6 **Bone char effects on soil: sequential fractionations and XANES**

7 **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 °C 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

46

47   At the global scale readily-available sources of phosphorus (P), a crucial macro-nutrient

48   element for agricultural production, are being faced with scarcity and overpricing (Scholz et

49   al., 2013; Van Vuuren et al., 2010). Environmental pollutions frequently arise from their

50   impurities (Cd, U) (Hartley et al., 2013; Kratz et al., 2016), and from over-fertilization (Rubaek

51   et al., 2013). Another problem is the quick formation of stable and inaccessible compounds that

52   limit plant P uptake (Shen et al., 2011). Many recent studies have targeted sustainable

53   agriculture through improving P-availability from applied fertilizers (Delgado et al., 2002;

54   Schröder et al., 2011), increasing P-uptake efficiency from organic and inorganic P-pools in the

55   soil (Kaur and Reddy, 2014) and developing new technologies for P-recycling from human and

56   animal waste (Siebers and Leinweber, 2013; Herzel et al., 2016).

57   As an economically and environmentally attractive example, pyrolyzed animal bone chips

58   branded as "bone char" (BC), a slow-release apatite-based P-fertilizer, have been surface-

59   modified by sulfur (S) compounds to enhance its solubility in neutral to alkaline soils.

60   Incubation and pot experiments confirmed that the surface-modification increased the P-release

61   in an incubation/leaching experiment (Morshedizad et al., 2016). Despite these attempts to raise

62   dissolution and use-efficiency of BC in supplying P for crop requirements, indeed a

63   considerable fraction of applied BC to the soil still remains insoluble in short term and is not

64   taken up by plants over the entire cropping period but this has not yet been explained by a

65   detailed P speciation.

66   Chemical speciation has often been described in terms of analytical identification of chemical

67   species of defined elements and measuring their quantities in the system (Templeton et al.,

68   2000). The precise characterization of variable P species in the soil as a dynamic response to

69   imposed non-equilibrium conditions such as fertilization can support a better understanding of

70   reactivity, stability and particularly plant-accessibility of different P-forms and provide a basis

71   for best management practices. Several techniques such as sequential fractionation (Dieter et

72   al., 2010; Condron and Newman, 2011), nuclear magnetic resonance (NMR) spectroscopy (Liu

73   et al., 2009; Vestergren et al., 2012; Ahlgren et al., 2013), Raman spectroscopy (Lanfranco,

74   2003; Vogel et al., 2013) and chromatography coupled to mass spectroscopy (De Brabandere

75   et al., 2008; Paraskova et al., 2015) have been developed for P speciation analysis in soil and

76   sediments. Each one of these techniques can offer specific advantages and disadvantages

77   depending on phase and complexity of sample matrixes (Kruse et al., 2015). Complementarily,

78   X-ray absorption near edge structure (XANES) spectroscopy is well-suited to identify various

79   P species through fingerprinting of molecular structures in solid and heterogeneous mediums

80   based on fine feature and position of absorbing edges (Kelly et al., 2008; Kizewski et al., 2011).

81   The advantages of XANES spectroscopy for soil samples have made it a promising technique

82   for direct and *in situ* P-speciation with no pretreatment and minimum sample manipulation

83   (Toor et al., 2006; Kelly et al., 2008).

84   According to the best of our knowledge, no studies have characterized P-speciation changes in

85   BC particles over non-equilibrium conditions in the soil system, and only few investigations

86   have been reported on the P-release from BC and alteration in P-species of the soil. Siebers et

87   al. (2013) investigated *K*-edge XANES spectroscopy on BC-incubated soil samples and

88   evidenced the increase in extractable Ca- and Mg-phosphate fractions were related to the

89   contribution of hydroxyapatite (HAP) increase after BC application. Accordingly, the objective

90   of this study was to provide applicable information on the fate and alteration of P-species in BC

91   and novel surface modified BC (BC[plus]) particles and their treated soils under incubation-

92   leaching and ryegrass cultivation practices, using sequential P-fractionation and P-XANES

93   spectroscopy.

