Bone char effects on soil: sequential fractionations and XANES spectroscopy

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Abstract. Acceptability of novel bone char fertilizers depends on their P release but reactions at bone char surfaces and impacts to soil P speciation are insufficiently known. By sequential fractionation and synchrotron-based X-ray absorption near edge structure (XANES) spectroscopy we investigated whether and how the chemical composition of bone char particles has been altered in soil and, consequently, has affected the P speciation of amended soils. Therefore, two different kinds of bone char particles (BC, produced by pyrolysis of degreased animal bone chips at 800 ºC and BC^{plus}, a BC enriched with reduced sulfur compounds) were manually separated from the soil at the end of two different experiments: “incubation-leaching” and “ryegrass cultivation”. Sequential P-fractionation of amended soils showed P-enrichment in all fractions as compared to the control. The most P increase between all treatments significantly occurred in the NaOH-P and resin-P fractions in response to BC^{plus} application in both, incubation-leaching and ryegrass cultivation experiments. This increase of the readily available P fraction in BC^{plus} treated soils was confirmed by linear combination fitting (LCF) analysis on P *K*-edge XANES spectra of BCs particles and amended soils. The proportion of Ca-hydroxyapatite decreased, whereas the proportion of $CaHPO_4$ increased in BC^{plus} particles after amended soils had been incubated and leached and cropped by ryegrass. Based on P XANES speciation as determined by LCF analysis, the proportion of inorganic $Ca(H_2PO_4)_2$ increased in amended soils after BC^{plus} application. These results indicated that soil amendment with BC^{plus} particles leads to elevated P concentration and maintains more soluble P species than BC particles even after 230-days of ryegrass cultivation.

1 Introduction

At the global scale readily-available sources of phosphorus (P), a crucial macro-nutrient element for agricultural production, are being faced with scarcity and overpricing (Scholz et al., 2013; Van Vuuren et al., 2010). Environmental pollutions frequently arise from their impurities (Cd, U) (Hartley et al., 2013; Kratz et al., 2016), and from over-fertilization (Rubaek et al., 2013). Further problems are the quick formation of stable and inaccessible compounds that limit plant P uptake (Shen et al., 2011) and the low agronomic efficiency of not more than 15% of fertilizer P in the first year of application (Schnug et al., 2003). Many recent studies have targeted sustainable agriculture through improving P-availability from applied fertilizers (Delgado et al., 2002; Schröder et al., 2011), increasing P-uptake efficiency from organic and inorganic P-pools in the soil (Fan et al., 2002; Kaur and Reddy, 2014) and developing new technologies for P-recycling from human and animal waste (Siebers and Leinweber, 2013; Herzel et al., 2016).

As an economically and environmentally attractive example, pyrolyzed animal bone chips branded as “bone char” (BC), a slow-release apatite-based P-fertilizer, have been surface modified by sulfur (S) compounds to enhance its solubility in neutral to alkaline soils. Incubation-leaching and pot experiments confirmed that surface-modification was an effective approach in P-release promotion from BC fertilizer (Morshedizad et al., 2016; Zimmer, D. and Panten, K., personal communication). Despite these attempts to raise dissolution and use-efficiency of BC in supplying P for crop requirements, a considerable fraction of applied BC-P to the soil remains insoluble in the short term and is not taken up by plants over the entire cropping period. A detailed P speciation can clarify the fate of insoluble P from BC which has not been done before.

Chemical speciation is described as analytical identification of chemical species of defined elements and measuring their quantities in the system (Templeton et al., 2000). The precise characterization of various P species in the soil as a dynamic response to non-equilibrium conditions imposed by human activities such as fertilization can support a better understanding of reactivity, stability and particularly plant-accessibility of different P-forms and provide a basis for best management practices. Several techniques such as sequential fractionation (Dieter et al., 2010; Condron and Newman, 2011), nuclear magnetic resonance (NMR) spectroscopy (Liu et al., 2009; Vestergren et al., 2012; Ahlgren et al., 2013), Raman spectroscopy (Lanfranco, 2003; Vogel et al., 2013) and chromatography coupled to mass spectroscopy (De Brabandere et al., 2008; Paraskova et al., 2015) have been developed for P speciation analysis in soil and sediments. Each one of these techniques can offer specific advantages and disadvantages depending on phase and complexity of sample matrixes (Kruse et al., 2015). Complementarily, X-ray absorption near edge structure (XANES) spectroscopy is well-suited for identification of various P species through fingerprinting of molecular structures in solid and heterogeneous mediums based on fine feature and position of absorbing edges (Kelly et al., 2008; Kizewski et al., 2011). The advantages of XANES spectroscopy for soil samples make it a promising technique for direct and *in situ* P-speciation with no pretreatment and minimum sample manipulation (Toor et al., 2006; Kelly et al., 2008).

According to the best of our knowledge, no studies have characterized P-speciation changes in BC particles over non-equilibrium conditions in the soil system, and only few investigations have been reported on the P-release from BC and alteration in P-species of the soil. Siebers et al. (2013) investigated *K*-edge XANES spectroscopy on BC-incubated soil samples and provided evidence that the increase in extractable Ca- and Mg-phosphate fractions were related to the contribution of hydroxyapatite (HAP) increase after BC application. Accordingly, the objective of this study was to provide practical information on the fate and alteration of P-species in BC and novel surface modified BC (BC^{plus}) particles and their treated soils under incubation-leaching and ryegrass cultivation practices, using sequential P-fractionation and P-XANES spectroscopy.

2 Materials and methods

2.1 Incubation-leaching experiment

Two particle size fractions (1-2 and 2-4 mm) of bone chars (BC, produced by pyrolysis of degreased animal bone chips at 800°C and BC^{plus} as a surface modified BC obtained by blending with reduced S-containing compounds composed of 60 % elemental S, 30 % calcium sulfate dehydrate and 10 % methansulfonate (Zimmer et al. unpublished results of S X-ray absorption near-edge fine structure spectroscopy) in a commercial biogas desulfurization process; patent application DE 212012000046U1; www.google.com/patents /DE212012000046U1?cl=en&hl=de) were incubated with a silt loam soil. The soil was classified as *Dystric Cambisol* (FAO) with pH of 4.7 (measured in 0.01 mol L^{-1} $CaCl_2$ solution) and total (digestion with HNO_3 and analyzed using ICP-OES; USEPA, 1997) and available (extracted by 1 mol L^{-1} NH_4NO_3 and analyzed using ICP-OES; He and Singh, 1993) P-contents of 1.6 g P kg^{-1} and 14 mg P kg^{-1}, respectively. The BC and BC^{plus} contained total P of 149 and 123 g kg^{-1}, total calcium (Ca) 185 and 265 g kg^{-1}, total S 6 and 199 g kg^{-1} and had average of pH_{CaCl2} values 7.8 and 4.9, respectively.

