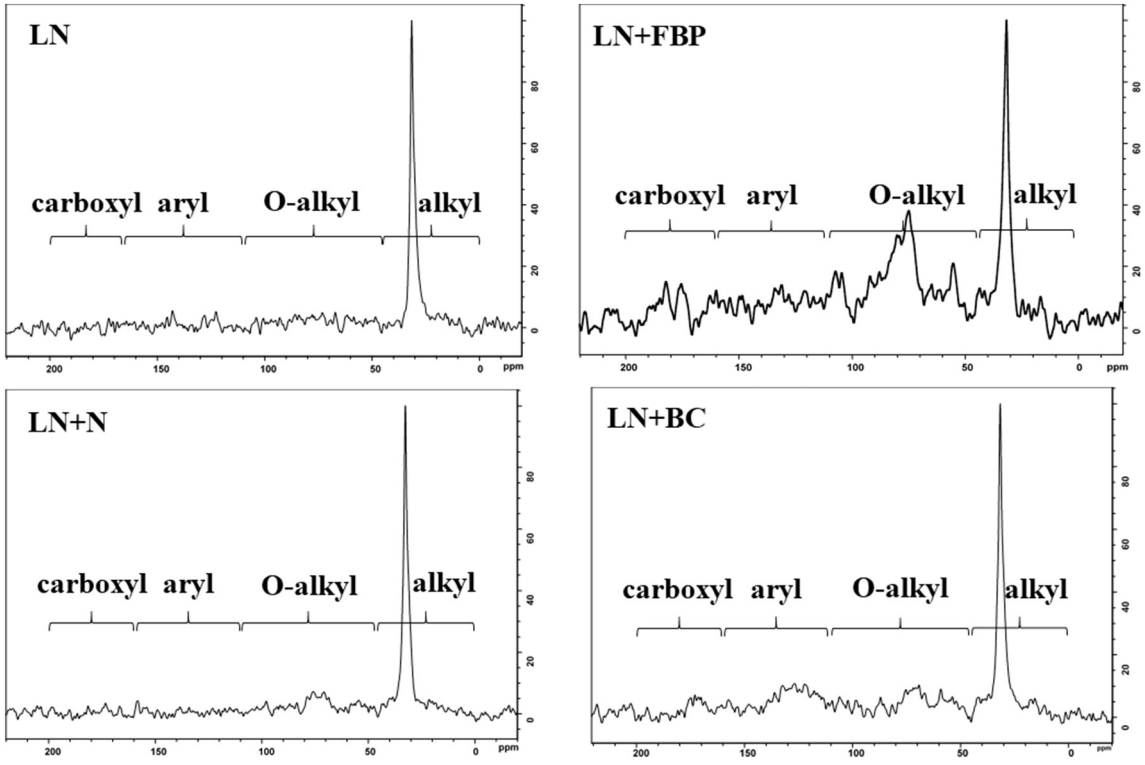

Fig. 1. Comparison of solid state ^{13}C NMR spectra of whole soil treated with bioenergy by-products (LN: Low N; LN + N: LN with extra fertilizer N; LN + FBP: Low N with fermentation by-product; LN + BC: Low N with biochar).

when extrapolating laboratory study results to field conditions. The differences in fate and behavior of fermentation by-products in the laboratory or field will also be altered by aspects mentioned in the foregoing discussion for biochar amendment.