94

95   **2 Materials and methods**

2.1 Incubation-leaching experiment

96

97 Two types of bone chars (BC, produced by pyrolysis of degreased animal bone chips at 800ºC

98 and BC[plus] as a result of surface-modification of BC through blending with reduced S

99 compounds; characterized in details by Morshedizad et al., 2016) in two particle size fractions

100 (1-2 and 2-4 mm) were incubated with a silt loam soil. The soil was classified as *Dystric*

101 *Cambisol* (FAO) with pH of 4.7 (measured in 0.01 mol L^{-1} CaCl$_2$ solution) and total (HNO$_3$)

102 and available (NH$_4$NO$_3$) P-contents of 1.6 g P kg^{-1} and 14 mg P kg^{-1}, respectively. The BC and

103 BC[plus] contained total P 149 and 123 g kg^{-1}, total calcium (Ca) 185 and 265 g kg^{-1}, total S 6 and

104 199 g kg^{-1} and had average of pH$_{CaCl2}$ values 7.8 and 4.9, respectively.

105 The BCs were added to 30 g of air dry soil (<2mm) at the levels of 0 mg P kg^{-1} soil (control)

106 and 500 mg P kg^{-1} soil in five replicates, homogenized and packed into glass columns with 10

107 cm length, inner diameter of 2 cm and a P-free filter placed at the bottom to avoid any particle

108 losses. The amended soils were incubated for 70 days at 20 ºC in the dark and constant soil

109 moisture between 60-70 % of soil water holding capacity. During the incubation period, the soil

110 columns were leached with three pore volumes of deionized water using a droplet irrigation

111 simulator system in five leaching steps, each one after 1, 5, 13, 34 and 70 days. The P-

112 concentrations in collected leachates were measured using inductively coupled plasma-optical

113 emission spectrometry (ICP-OES). Outcomes of the leaching experiment were described in

114 Morshedizad et al. (2016). After the incubation-leaching experiment, the treated soil samples

115 were carefully removed from the glass columns, air dried and BCs particles were manually

116 separated from the soils very gently. The BCs particles were delicately washed with deionized

117 water to remove adhered soil particles, allowed to dry completely at ambient conditions and

118 finely ground for further analyses.

119

2.2 Pot experiment with annual ryegrass

120

121 The same BC and BC^{plus} as described for the incubation-leaching experiment were used in

122 original sizes (mostly between 1 to 5 mm) for P fertilization of annual ryegrass in a pot

123 experiment. The experiment was set-up using an acidic sandy silt soil with available P-content

124 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

125 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

126 and in four replicates arranged in a complete randomized block. After four weeks of incubation

127 at field capacity water content and natural temperature conditions, 30 seeds of annual ryegrass

128 per each pot were sown on 13th of May 2016. The experiment was conducted in a glasshouse

129 under natural air and temperature conditions and the soil moisture was maintained at field

130 capacity during the whole experiment. All other essential nutrients were sequentially added at

131 sufficient levels before seeding and after each six cuts of ryegrass between 23rd of June and 3rd

132 of November 2016. Finally, after the last harvest (7th), plant parts (shoots and roots) were dried

133 at 60 °C and BCs particles were manually separated from the soils, gently washed and

134 subsequently all samples were dried and ground to fine powders for further analyses.

135

2.3 Sequential phosphorus fractionation

137 Soil samples were sequentially extracted based on chemical solubility in order according to a

138 modified Hedley et al. (1982) procedure. After BC particles detachment, duplicate 0.5 g fine-

139 ground and air-dried soil samples were weighed into 50-mL centrifuge tubes. In summary,

140 chemical P fractionation includes the following steps:

141 1) The mobile and readily available P fraction was extracted with resin strips (saturated in 0.5

142 M NaHCO$_3$) after 18 hours end-over-end shaking in 30 mL deionized water. The resin strips

143 were separated from solids/solution and washed using 50 mL of 1 M HCl to remove absorbed

144 P. The soil suspension was centrifuged at 2500 × g for 20 minutes and the supernatant was

145 decanted.