The BCs were added to 30 g of air dry soil (< 2 mm) at the levels of 0 mg P kg^{-1} soil (control) and 500 mg P kg^{-1} soil in five replicates. The soil and BCs mixture was homogenized and packed into glass columns with 10 cm length and inner diameter of 2 cm. A P-free filter (MN 616 G; Macherey-Nagel GmbH & Co., KG Düren, Germany) was placed at the bottom of each column to avoid any particle losses. The amended soils were incubated for 70 days at 20 °C in the dark and constant soil moisture between 60-70 % of soil water holding capacity. During the incubation period, the soil columns were leached with three pore volumes of deionized water added by a droplet irrigation simulator system. The leaching process was repeated in five steps, each one after 1, 5, 13, 34 and 70 days. The P-concentrations in collected leachates were measured using inductively coupled plasma-optical emission spectrometry (ICP-OES). Outcomes of the leaching experiment were described in Morshedizad and Leinweber (2017). After the incubation-leaching experiment, the treated soil samples were carefully removed from the glass columns, air dried and BCs particles were manually separated from the soils very gently. The BCs particles were delicately washed with deionized water to remove adhered soil particles, allowed to dry completely at ambient conditions and finely ground for further analyses.

2.2 Pot experiment with annual ryegrass

The same BC and BC^{plus} as described for the incubation-leaching experiment were used in original sizes (mostly between 1 to 5 mm) for P fertilization of annual ryegrass in a pot experiment. The experiment was set-up using an acidic sandy silt soil with available P-content of 24.2 mg P kg^{-1} and pH of 5.2. The pot experiment was set up by adding BC and BC^{plus} at the levels of 0 mg P kg^{-1} (control) and 280 mg P kg^{-1} into the 6 kg of the soil dry matter in each pot and in four replicates arranged in a complete randomized block. After four weeks of incubation at field capacity water content and ambient temperature conditions, 30 seeds of annual ryegrass per each pot were sown on 13th of May 2016. The experiment was conducted in a glasshouse under ambient air and temperature conditions and the soil moisture was maintained at field capacity during the whole experiment. All other essential nutrients were sequentially added at sufficient levels before seeding and after each six cuts of ryegrass between 23rd of June and 3rd of November 2016. Finally, after the last harvest (7th), plant parts (shoots and roots) were dried at 60 °C and BCs particles were manually separated from the soils as they could be detected visually by their size and dark color very gently, using tweezers. Then these particles were washed delicately with deionized water to remove attached soil particles, allowed to dry completely at ambient conditions and finely ground to fine powders for further analyses.

2.3 Sequential phosphorus fractionation

Soil samples were sequentially extracted based on chemical solubility in order according to a modified Hedley et al. (1982) procedure. After BC particles detachment, duplicate 0.5 g fine-ground and air-dried soil samples were weighed into 50-mL centrifuge tubes. In summary, chemical P fractionation includes the following steps:

1) The mobile and readily available P fraction was extracted with resin strips (saturated in 0.5 M $NaHCO_3$) after 18 hours end-over-end shaking in 30 mL deionized water. The resin strips were separated from solids/solution and washed using 50 mL of 1 M HCl to remove absorbed P. The soil suspension was centrifuged at 2500 × *g* for 20 minutes and the supernatant was decanted.

2) Next, the labile inorganic and organic fractions weakly absorbed to mineral surfaces and some microbial P were extracted by 30 mL of 0.5 M $NaHCO_3$, 18 hours end-over-end shaking and centrifugation at 2500 × *g* for 20 minutes. The supernatant was filtered (Whatman no. 42 filter) and collected for measurements.

3) The inorganic P adsorbed and bound to Al- and Fe-oxide minerals and organic P from humic substances were extracted using 30 mL of 0.1 M NaOH solution and repeating the second step procedure as described above.

4) The relatively insoluble fraction of P bound to Ca and Mg minerals and apatite was extracted by 30 mL of 1 M HCl in the same way as for the previous steps.

Total P concentrations (P_t) and inorganic P (P_i) in all extracts were measured by ICP-OES and colorimetrically (molybdenum blue method; Murphy and Riley, 1962), respectively. The organic P (P_o) concentrations were calculated by $P_t - P_i$.

2.4 Phosphorus *K*-edge XANES analysis

The XANES data collection for characterizing P-species in all soil samples and BCs particles was acquired at the Synchrotron Light Research Institute (SLRI) in Nakhon Ratchasima, Thailand, on the beamline 8 (BL8) of the electron storage ring with a covering photon energy from 1.25 to 10 KeV, electron energy operated at 1.2 GeV and beam current of 80-150 mA (Klysubun et al., 2012). The P *K*-edge XANES spectra were collected from dried and very finely ground treated soils and particulate BCs samples which had been diluted to P concentrations < 10 mg P kg^{-1} with SiO_2 powder (to eliminate self absorption effects; Prietzel et al., 2013), again ground in agate stone mini-mortar and spread uniformly as a thin layer on P-free kapton tape (Lanmar Inc., Northbrook, IL, USA). Data collection was operated in standard conditions comprising the energy calibration by standard pure elemental P and allocating the reference energy (E_0) at 2145.5 eV using the maximum peak of spectrum. All spectra were recorded at photon energies between 2045.5 and 2495.5 eV in step sizes of 5 eV (2045.5 to 2105.5 eV and 2245.5 to 2495.5 eV), 1 eV (2105.5 to 2135.5 eV and 2195.5 to 2245.5 eV) and 0.25 eV (2135.5 to 2195.5 eV) with a 13-channel germanium detector in fluorescence mode. At least three scans were collected and averaged for each sample.

The P-XANES spectra were normalized and after merging replicates, a linear combination fitting (LCF) was performed using the ATHENA software package (Ravel and Newville, 2005). All XANES spectral data were baseline corrected in the pre-edge region between 2115–2145 eV and normalized in the post-edge region of 2190–2215 eV similar to those were used for the reference P *K*-edge XANES spectra (Prietzel et al. 2016) to achieve consistency in the following fitting analysis. To achieve the best compatible set of references with each specified sample spectrum, LCF analysis was performed in the energy range between -20 eV and +30 eV relative to the E_0 using the combinatorics function of ATHENA software to attain all possible binary, ternary and at most quaternary combinations between all nineteen P reference spectra. The following set of reference P *K*-edge XANES spectra, all recorded in SLRI under the same adjustments by Werner and Prietzel (2015) and Prietzel et al. (2016), were used for fitting and calculations; Ca-, Al- and Fe-phytate, non-crystalline and crystalline $AlPO_4$, non-crystalline and crystalline $FePO_4.2H_2O$, Ca-hydroxyapatite ($Ca_5(OH)(PO_4)_3$), inositol hexakisphosphate (IHP), ferrihydrite-IHP, montmorillonite-Al-IHP, soil organic matter (SOM)-Al-IHP, ferrihydrite-orthophosphate, montmorillonite-Al-orthophosphate, SOM-Al-orthophosphate, bohemite-IHP, bohemite-orthophosphate, $CaHPO_4$, $Ca(H_2PO_4)_2$ and $MgHPO_4$. To select the best possible combination fit between the sample spectrum and the P-reference spectra, the lowest reduced chi value (χ^2) and *R*-factor were chosen.