The above discussion undoubtedly indicated the differential stability of amended C which is directly related to labile and recalcitrant pools of SOC. Estimation of labile SOC pools has been adopted successfully through sequential extraction schemes in the past (Ghani et al., 2003; Silveira et al., 2008, Akinsete and Nkongolo, 2016; Sihi et al., 2016). In the present study, the fractionation scheme including CWC, HWC and AEC allowed us to investigate the labile SOC in detail (Akinsete and Nkongolo, 2016; Sihi et al., 2016). Cold and hot water are considered as a mild agent that can affect the carbon fractions involved in the short-term binding of aggregates. Cold water extracts the most labile carbon pools, followed by hot water extraction where the extracted carbon is strongly related to soil microbial biomass, respiration and micro aggregation (Haynes and Francis, 1993; Ghani et al., 2003). Cold and hot-water extractions have been used to determine readily decomposable fractions of soil organic matter and thus are used as fractions of labile SOC pools (Balaria et al., 2009; Gregorich et al., 2006). Acid extractant (6 N HCl) is considered a stronger extractant compared to cold and hot water and able to extract significantly greater amounts of SOC (Silveira et al., 2008). Greater acid extractable labile SOC content than CWC and HWC for all the treatments in the present study is in agreement with the previously reported results (Rovira and Vallejo, 2002; Silveira et al., 2008). The information on the extractable C in soil amended with the fermentation by-product or biochar is limited. In a recent study, Lin et al. (2012) reported water extractable organic C content was at ppb levels in a series of biochars. Therefore, the contribution of biochar to extractable SOC would be inconsequential. Insignificant

Table 4
^{13}C Nuclear Magnetic Resonance functional groups (percent of total organic C and the absolute value) of soil following three annual applications of N or bioenergy by-products. The groups were assigned similarly as Knicker (2011).

Sample Treatment		*alkyl*	*O-alkyl*			*aryl*		Carboxyl	Total	Total C (g kg^{-1})
		Chemical shift region (ppm)								
		0–45	45–60	60–90	90–110	110–140	140–165	165–220	220–0	
		Aliphatic C, methyl, methylene, methine ($-CH_3$, $-CH_2$)	Methoxyl ($-OCH_3$)	C—O of carbohydrate and cellulose	Anomeric C, cellulose C^{-1}	aromatic-C; Aromatic lignins	Lignins, phenols Aromatic ethers	Carboxyl-C C - in amidic groups, esters		
LN	(%)	100	ND	ND	ND	ND	ND	ND	100	8.8
LN + N	(%)	86.0	ND	14.0	ND	ND	ND	ND	100	10.1
LN + FBP	(%)	24.9	10.9	33.0	10.9	12.9	5.9	1.4	100	12.1
LN + BC	(%)	40.7	6.5	13.9	6.4	21.1	6.2	5.2	100	32.6

(LN: Low N; LN + N: LN with extra fertilizer N; LN + FBP: Low N with fermentation by-product; LN + BC: Low N with biochar. ND = No NMR spectroscopy signal was detectable for the assigned groups).

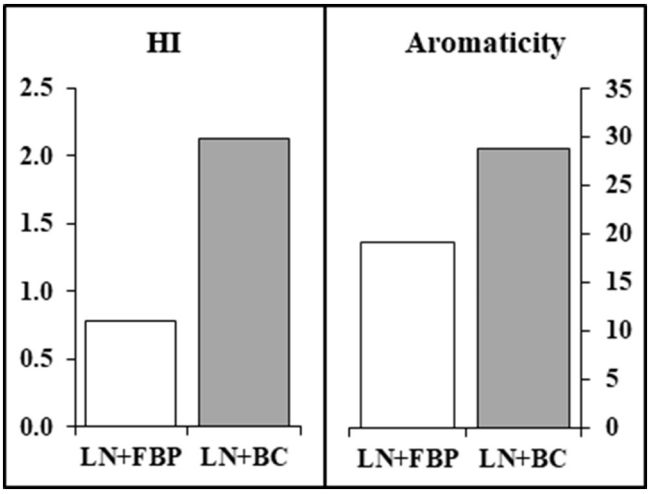

Fig. 2. Comparison of hydrophobicity index (HI) and aromaticity of bioenergy by-products amended soil (LN + FBP: Low N with fermentation by-product; LN + BC: Low N with biochar).

influence of biochar on water extractable C is also supported by our results (Table 3). Fermentation by-product contains large amounts of extractable C as compared to biochar. Johnson et al. (2004) found fermentation by-product (similar to that used in the present investigation) to contain 70% acid extractable C. Therefore, extractable C content in bioenergy by-product amended soil will be greater, as found in the present study (Table 3). Likewise, Cayuela et al. (2010) found an 86% increase in extractable C in bioenergy by-product amended soil compared to unamended soil.