146   2) Next, the labile inorganic and organic fractions weakly absorbed to mineral surfaces and

147   some microbial P were extracted with 30 mL of 0.5 M NaHCO$_3$, 18 hours end-over-end shaking

148   and centrifugation at 2500 $\times$ g for 20 minutes. The supernatant was filtered and collected for

149   measurements.

150   3) The inorganic P adsorbed and bound to Al- and Fe- oxide minerals and organic P from humic

151   substances were extracted using 30 mL of 0.1 M NaOH solution and repeating the second step

152   procedure as described above.

153   4) The relatively insoluble fraction of P bound to Ca and Mg minerals and apatite was extracted

154   with 30 mL of 1 M HCl in the same way as for the previous steps.

155   Total P concentrations (P$_t$) and inorganic P (P$_i$) in all extracts were measured by ICP-OES and

156   colorimetrically (molybdenum blue method; Murphy and Riley, 1962), respectively. The

157   organic P (P$_o$) concentrations were calculated by P$_t$ – P$_i$.

158

2.4 Phosphorus *K*-edge XANES analysis

160   The XANES data collection for characterizing P-species in all soil samples and BCs particles

161   was acquired at the Synchrotron Light Research Institute (SLRI) in Nakhon Ratchasima,

162   Thailand, on the beamline 8 (BL8) of the electron storage ring with a covering photon energy

163   from 1.25 to 10 KeV, electron energy operated at 1.2 GeV and beam current of 80-150 mA

164   (Klysubun et al., 2012). The P *K*-edge XANES spectra were collected from dried and very

165   finely ground particulate samples which had been diluted to lower P concentrations than <10

166   mg P kg^{-1} with SiO$_2$ powder (to eliminate self absorption effects; Prietzel et al., 2013), again

167   ground in agate stone mini-mortar and spread uniformly as a thin layer on P-free kapton tape

168   (Lanmar Inc., Northbrook, IL, USA). Data collection was operated in standard conditions

169   comprising the energy calibration by standard pure elemental P and allocating the reference

170   energy (E$_0$) at 2145.5 eV using the maximum peak of spectrum. All spectra were recorded at

171   photon energies between 2045.5 and 2495.5 eV in step sizes of 5 eV (2045.5 to 2105.5 eV and

172   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

173   to 2195.5 eV) with a 13-channel germanium detector. At least three scans were collected and

174   averaged for each sample.

175   The P-XANES spectra were normalized and after merging replicates, a linear combination

176   fitting (LCF) was performed using the ATHENA software package (Ravel and Newville, 2005).

177   The following set of reference P K-edge XANES spectra, all recorded in SLRI under the same

178   adjustments by Werner and Prietzel (2015) and Prietzel et al. (2016), were used for fitting and

179   calculations; Ca-, Al- and Fe-phytate, non-crystalline and crystalline $AlPO_4$, non-crystalline

180   and crystalline $FePO_4.2H_2O$, Ca-hydroxyapatite ($Ca_5(OH)(PO_4)_3$), inositol hexakisphosphate

181   (IHP), ferrihydrite-IHP, montmorillonite-Al-IHP, soil organic matter (SOM)-Al-IHP,

182   ferrihydrite-orthophosphate, montmorillonite-Al-orthophosphate, SOM-Al-orthophosphate,

183   bohemite-IHP, bohemite-orthophosphate, $CaHPO_4$, $Ca(H_2PO_4)_2$ and $MgHPO_4$. To select the

184   best possible combination fit between the sample spectrum and the P-reference spectra, the

185   lowest reduced chi value (χ^2) and R-factor were chosen.

186

3 Results

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

189   After 70 days of incubation-leaching, the sequential P-fractionation of amended soils showed

190   variations in the amount and distribution of various P-fractions between different treatments

191   (Table 1). For all treatments, NaOH extracted the majority of fractionated P (62.4 to 66.5 % of

192   total fractionated P), followed by the labile P fraction ($NaHCO_3$, 19.2 to 20.0 %), HCl-P (9.5

193   to 13.9 %) and the readily available P (resin strips, 4.1 to 4.8 %). The BCs addition increased

194   the soil P pools although the difference was significant only for the $BC^{plus}_{1-2\ mm}$ and $BC_{1-2\ mm}$