3 Results

3.1 Effect of BCs on sequentially extracted P after incubation-leaching

After 70 days of incubation-leaching, the sequential P-fractionation of amended soils showed variations in the amount and distribution of various P-fractions between different treatments (Table 1). For all treatments, NaOH extracted the majority of fractionated P (62.4 to 66.5 % of total fractionated P), followed by the labile P fraction ($NaHCO_3$, 19.2 to 20.0 %), HCl-P (9.5 to 13.9 %) and the readily available P (resin strips, 4.1 to 4.8 %). The BCs addition increased the total soil P pools although the difference was significant only for the $BC^{plus}_{1\text{-}2\,mm}$ and $BC_{1\text{-}2mm}$ treatments. The largest increase in total fractionated P_t (resin-P_t + $NaHCO_3$-P_t + NaOH-P_t + HCl-P_t) occurred in $BC^{plus}_{1\text{-}2\,mm}$ (133.8 mg P kg^{-1} soil) followed by $BC_{1\text{-}2\,mm}$

(118.6 mg P kg^{-1} soil), $BC_{2\text{-}4\ mm}$ (67.1 mg P kg^{-1} soil) and $BC^{plus}_{2\text{-}4\ mm}$ (35.7 mg P kg^{-1} soil), compared to the control soil.

INSERT Table 1 HERE

The proportion of P-enrichment in each fraction varied between different treatments in the order NaOH-P > HCl-P > $NaHCO_3$-P > resin-P for $BC^{plus}_{1\text{-}2\ mm}$ and $BC^{plus}_{2\text{-}4\ mm}$. For the $BC_{1\text{-}2\ mm}$ and $BC_{2\text{-}4\ mm}$ treatment the order was HCl-P > NaOH-P > $NaHCO_3$-P > resin-P. In all treatments, the P_i proportions in each of the P fractions were greater than the P_o proportions. Compared to the control soil, the most P_i increase was observed in NaOH-P_i and resin-P_i in response to $BC^{plus}_{1\text{-}2\ mm}$ application (Table 1). Moreover, after 70 days of incubation-leaching, soil pH increased in BC treatments whereas BC^{plus} amendments had an acidifying effect. Soil pH levels of $BC_{1\text{-}2\ mm}$ and $BC_{2\text{-}4\ mm}$ increased by 0.07 and 0.05 units and decreased for $BC^{plus}_{1\text{-}2\ mm}$ and $BC^{plus}_{2\text{-}4\ mm}$ treatments by 0.21 and 0.15 units compared to unamended control soil (pH = 5.06).

3.2 Effect of BCs on sequentially extracted P after ryegrass cropping

Sequentially extracted P fractions in soil varied between different treatments after 230 days of ryegrass cropping (Table 1). In all treatments (control, BC and BC^{plus}), NaOH-P was the largest P pool mainly associated with Al- and Fe-oxide minerals and humic substances (65.0 to 67.5 % of total fractionated P) followed by the $NaHCO_3$-P (18.2 to 19.0 %), HCl-P (11.5 to 12.6 %) and resin-P (2.2 to 3.4 %) fractions. Enrichments of P fractions in BC^{plus} treatments were more pronounced than in treated soils with BC particles. In this treatment the concentrations of readily available and labile inorganic P fractions were insignificantly smaller than in the control. Additionally, a significant increase in P concentration was obtained only in resin-P_i and NaOH-P_i fractions of the BC^{plus} treated soil (Table 1). The maximum increase of total fractionated P was obtained in BC^{plus} treatment (37.6 mg P kg^{-1} soil). In comparison to incubation-leaching results, a similar sequence was observed for the order of increasing magnitude of P fractions in response to BC and BC^{plus} amendments (NaOH-P > $NaHCO_3$-P > HCl-P > resin-P). However, for BC treatment, the total P extracted by resin strips was lowered in comparison with the control. In the control and BC treatments, P_o was the predominant form in NaOH-P and $NaHCO_3$-P fractions while for BC^{plus} it was only in the NaOH-P fraction. Each P fraction was highest under BC^{plus} application, except for $NaHCO_3$-P_o of BC treatment.

Separately, the effect of BC and BC^{plus} application on ryegrass yield parameters was examined in the 230-days pot experiment. The results indicated that P uptake, ryegrass yield and apparent nutrient recovery efficiency (ANR) of BC^{plus} treatments exceeded that of BC and control treatments and increased to values comparable with triple super phosphate (TSP) fertilizer (Zimmer, D. and Panten, K., personal communication). Addition of BC and BC^{plus} did not significantly change the bulk soil pH, although local acidification around BC^{plus} particles (pH 4.9; Morshedizad and Leinweber, 2017) probably can lower soil pH in small scale areas compared to BC treatments (pH about 8).

3.3 XANES analysis of BCs particles

All spectra from BCs were characterized by an intense white-line peak, post-edge position and without a distinct pre-edge which corresponded to calcium phosphate compounds including Ca-hydroxyapatite, dicalcium phosphate ($CaHPO_4$) and Ca-phytate (Fig. 1). The P *K*-edge XANES results indicated no obvious alterations in spectral features of BCs particles after the incubation-leaching experiment. After 70-days of incubation-leaching, the BC spectra were shifted towards Ca-hydroxyapatite, and this was more pronounced for the 2-4 mm than for the 1-2 mm BC-particles. The opposite trend was the case for BC^{plus} particles where the white-line signal intensity decreased after incubation-leaching period and the post-edge of spectra tended more to dicalcium phosphate. This effect was stronger for BC^{plus}-particle size reduction from 2-4 mm to 1-2 mm.

INSERT Figure 1 HERE

To quantify the P speciation of BC and BC^{plus} particles, LCF analyses using all possible combinations were performed on all P *K*-edge XANES spectra (Table 2). The fitting results indicated that untreated BC and BC^{plus} particles before the experiment contained on average 61 and 60 % Ca-hydroxyapatite, 22 and 30 % $CaHPO_4$ and 18 and 10 % Ca-phytate. After 70-days of incubation-leaching, the proportion of Ca-hydroxyapatite increased to the average of 80% in BC while it remained unchanged in BC^{plus}particles. The $CaHPO_4$ proportion increased in BC^{plus} particles to the average of 34 % whereas the lower content was assigned in the spectra of BC particles accounting for 10 % of total P species. Moreover, the Ca-phytate proportion decreased slightly in BC and BC^{plus} particles from about 18 and 10% to averages of 11 and 7 %, respectively.