^{13}C solid state NMR has been used to identify SOC functional groups: carboxyl, aryl, O-alkyl, and alkyl based on chemical peak shifts (Baldock et al., 1997; Knicker, 2011). The identified SOC functional groups vary in their molecular composition which in turn determines the chemical extractability, microbial decomposability or lability and persistence of SOC (Baldock et al., 1997; Silveira et al., 2008; Knicker, 2011). Generally, the extractable C (water and acid) was related to alkyl, O-alkyl and carboxylic C (0–110 and 165–200 ppm) while the recalcitrant C was mostly linked with aryl C (165–220 ppm) and to a lesser extent with O-alkyl C (Silveira et al., 2008). The extractable C includes mostly carbohydrates, proteins and amino acids while the recalcitrant C includes lignin, lipids, waxes and anomeric cellulosic C (Silveira et al., 2008). The analogous C functional group composition of SOC in LN and LN + N suggested

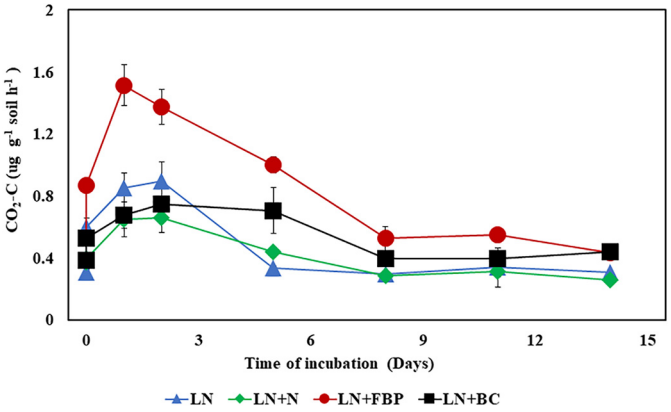

Fig. 3. Rate of CO_2-C production as a result of organic C mineralization from soil amended with N fertilizer or bioenergy by-products [LN: Low N (▲); LN + N: LN with extra fertilizer N (♦); LN + FBP: Low N with fermentation by-product (●); LN + BC: Low N with biochar (■)].

insignificant change in extractable C and microbial response due to mineral N fertilizer. Primarily, the experimental soil was sandy and strictly C limited (~10.0 g kg^{-1} soil). Under C-limited conditions, mineral N fertilization or anthropogenic N deposition was reported to have little effect on soil CO_2 production through microbial respiration (Chen et al., 2014). Thus, extra N fertilization resulted in similar extractable C content and microbial activity as in low C soil. Greater alkyl C and O-alkyl C in fermentation by-product amended soil may have directly originate from amendment and resulted in greater extractable C and microbial responses, as indicated by respired CO_2 and enzyme activity. However, contrary to our expectation, biochar amended soil had lower extractable C and microbial response regardless of having greater alkyl C (40% of SOC) which indicates that mere functional group detection would not be enough to predict extractable C and microbial response. Use of an index based on relative proportion of identified C functional groups could be of help in explaining the effect of organic C-rich amendments (Spaccini et al., 2002). The greater HI index and aromaticity of biochar amended soil confirmed that the biochar amendment supplied more recalcitrant C than labile C despite having greater alkyl C. The HI and aromaticity have been used frequently as interpreters of extractable C and microbial activity in aerobic and peat soil (Spaccini et al., 2002, Normand et al., 2017).