195   treatments. The largest increase in total fractionated P_t (resin-P_t + $NaHCO_3$-P_t + NaOH-P_t +

196   HCl-P_t) occurred in $BC^{plus}_{1-2\ mm}$ (133.8 mg P kg^{-1} soil) followed by $BC_{1-2\ mm}$ (118.6 mg P kg^{-1}

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

198   control soil. The proportion of P-enrichment in each fraction varied between different

199   treatments in the order NaOH-P > HCl-P > $NaHCO_3$-P > resin-P for $BC^{plus}_{1-2\ mm}$ and BC^{plus}_{2-}

200    $_{4\ mm}$. For the $BC_{1-2\ mm}$ and $BC_{2-4\ mm}$ treatment the order was HCl-P > NaOH-P > $NaHCO_3$-P >

201   resin-P. In all treatments, the P_i proportions in each of the P fractions were greater than the P_o

202   proportions. Compared to the control soil, the most P_i increase was observed in NaOH-P_i and

203   resin-P_i in response to $BC^{plus}_{1-2\ mm}$ application.

204

205   **INSERT Table 1 HERE**

206

207   In leachate samples, the two particle sizes of BC and BC^{plus} gave a significant rise in P-

208   concentration after 1, 5, 13, 34 and 70 days of incubation (Morshedizad and Leinweber, 2017).

209   Results of whole incubation-leaching period revealed that the averaged increase in

210   accumulative leached P concentrations were 4.66 mg P kg^{-1} soil (BC_{1-2mm}), 4.21 mg P kg^{-1} soil

211   (BC_{2-4mm}), 3.68 mg P kg^{-1} soil (BC^{plus}_{1-2mm}) and 3.19 mg P kg^{-1} soil (BC^{plus}_{2-4mm}) compared to

212   the control soil. Soil pH increased in BC treatments after 70 days of incubation-leaching

213   whereas BC^{plus} amendments had an acidifying effect. Soil pH levels of BC_{1-2mm} and BC_{2-4mm}

214   increased by 0.07 and 0.05 units and decreased for BC^{plus}_{1-2mm} and BC^{plus}_{2-4mm} treatments by

215   0.21 and 0.15 units compared to unamended control soil (pH = 5.06).

216

217   **3.2 Effect of BCs on sequentially extracted P after ryegrass cropping**

218   Sequentially extracted P fractions in soil varied between different treatments after 230 days of

219   ryegrass cropping (Table 1). In all treatments (control, BC and BC^{plus}), the largest P pool was

220   mainly associated with Al- and Fe-oxide minerals and humic substances (NaOH-P, 65.0 to 67.5

221 % of total fractionated P) followed by the $NaHCO_3$-P (18.2 to 19.0 %), HCl-P (11.5 to 12.6 %)

222 and resin-P (2.2 to 3.4 %) fractions. Both BC and BC^{plus} amendments led to enrichments of P

223 fractions, except for the readily available and labile inorganic P concentrations in the BC

224 treatment. The maximum increase of total fractionated P was obtained in BC^{plus} treatment (37.6

225 mg P kg^{-1} soil). In comparison to incubation-leaching results, a similar sequence was observed

226 for the order of increasing magnitude of P fractions in response to BC and BC^{plus} amendments

227 (NaOH-P > $NaHCO_3$-P > HCl-P > resin-P). However, for BC treatment, the total P extracted

228 by resin strips was lowered in comparison with the control. In the control and BC treatments,

229 P_o was the predominant form in NaOH-P and $NaHCO_3$-P fractions while for BC^{plus} it was only

230 in the NaOH-P fraction. Each P fraction was highest under BC^{plus} application, except for

231 $NaHCO_3$-P_o of BC treatment. Additionally, a significant increase in P concentration was

232 obtained only in resin-P_i and NaOH-P_i fractions of the BC^{plus} treated soil (Table 1).

233 Separately, the effect of BC and BC^{plus} application on ryegrass yield parameters was examined

234 in the 230-days pot experiment. The results indicated that P uptake, ryegrass yield and apparent

235 nutrient recovery efficiency (ANR) of BC^{plus} treatments exceeded that of BC and control

236 treatments and increased to values comparable with triple super phosphate (TSP) fertilizer.