INSERT Table 2 HERE

Spectra of BC and BC^{plus} particles, before and after 230-days of ryegrass cultivation, were characterized by a sharp white-line followed by a shoulder and then a post-edge feature between 2160 and 2175 eV which was divided into two peaks (Fig. 2). These features were most similar to P *K*-edge XANES spectra of Ca-hydroxyapatite, $CaHPO_4$ and Ca-phytate standard compounds. Treated BC particles had a white-line with higher intensity which appeared more similar to the Ca-hydroxyapatite spectrum. In contrast, BC^{plus} particles under ryegrass cultivation showed a weaker white-line exhibiting the shoulder and post-edge feature more comparable to the $CaHPO_4$ spectrum.

INSERT Figure 2 HERE

Some differences in proportions of P species observed between BCs particles before and after the cropping period in the ryegrass pot experiment are presented in Table 3. The LCF results revealed overall contributions of 63 and 70 % Ca-hydroxyapatite, 29 and 29% $CaHPO_4$ and 8 and 1 % Ca-phytate in the original BC and BC^{plus}, respectively. After the cropping period, the percentage of Ca-hydroxyapatite was increased in BC particles. In the BC^{plus} treatment, the percentage of $CaHPO_4$ increased from 29 to 43, while the percentage of Ca-hydroxyapatite was reduced from 70 to 49 %. The Ca-phytate proportion remained unchanged in BC particles while that of BC^{plus} increased from 1 to 8 % after the ryegrass cultivation period.

INSERT Table 3 HERE

3.4 XANES analysis of soil samples

The P *K*-edge XANES spectra of soil samples from the incubation-leaching experiment showed two dominant features including 1) a strong white-line lacking pre-edge and shoulder and 2) a tailed post-edge feature (Fig. 3). The most similarity to these features was seen in XANES spectra of amorphous $AlPO_4$, $FePO_4$ and SOM-Al-IHP compounds. Distinct differences appeared between the control and treated soil with BC^{plus}, not with BC treatments. This was reflected by slightly lower intensities of both white-line and post-edge features.

INSERT Figure 3 HERE

The P species of treated soils in the incubation-leaching experiment were determined by LCF analysis to select at most four reference compounds in combinatorics of all possible fitting combinations (Table 4). The fitting results indicated that P in the control soil and BC treatments occurred dominantly as $AlPO_4$ amorphous (≈ 40 %), $FePO_4$ (≈ 30 %) and SOM-Al-IHP (≈ 20 %) compounds. In BC^{plus} treated soils, the average proportion of amorphous $AlPO_4$ decreased to 26 % and instead, $Ca(H_2PO_4)_2$ was identified with an average of 25 % which did not appear in the control and BC treatments. The LCF results showed that the soil treated with BC^{plus} had no detectable Ca-hydroxyapatite which was found in the control and BC treatments.

INSERT Table 4 HERE

The XANES spectra recorded from treated soil samples in the ryegrass pot experiment showed the presence of an intense white-line in the energy range of 2152 to 2158 eV and a stretched post-edge feature approximately from 2165 to 2178 eV (Fig. 4). Decreases in white-line and post-edge intensities of the soil samples appeared as an effect of BC^{plus} application. Visual inspection of P *K*-edge spectra revealed no indication of specific alteration in spectral features in response to the BC treatment.

INSERT Figure 4 HERE

Amorphous $AlPO_4$ was identified by LCF analysis as dominant component (≈ 35 %) in all treated soil samples from the ryegrass pot experiment (Table 5). The second major P form in the control soil was IHP (29 %) followed by Ca-phytate (27 %), with the latter also as pronounced as that observed for BC and BC^{plus} treatments. All treated soils varied in proportions of free or bound IHP forms. The Mont-Al-PO_4 and $Ca(H_2PO_4)_2$ compounds were only assigned in the control and BC^{plus} treatments, respectively.

INSERT Table 5 HERE

4 Discussion

4.1 P availability as revealed by sequential fractionation

The sequence of P distribution between sequentially extracted P-fractions was in accordance with findings by many studies (Cross and Schlesinger, 1995; McDowell and Stewart, 2006; Hashimoto and Watanabe, 2014), reflecting the general status of different P-pools in acidic soils. The results indicated that the largest P proportion was found in the NaOH fraction reflecting P fixed to Fe- and Al-oxides as followed by the $NaHCO_3$-P fraction assigned to weakly P absorbed on crystalline Fe- and Al-oxides or surface of minerals. Guo et al. (2000) reported that the NaOH-P fraction may support the labile $NaHCO_3$-P fraction as a buffering P-pool in highly weathered and acidic soils. According to soil pH values (4.7 and 5.2), the larger proportions of $NaHCO_3$-P even than HCl-P can be explained by the abundance and surface loadings of Fe- and Al-oxides that support the electrostatic binding of phosphate ions and a scarcity of Ca and Mg minerals or soluble ions. As expected, the lowest P proportions were found in the mobile and readily available P fraction extracted by resin strips in agreement to many comparable studies (Cross and Schlesinger, 1995; Bauchemin et al., 2003; Sharpley et al., 2004; Siebers et al., 2013). Among the two soils which were used in the two different experiments, the largest proportions of inorganic P were achieved in the soil after incubation-leaching experiment, while the organic P forms were considerably more abundant in the soil samples after ryegrass cultivation (Table 1). These differences may be due to the microbial activities in the rhizosphere of grasses and transformation of P_i to more stable P_o fractions during longer plant cultivation period (230 days) than in the non-cropped incubation-leaching experiment (70 days).

In general, all P fraction concentrations were elevated by adding BC and BC^{plus} particles which appeared to follow the same pattern in both soils under two different experimental conditions. However, significant differences were found only between the control and BC^{plus} treated soils (1-2 mm in the incubation-leaching experiment) for the resin-P and NaOH-P fractions. Since the BCs particles were separated from the soils before chemical analysis, it was expected that partly dissolved BCs would have a limited impact on different P-fractions rather than totally ground and mixed BCs. This is consistent with the study of Siebers et al. (2013) according to which the BC application (<90 µm BC thoroughly mixed to soil) significantly increased the insoluble P proportion (H_2SO_4-P). Additionally, our study confirmed previous findings concerning the effect of particle sizes on the P release from BCs (Morshedizad and Leinweber, 2017) and consequently the P status of treated soils (Ma and Matsunaka, 2013). Sequentially extracted P contents increased with decreasing size of BC particles whereby BC^{plus} treatments appeared more dependent on particle size than BC treatments. The results of sequential P fractionation of BC^{plus} treatments in the incubation-leaching experiment indicated that the P increase was more pronounced for P fixed to Al- and Fe-oxides (NaOH-P) than other fractions, whereas for BC treatments the largest increase occurred in P bound to Ca and Mg minerals (HCl-P). It seems that local pH changes in soil associated with BC and BC^{plus} amendments could eventually lead to a different distribution of released P into differently soluble or insoluble P-pools which are generally controlled by pH (Arai and Sparks, 2007). However, due to lower fertilization level and longer period of experiment in ryegrass cultivation compared to incubation-leaching, it appears that the chemical equilibrium has been established in the soil (no significant change in bulk soil pH) and, accordingly, the soil P fractions were altered minimal.