4.2. Soil microbial respiration and extracellular enzyme activities: Legacy impact of bioenergy by-product application

Fermentation residue amendment increased the CO_2-C production rate and resulting total CO_2-C produced in the present experiment, as also reported in previous studies (Johnson et al., 2004; Johnson et al., 2007; Cayuela et al., 2010; Cayuela et al., 2014). The principal reason for greater microbial activity could be linked to greater labile C availability in LN + FBP soil because of fermentation by-product amendment (Cayuela et al., 2010). Previously, a 0–175% increase in CO_2-C respiration rate was observed in different C content soils for fermentation by-product amendment with application rates ranging from 1 to 100 Mg ha^{-1} (Johnson et al., 2007). A fermentation by-product amendment rate of 10 Mg ha^{-1}, like our experiment (9 Mg ha^{-1} y^{-1}), produced only 20–50% greater CO_2 than the control (Johnson et al., 2007). The earlier mentioned studies used laboratory incubation of soil rather than involving field soil as in the present study with fermentation by-product application for three consecutive years. Thus, the present study provided the unique opportunity of understanding the midterm influence of field applying fermentation by-product on soil microbial responses. Additionally, air-dried soil was remoistened at 70% WHC to reflect natural field conditions in which soils go through a cycle of drying and rewetting due to summer fallow following rainfall and/or irrigation. The labile and recalcitrant SOC is the most important aspect determining soil microbial activity in rewetted air-dried soil (Sun et al., 2015). Though soil drying has been frequently reported to alter the soil microbial responses (Birch, 1958), MBC and enzyme activity of rewetted air-dried soils were measured to determine the impact of C amendment in soil previously (Sun et al., 2015; Zhang and Marschner, 2016). The greater MBC in LN + FBP soil was expected since the labile C (measured by extractable C and NMR spectroscopy) increased. Increased availability of labile SOC exploded the proliferation of 'r-strategist' microbes that are adapted to respond quickly to newly available C with greater respiration and enzyme activity (Zimmerman et al., 2011). Surprisingly, despite having comparable MBC to LN + FBP, LN + BC soil with lower CO_2-C production rate indicated the likelihood of a different microbial habitat. It is tempting to speculate on the existence of "biochar-sphere" (Lehmann et al., 2011) in explaining the greater MBC with lower enzyme activity in LN + BC soil. "Biochar-sphere" is characterized by the co-localization of substrate, microbes and enzymes. In a co-localized habitat, microbes, substrate and enzymes are usually immobilized under an optimal environment on solid surfaces. The optimum environment leads to lowered activity of C-hydrolyzing enzymes and increases

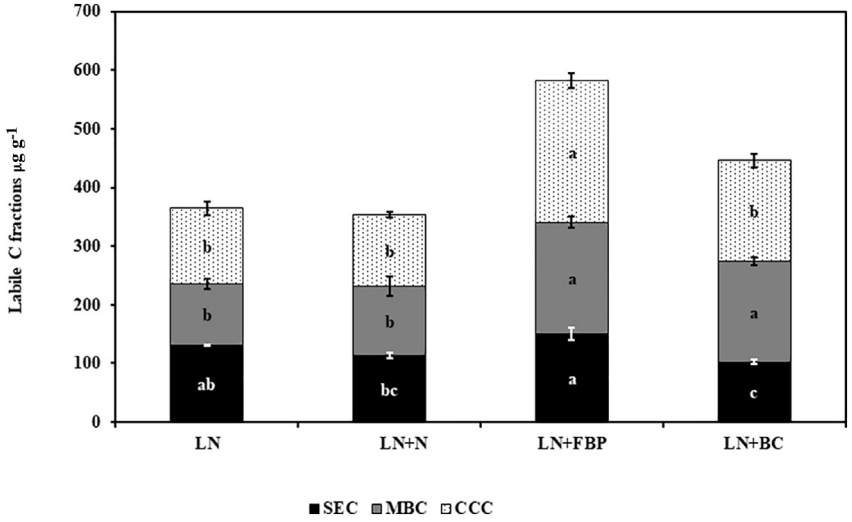

Fig. 4. Labile C content of soil following three annual applications of N or bioenergy by-products. LN: Low N; LN + N: LN with extra fertilizer N; LN + FBP: Low N with fermentation by-product; LN + BC: Low N with biochar. Values within each labile C fractions with same letter are statistically similar based on Tukey honest significant difference test ($p < 0.05$). SEC: 05 M K_2SO_4 extractable C; MBC: Soil microbial biomass C; and CCC: cumulative CO_2-C production over 2 weeks incubation period.

the C use efficiency and enzyme activity efficiencies of microbes, but maintains greater microbial biomass (Lehmann et al., 2011).