237 Addition of BC and BC^{plus} did not significantly change soil pH, although likely occurrence of

238 local acidification around BC^{plus} particles lowered soil pH by 0.01 to 0.05 units (insignificant)

239 compared to BC treatments at harvest time (Zimmer, D. and Panten, K., personal

240 communication).

241

242 **3.3 XANES analysis of BCs particles**

243 All spectra from BCs were characterized by an intense white-line peak, post-edge position and

244 without a distinct pre-edge which corresponded to calcium phosphate compounds including Ca-

245 hydroxyapatite, dicalcium phosphate ($CaHPO_4$) and Ca-phytate (Fig. 1). The P K-edge XANES

246   results indicated no obvious alterations in spectral features of BCs particles after the incubation-

247   leaching experiment. After 70-days of incubation-leaching, the BC spectra were shifted towards

248   Ca-hydroxyapatite, and this was more pronounced for the 2-4 mm than for the 1-2 mm BC-

249   particles. The opposite trend was the case for BC[plus] particles where the white-line signal

250   intensity decreased after incubation-leaching period and the post-edge of spectra tended more

251   to dicalcium phosphate. This effect was stronger for BC[plus]-particle size reduction from 2-4 mm

252   to 1-2 mm.

253

254   **INSERT Figure 1 HERE**

255

256   To quantify the P speciation of BC and BC[plus] particles, LCF analyses using all possible

257   combinations were performed on all P K-edge XANES spectra (Table 2). The fitting results

258   indicated that untreated BC and BC[plus] particles before the experiment contained on average 61

259   and 60 % Ca-hydroxyapatite, 22 and 30 % $CaHPO_4$ and 18 and 10 % Ca-phytate. After 70-days

260   of incubation-leaching, the proportion of Ca-hydroxyapatite increased to the average of 80 %

261   in BC while it remained unchanged in BC[plus] particles. The $CaHPO_4$ proportion increased in

262   BC[plus] particles to the average of 34 % whereas the lower content was assigned in the spectra

263   of BC particles accounting for 10 % of total P species. Moreover, the Ca-phytate proportion

264   decreased slightly in BC and BC[plus] particles from about 18 and 10 % to averages of 11 and 7

265   %, respectively.

266

267   **INSERT Table 2 HERE**

268

269   Spectra of BC and BC[plus] particles, before and after 230-days of ryegrass cultivation, were

270   characterized by a sharp white-line followed by a shoulder and then a post-edge feature between

271   2160 and 2175 eV which was divided into two peaks (Fig. 2). These features were most similar

272   to P K-edge XANES spectra of Ca-hydroxyapatite, $CaHPO_4$ and Ca-phytate standard

273   compounds. Treated BC particles had a white-line with higher intensity which appeared more

274   similar to the Ca-hydroxyapatite spectrum. In contrast, BC^{plus} particles under ryegrass

275   cultivation showed a weaker white-line exhibiting the shoulder and post-edge feature more

276   comparable to the $CaHPO_4$ spectrum.

277

278   **INSERT Figure 2 HERE**

279

280   Some differences in proportions of P species observed between BCs particles before and after

281   the cropping period in ryegrass pot experiment are presented in Table 3. The LCF results

282   revealed overall contributions of 63 and 70 % Ca-hydroxyapatite, 29 and 29 % $CaHPO_4$ and 8

283   and 1 % Ca-phytate in the original BC and BC^{plus}, respectively. After the cropping period, the

284   percentage of Ca-hydroxyapatite was increased in BC particles. In the BC^{plus} treatment, the

285   percentage of $CaHPO_4$ increased from 29 to 43, while the percentage of Ca-hydroxyapatite was

286   reduced from 70 to 49 %. The Ca-phytate proportion remained unchanged in BC particles while

287   that of BC^{plus} increased from 1 to 8 % after the ryegrass cultivation period.