4.2 P speciation of BCs particles by XANES

The predominance of Ca-hydroxyapatite in BCs as evidenced by P *K*-edge XANES analysis is consistent with findings reported by previous studies (Warren et al., 2009; Siebers et al., 2013). The mineral phase of bone consists mainly of hydroxyapatite, and its contribution to bone and bone char compositions depends on species and the age of animals (Wu et al., 2003) and carbonization temperature and residence time (Novotny et al., 2012). Bone crystallinity might be improved through structural modifications on poorly crystalline fresh bone samples (such as mineral maturity over periods of time or intensive carbonization) which can also result in increased proportions of hydroxyapatite and accordingly a decrease in P solubility (Novotny et al., 2012). Based on LC fittings, the second major component of BCs particles was $CaHPO_4$, in good agreement with the results of Rajendran et al. (2013) who indicated the heated bones at 400 ºC contained some more soluble phosphates such as $CaHPO_4$ and CaH_2PO_4 in addition to the hydroxyapatite fraction. The authors reported that spectra of calcined bone samples at 700 ºC had white-line at 2154 eV and two post-edge peaks at 2162 eV and 2169 eV with no pre-edge peaks and appeared similar to $CaHPO_4$ and CaH_2PO_4 spectra. Our LCF also assigned Ca-phytate in BCs samples which seems to be controversial as a component of animal bone materials. The P *K*-edge spectrum of Ca-phytate is very similar to other Ca-bound P compounds with a distinct white-line and lack of a pre-edge feature, although it is likely distinguishable due to specific shape of white-line tailing and absence of post-edge signal at 2164 eV (Prietzel et al., 2016). Moreover, some inaccuracies in LCF estimations have to be considered because of 1) uncertainty in speciation of organic P forms by *K*-edge XANES, 2) lack of reference compounds representing all P forms in BCs and 3) smaller Ca-phytate proportions than proposed 10 to 15 % of P_t as detection limit for reliable XANES fittings (Beauchemin et al., 2003). Therefore, the P proportions assigned to Ca-phytate also could originate from a range of other CaP compounds.

In both experiments, incubation-leaching and ryegrass cropping, changes in proportions of Ca-hydroxyapatite and $CaHPO_4$ in BC particles followed an opposite trend than in BC^{plus} particles. After placement of BC particles in the soil, Ca-phosphate seemed to be released gradually over time which provides a locally lime-saturated condition. Due to elevated pH surrounding the BC particles, dissolved P can be resorbed to maintain solubility and the Ca-P equilibrium constant which likely resulted in a decreased proportion of soluble $CaHPO_4$ and possibly the transformation into the relatively insoluble Ca-hydroxyapatite fraction. In contrast, if BC^{plus} particles were applied to soils, larger proportions of $CaHPO_4$ at the expense of Ca-hydroxyapatite could be explained by soil acidification through the microbial oxidation of released S. This effect was more pronounced over the longer time period in the ryegrass cropping pot experiment, favoring a greater $CaHPO_4$ than Ca-hydroxyapatite fraction. This implies that BC^{plus} can actively supply P with predominance of soluble over insoluble P forms in the long-term and, thus, meet crop requirements.

4.3 P speciation of treated soils by XANES

Differences between characteristics of two soils, dissimilar mechanisms of incubation-leaching and plant uptake besides different experiment time durations complicate the joint interpretation of the P-XANES data. In unfertilized soil of the incubation-leaching experiment, the proportions of P species followed the order $AlPO_4$ > $FePO_4$ > SOM-Al-IHP > Ca-hydroxyapatite which did not vary despite partial changes in some proportions after application of both size fractions of BC particles. In general, these results concur with the findings by Siebers et al. (2013) that Ca-hydroxyapatite proportion was slightly increased by

BC application. This could be attributed to irreversibly mixing finely-ground BC to the soil samples whereas in the present experiments the BC particles were separated from the soils before P speciation. Furthermore, these XANES data (Table 4 and Table 5) are in agreement with sequential P fractionation results (Table 1) which indicated the dominance of inorganic over organic P forms and showed the P fractions almost unchanged after BC application. Implications of low solubility of BC particles observed in this work are consistent with previous studies showing a slow release P from BCs (Warren et al., 2009; Siebers et al., 2013; Morshedizad et al., 2016). Besides reducing the $AlPO_4$ and Ca-hydroxyapatite proportions, BC^{plus} particles introduced highly soluble $Ca(H_2PO_4)_2$ to soils in the incubation-leaching experiment. These results imply that considerable changes in P speciation were more attributed to pH reductions and, accordingly, leaching out solubilized P-forms compared with P enrichment by BC^{plus} dissolution. This is supported by results from a previous publication in which two particle sizes of BC^{plus} gave a significant rise in the leached P-concentration after 1, 5, 13, 34 and 70 days of incubation along with reductions in soil pH (Morshedizad and Leinweber, 2017). This is in line with Sato et al. (2005) who found that increasing soil pH in a naturally acidic soil (pH = 4.32) was an effective approach to minimize P leaching, while pH decrease resulted in transformation of stable to soluble and more leachable P species. Regarding the XANES results of the ryegrass cultivation experiment (Table 5), the effect of BC^{plus} treatment can be explained better. In the control soil, the presence of $AlPO_4$ and increasing abundance of organic P-forms (Ca-phytate and IHP compounds) (Table 5) were consistent with the appearance of NaOH-P and HCl-P fractions by sequential extraction (Table 1). In the BC treatment the proportions of $AlPO_4$ and Ca-phytate did not change compared to the control but the contribution of organic P increased by Mont-Al-IHP formation. The stability of different P fractions can be favored by the pH effect (Gustafsson et al., 2012) likewise the dependence of BC particles solubility on the soil pH (Siebers et al., 2013). In agreement with incubation-leaching results (Table 4), $Ca(H_2PO_4)_2$ was detected as a result of BC^{plus} amendment even though similar proportions of $AlPO_4$ and Ca-phytate were observed between the control and BC^{plus} treatment. The results of sequential P fractionation and XANES analyses on treatments in the two different experiments presented here demonstrated that surface modification of BC particles effectively improved soluble P fractions in BC^{plus} particles and, consequently, in amended soils.