Greater β-glucosidase, *N*-acetyl glucosaminidase, Leucine aminopeptidase and phosphomonoesterase activity of LN + FBP among the treatments confirmed the impact of added available substrate through fermentation by-product. To be specific, greater hydrolytic enzyme activities related to C, N and P mineralization were directly correlated to extractable C content in soil (Fig. 6). In LN + FBP soil, the greater glucosidase activity was indicative of increased availability of substrate, i.e. labile C. Previously, increased glucosidase activity was linked to increased substrate availability through either plant litter or organic C substrate addition (Allison and Vitousek, 2005). Similarly, the greater peptidase activity being highly correlated to AEC (Fig. 6) was indicative of positive response to available substrate rather than 'substrate limitation'. Commonly, the phosphatase activity was connected to 'substrate limitation' (Sinsabaugh et al., 2009). Thus, greater APA activity in the LN + FBP could be indicative of available P deficiency even though 486 kg of Mehlich1-P had been added through 27 Mg of FBP in 3 years. The present experimental soil was adequately supplied with available P (Mehlich-1 P: 64.7 mg kg^{-1}), making the interpretation of APA activity

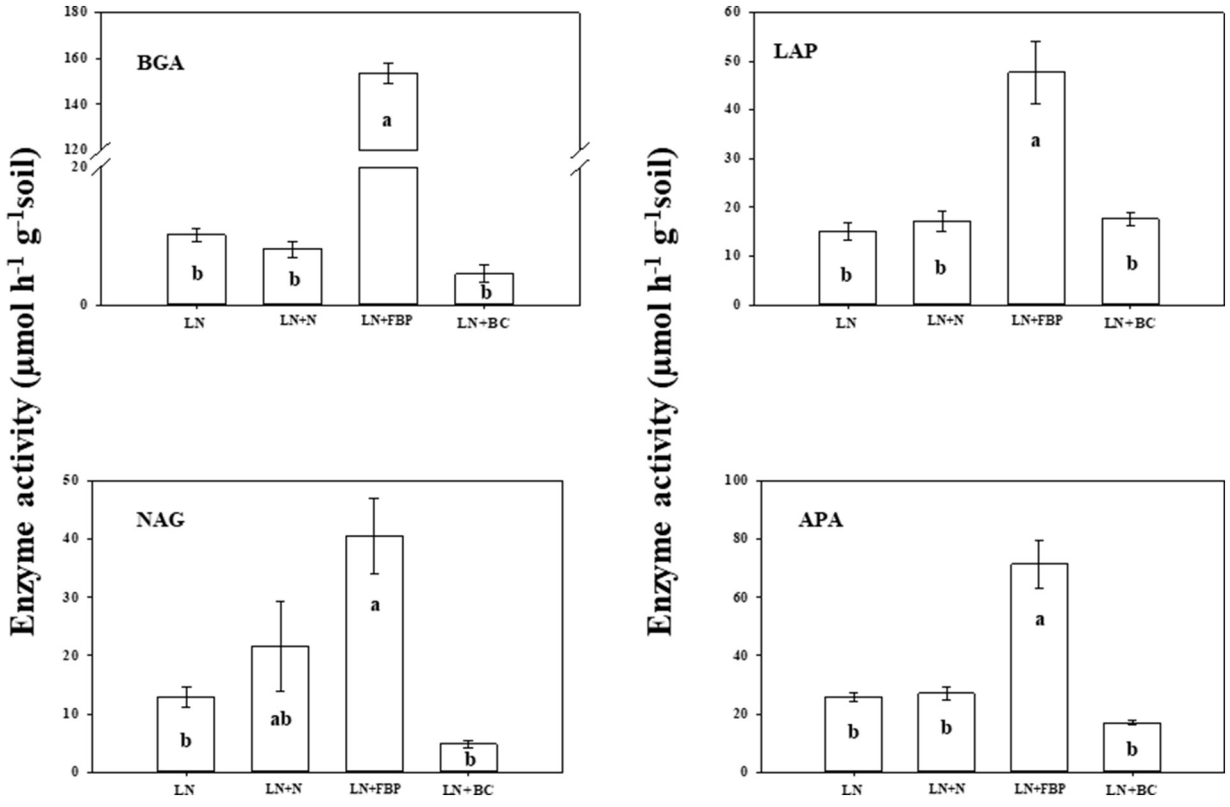