5 Conclusions

In the present study, the P speciation by a sequential P fractionation and P *K*-edge XANES spectroscopy revealed the noticeable alteration in the P-pools of treated soil samples. Results of incubation-leaching and ryegrass cultivation experiments indicated that BC^{plus} produced by surface-modification of BC through addition of S compounds provided more soluble and plant-available P than non-modified BC during the growth season. The S oxidation and thereby the soil pH decrease seems to stimulate the P release from BC^{plus} particles. The P *K*-edge XANES analysis of BC^{plus} particles revealed more soluble $CaHPO_4$ than in BC particles at the expense of Ca-hydroxyapatite. This was associated with $Ca(H_2PO_4)_2$ addition to amended soils, as identified by sequential P fractionation and XANES analyses. Future studies will be directed to validate the beneficial effects of BC^{plus} at the field scale with different soils and to optimize the surface modification of BCs.

Data availability. All compiled data of this study are published in figures and tables. Detailed primary data including the incubation-leaching and ryegrass cultivation results and the acquired XANES spectra will be saved and published in the BonaRes Data Center (https://www.bonares.de/research-data) and get the BonaRes DOI prefix (10.20387).

Competing interests. The authors declare that they have no conflict of interest.

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Table 1. Distribution of inorganic P (P_i), organic P (P_o) and total P (P_t) concentrations (mg P kg^{-1} soil) of sequentially extracted P-fractions in the soils as affected by different treatments (treated with two particle size fractions (1-2 and 2-4 mm) and original sizes of BC and BC^{plus} or unfertilized soils (control) after incubation-leaching and ryegrass cultivation experiments.

Treatment	Resin-P			$NaHCO_3$-P			NaOH-P			HCl-P		
	P_i	P_o	P_t	P_i	P_o	P_t	P_i	P_o	P_t	P_i	P_o	P_t
Incubation-leaching												
Control	47	5	52	160	99	259	565	294	859	113	10	123
BC$_{1-2}$	56^{NS}	7^{NS}	63^{NS}	163^{NS}	108^{NS}	271^{NS}	578^{NS}	303^{NS}	881^{NS}	140^{NS}	56^{NS}	196^{NS}
BC$_{2-4}$	50^{NS}	7^{NS}	57^{NS}	161^{NS}	105^{NS}	266^{NS}	574^{NS}	301^{NS}	875^{NS}	121^{NS}	40^{NS}	161^{NS}
BC$^{plus}_{1-2}$	61^{*}	7^{NS}	68^{NS}	172^{NS}	111^{NS}	283^{NS}	593^{*}	313^{NS}	906^{*}	131^{NS}	37^{NS}	170^{NS}
BC$^{plus}_{2-4}$	50^{NS}	7^{NS}	57^{NS}	160^{NS}	104^{NS}	264^{NS}	574^{NS}	298^{NS}	872^{NS}	115^{NS}	21^{NS}	135^{NS}
Ryegrass cropping experiment												
Control	4	4	8	25	27	52	75	121	196	28	5	33
BC	2^{NS}	5^{NS}	7^{NS}	24^{NS}	32^{NS}	56^{NS}	79^{NS}	125^{NS}	204^{NS}	30^{NS}	6^{NS}	36^{NS}
BC^{plus}	6^{*}	5^{NS}	11^{*}	35^{NS}	27^{NS}	62^{NS}	85^{**}	128^{NS}	213^{NS}	34^{NS}	7^{NS}	41^{NS}

* Significant at $P<0.05$; ** Significant at $P< 0.01$; ^{NS} Non-significant difference (treatment vs. control; Tukey-Test)

Table 2. Results of linear combination fitting (LCF) conducted on P *K*-edge XANES spectra of bone char (BC) and surface modified bone char (BC^{plus}) particles before and after a 70-days incubation-leaching period.

Reference compound	Before experiment				After 70 days incubation-leaching			
	BC		BC^{plus}		BC		BC^{plus}	
	1-2 mm	2-4 mm	1-2 mm	2-4 mm	1-2 mm	2-4 mm	1-2 mm	2-4 mm
Ca-hydroxyapatite (%)	58± 6	64±5	62± 5	58± 5	75± 4	85± 3	59± 5	60± 6
$CaHPO_4$ (%)	24± 5	19± 4	28± 4	32± 6	14± 3	5± 2	33± 4	35± 5
Ca-phytate (%)	18± 4	17± 4	10± 3	10± 3	11± 3	10± 2	8± 4	5± 4
R-factor	0.012	0.008	0.007	0.009	0.005	0.002	0.009	0.010

Table 3. Results of linear combination fitting (LCF) conducted on P *K*-edge XANES spectra of bone char (BC) and surface modified bone char (BC^{plus}) particles before and after 230-days of ryegrass cultivation in a pot experiment.

Reference compound	Before experiment		After 230 days ryegrass cultivation	
	BC	BC^{plus}	BC	BC^{plus}
Ca-hydroxyapatite (%)	63± 6	70± 4	75± 4	49± 8
$CaHPO_4$ (%)	29± 5	29± 3	17± 4	43± 6
Ca-phytate (%)	8± 4	1± 3	8± 3	8± 5
R-factor	0.012	0.005	0.006	0.018

Table 4. Results of linear combination fitting (LCF) conducted on P *K*-edge XANES spectra of unfertilized (control) and fertilized soils with bone char (BC) and surface modified bone char (BC^{plus}) particles in the 70-days incubation-leaching experiment.

Reference compound	Control	BC treatment		BC^{plus} treatment	
		1-2 mm	2-4 mm	1-2 mm	2-4 mm
Ca-hydroxyapatite (%)	8± 1	4± 1	8± 1	0	0
$AlPO_4$ amorphous (%)	42± 1	42± 2	40± 1	27± 1	24± 1
$FePO_4$ (%)	29± 1	31± 2	31± 1	27± 1	26± 1
SOM-Al-IHP (%)	21± 2	23± 4	21± 3	26±2	21± 1
$Ca(H_2PO_4)_2$ (%)	0	0	0	20± 1	29± 1
R-factor	0.0003	0.0007	0.0003	0.0005	0.0004

Table 5. Results of linear combination fitting (LCF) conducted on P *K*-edge XANES spectra of unfertilized (control) and fertilized soils with bone char (BC) and surface modified bone char (BC^{plus}) particles under 230-days of ryegrass cultivation in a pot experiment.

Reference compound	Control	BC treatment	BC^{plus}treatment
$AlPO_4$ amorphous (%)	35± 3	35± 3	34± 1
Ca-phytate (%)	27± 3	28± 3	27± 1
IHP (%)	29± 5	21± 7	0
Mont-Al-IHP (%)	0	16± 1	0
SOM-Al-IHP (%)	0	0	25± 2
Mont-Al-PO_4 (%)	9± 1	0	0
$Ca(H_2PO_4)_2$ (%)	0	0	14± 2
R-factor	0.0006	0.0008	0.0006

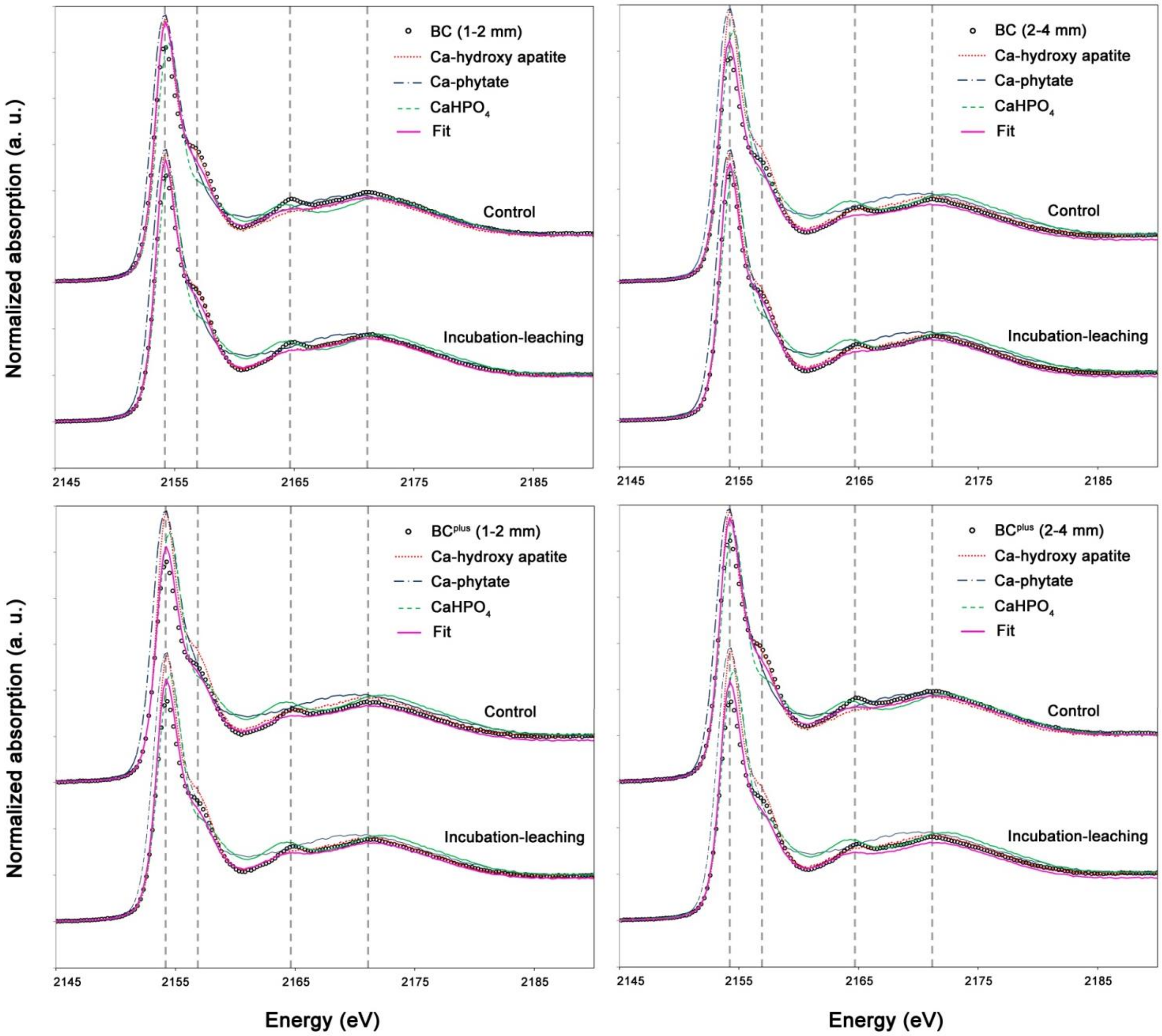
Figure captions

Figure 1. Normalized P *K*-edge XANES spectra of different BC and BC^{plus} particle sizes (1-2 and 2-4 mm) before (control) and after 70-days incubation-leaching experiment compared to the reference compounds selected using LCF method.

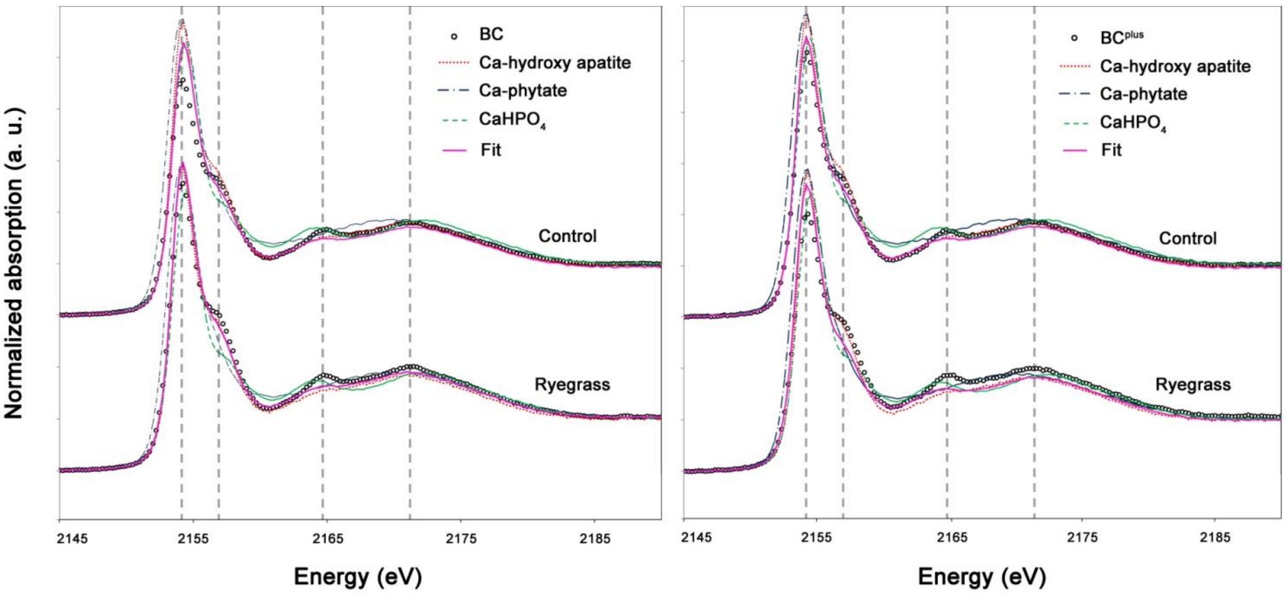
Figure 2. P *K*-edge XANES spectra of BC and BC^{plus} particles before (control) and after 230-days of ryegrass cultivation compared to the reference compounds selected by LCF method.

Figure 3. P *K*-edge XANES spectra of unfertilized (control) and fertilized soils with BC and BC^{plus} particles under 70-days incubation-leaching experiment compared to the reference compounds selected by LCF method.