Fig. 5. Extracellular enzyme activity in soil treated with N fertilizer or bioenergy by-products (LN: Low N; LN + N: LN with extra fertilizer N; LN + FBP: Low N with fermentation by-product; LN + BC: Low N with biochar). Values for each enzyme with same letter are statistically similar based on Tukey honest significant difference test ($p < 0.05$) BGA: β-Glucosidase activity; APA: Acid phosphatase activity; LAP: Leucine Aminopeptidase activity; NAG: *N*-acetyl-β-D-glucosaminidase activity.

Table 5
Mean enzymatic activities normalized to soil microbial biomass C (mmol g^{-1} MBC h^{-1}) and enzyme activity ratios in soil following three annual applications of N or bioenergy by-products.

	BGA: MBC	LAP: MBC	NAG: MBC	AP: MBC	C:N enzyme activity ratio	C:P enzyme activity ratio	LAP: NAG
					BGA:(NAG + LAP)	BGA: AP	
LN	98 ± 1^{b}	144 ± 8^{ab}	128 ± 27^{ab}	252 ± 29^{a}	0.37 ± 0.04^{b}	0.40 ± 0.05^{b}	1.23 ± 0.25^{b}
LN + N	69 ± 3^{b}	157 ± 43^{ab}	185 ± 56^{a}	239 ± 38^{a}	0.23 ± 0.07^{b}	0.31 ± 0.06^{b}	0.97 ± 0.27^{b}
LN + FBP	812 ± 46^{a}	252 ± 34^{a}	211 ± 29^{a}	375 ± 37^{a}	1.80 ± 0.22^{a}	2.20 ± 0.23^{a}	1.21 ± 0.13^{b}
LN + BC	26 ± 7^{b}	103 ± 10^{b}	28 ± 5^{b}	99 ± 8^{b}	0.20 ± 0.05^{b}	0.27 ± 0.08^{b}	3.79 ± 0.51^{a}

(LN: Low N; LN + N: LN with extra fertilizer N; LN + FBP: Low N with fermentation by-product; LN + BC: Low N with biochar. MBC: microbial biomass C; BGA: β-Glucosidase activity; AP: Acid phosphatase activity; LAP: Leucine Aminopeptidase activity; NAG: *N*-acetyl-β-D-glucosaminidase activity. Values within each column followed by same letter are statistically similar based on Tukey honest significant difference test ($p < 0.05$)).