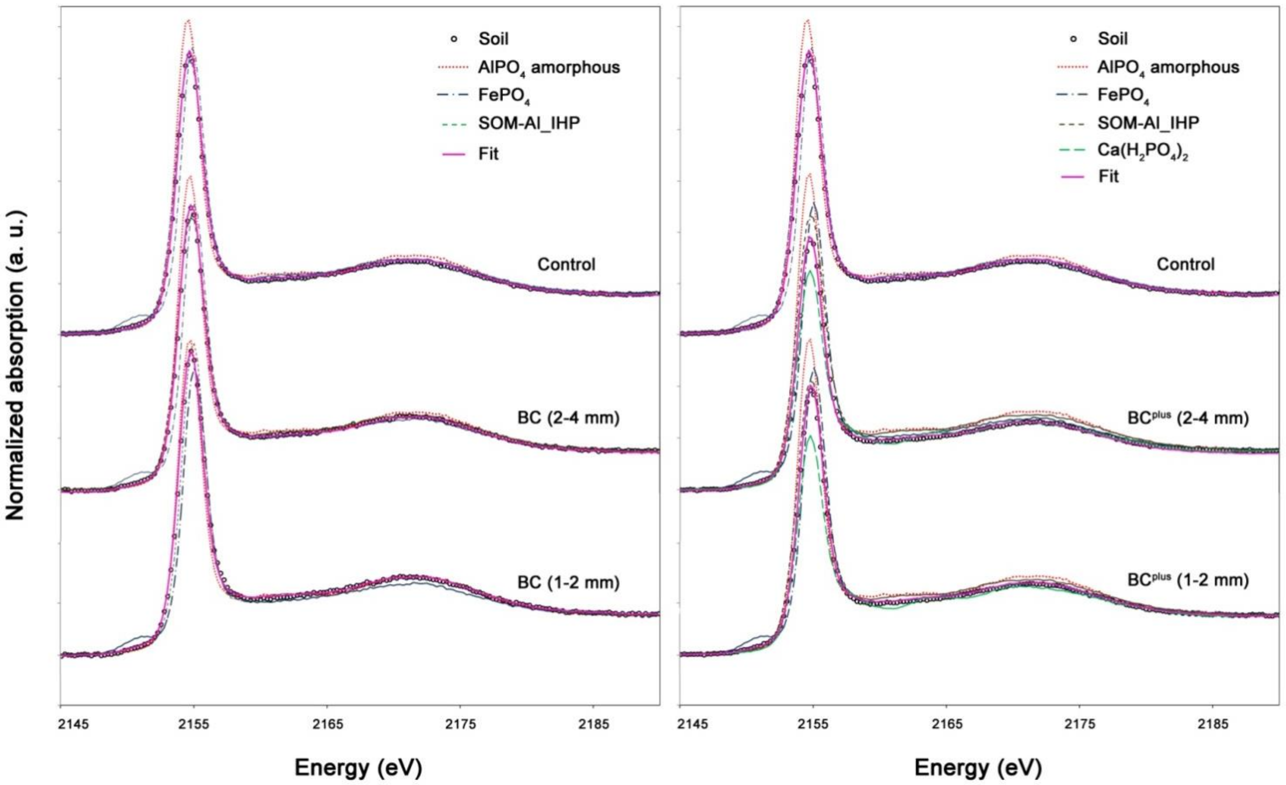
Figure 4. P *K*-edge XANES spectra of unfertilized (control) and fertilized soils with BC and BC^{plus} particles under 230-days of ryegrass cultivation compared to the reference compounds selected by LCF method.



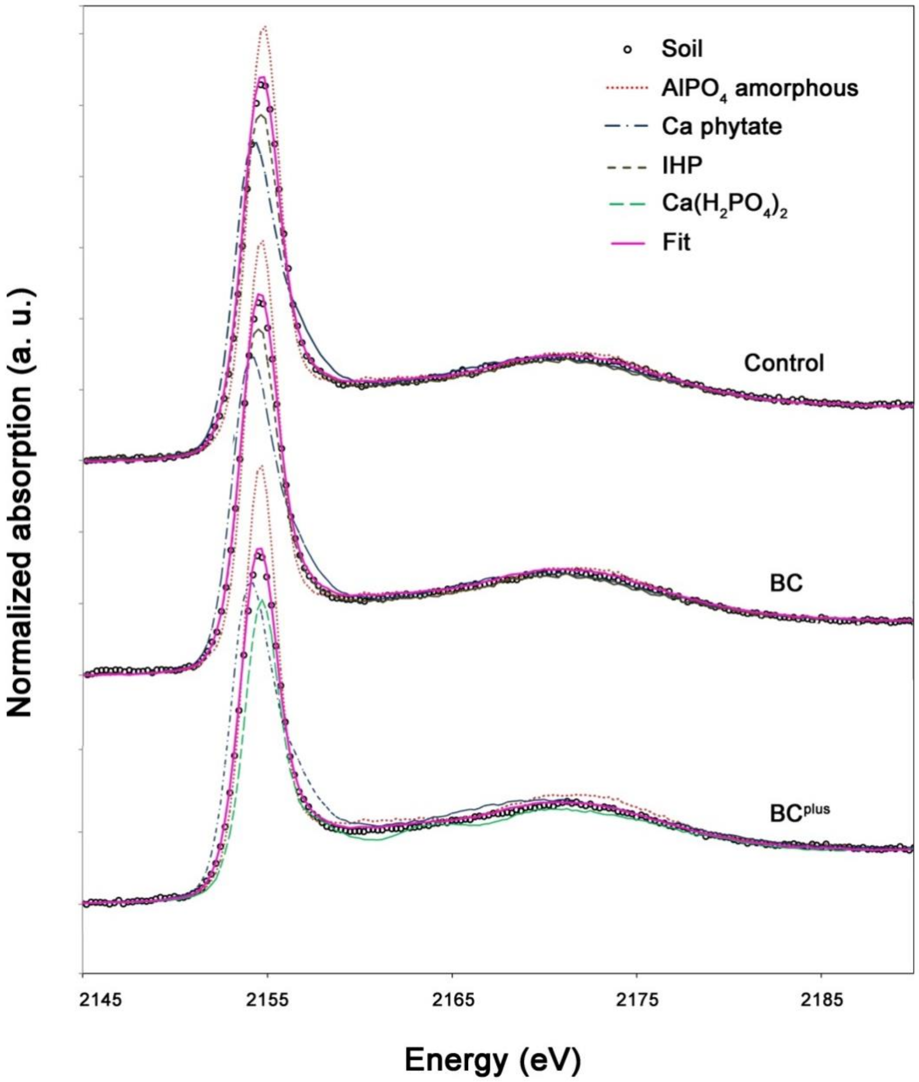
Morshedizad et al., Figure 1



Morshedizad et al., Figure 2



Morshedizad et al., Figure 3



Morshedizad et al., Figure 4