more complex. Subsequently, the interpretation of APA activity in C-rich amended soil needed greater attention from the investigator to clearly understand the ecology of APA. Primarily, the lower enzyme activity in LN + BC soil could be explained by 'biochar-sphere', as previously discussed. Secondly, the decreased enzyme activity in LN + BC soil could also be caused by substrate sorption on BC surfaces (Lehmann et al., 2011). In the present study, sorption-driven reductions in enzyme activities were not likely to occur as enzyme activity measurement was performed using fluorescence-based substrate, as suggested by Bailey et al. (2010). Furthermore, the specific enzyme activity analysis also suggested noteworthy influence of fermentation by-product on soil glucosidase activity. Among the enzyme activity ratios, a prominent difference was recorded for LAP: NAG in LN + BC soil because of very low NAG activity. Typically, NAG activity is an indicator of greater fungal biomass than bacterial biomass. Biochar amendment was previously reported to alter the microbial composition (Lehmann et al., 2011), but lack of information on microbial composition in the present study makes it hard to reach a definite conclusion. Nevertheless, the difference in both bioenergy by-products' effects on SOC triggered varied microbial responses. The PCA analysis invariably suggested that fermentation by-product mostly increased the labile C fraction in soil which in turn stimulated the microbial activity. The biplot of first and second principal component explaining 91% total variability in the analyzed data set clearly separated out the LN + FBP from the other three treatments (Fig. 6). The clustering of LN + BC soil opposite to LN + FBP in the biplot was also evidence of their contradictory soil responses. The clustering of LN and LN + N together away from LN + FBP and LN + BC suggested a negligible effect of mineral N fertilizer on the soil properties studied related to SOC and microbial biomass.

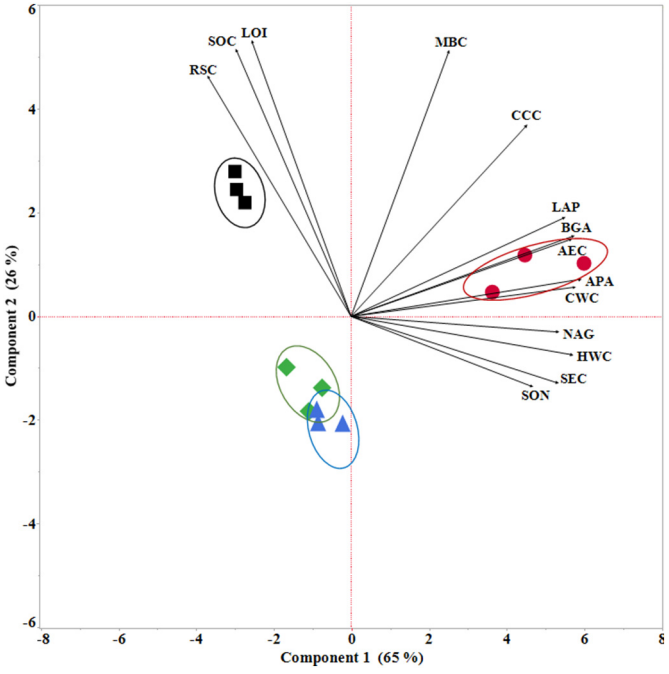

Fig. 6. Correlation biplots based on principal component analysis (PCA) depicting the relationship among the physicochemical and microbial properties of soil amended with N fertilizer or bioenergy by-products [LN: Low N (▲); LN + N: LN with extra fertilizer N (♦); LN + FBP: Low N with fermentation by-product (●); LN + BC: Low N with biochar (■)].The length of the arrows indicates the significance for sample differentiation. For other soil characteristics abbreviations refer 'Materials and methods.'

5. Conclusions

Bioenergy by-product application unsurprisingly improved SOC content compared to either LN or LN + N. The increase in SOC by LN + BC was 270% greater than LN alone. LN + FBP had greater soil microbial biomass, labile C and soil respiration compared to LN. Biochar increased the aromatic and lignin (recalcitrant forms of) C in soil, whereas fermentation by-product increased aliphatic, methoxyl and cellulosic (labile forms of) C in soil, as compared to LN. Biochar reduced the enzyme activity compared to all other treatments. Thus, soil application can be a part of sustainable bioenergy by-products management. Depending on the type of bioenergy by-product, influences on labile and recalcitrant C contents will differ in soil. Fermentation by-product (similar to that studied here) application can increase soil C, mostly in the labile fraction, facilitating soil microbial activity and nutrient bioavailability. Biochar will also increase soil C, mostly in the recalcitrant fraction, facilitating increased C storage in the soil over time.

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