288

289   **INSERT Table 3 HERE**

290

291   **3.4 XANES analysis of soil samples**

292   The P K-edge XANES spectra of soil samples from the incubation-leaching experiment showed

293   two dominant features including 1) a strong white-line lacking pre-edge and shoulder and 2) a

294   tailed post-edge feature (Fig. 3). The most similarity to these features was seen in XANES

295   spectra of amorphous $AlPO_4$, $FePO_4$ and SOM-Al-IHP compounds. Distinct differences

296 appeared between the control and treated soil with BC^{plus}, not with BC treatments. This was

297 reflected by slightly lower intensities of both white-line and post-edge features.

298

299 **INSERT Figure 3 HERE**

300

301 The P species of treated soils in the incubation-leaching experiment were determined by LCF

302 analysis to select at most four reference compounds in combinatorics of all possible fitting

303 combinations (Table 4). The fitting results indicated that P in the control soil and BC treatments

304 occurred dominantly as AlPO$_4$ amorphous ($\approx$ 40 %), FePO$_4$ ($\approx$ 30 %) and SOM-Al-IHP ($\approx$ 20

305 %) compounds. In BC^{plus} treated soils, the average proportion of amorphous AlPO$_4$ decreased

306 to 26 % and instead, Ca(H$_2$PO$_4$)$_2$ was identified with an average of 25 % which did not appear

307 in the control and BC treatments. The LCF results showed that the soil treated with BC^{plus} had

308 no detectable Ca-hydroxyapatite which was found in the control and BC treatments.

309

310 **INSERT Table 4 HERE**

311

312 The XANES spectra recorded from treated soil samples in the ryegrass pot experiment showed

313 the presence of an intense white-line in the energy range of 2152 to 2158 eV and a stretched

314 post-edge feature approximately from 2165 to 2178 eV (Fig. 4). Decreases in white-line and

315 post-edge intensities of the soil samples appeared as an effect of BC^{plus} application. Visual

316 inspection of P K-edge spectra revealed no indication of specific alteration in spectral features

317 in response to the BC treatment.

318

319 **INSERT Figure 4 HERE**

320

321 Amorphous $AlPO_4$ was characterized by LCF analysis as dominant component (≈ 35 %) in all

322 treated soil samples from the ryegrass pot experiment (Table 5). The second major P form in

323 the control soil was IHP (29 %) followed by Ca-phytate (27 %), with the latter also as

324 pronounced as that observed for BC and BC[plus] treatments. All treated soils varied in

325 proportions of free or bound IHP forms. The Mont-Al-PO_4 and $Ca(H_2PO_4)_2$ compounds were

326 only assigned in the control and BC[plus] treatments, respectively.

327

328 **INSERT Table 5 HERE**

329

330

331 **4 Discussion**

332 **4.1 P availability as revealed by sequential fractionation**

333 The sequence of P distribution between sequentially extracted P-fractions was in accordance

334 with findings by many studies (Cross and Schlesinger, 1995; McDowell and Stewart, 2006;

335 Hashimoto and Watanabe, 2014), reflecting the general status of different P-pools in acidic

336 soils. The results indicated that the largest P proportion was found in the NaOH fraction

337 reflecting P fixed to Fe- and Al-oxides as followed by the $NaHCO_3$-P fraction assigned to

338 weakly P absorbed on crystalline Fe- and Al-oxides or surface of minerals. Guo et al. (2000)

339 reported that the NaOH-P fraction may support the labile $NaHCO_3$-P fraction as a buffering P-

340 pool in highly weathered and acidic soils. According to soil pH values (4.7 and 5.2), the larger

341 proportions of $NaHCO_3$-P even than HCl-P can be explained by the abundance and positive

342 loadings of Fe- and Al-oxides and a scarcity of Ca and Mg minerals or soluble ions. As

343 expected, the lowest P proportions were found in the mobile and readily available P fraction

344 extracted by resin strips in agreement to many comparable studies (Cross and Schlesinger,

345 1995; Bauchemin et al., 2003; Sharpley et al., 2004; Siebers et al., 2013). Among the two soils

346   which were used in the two different experiments, the most inorganic P fractions were achieved

347   in the soil after incubation-leaching experiment, while the organic P forms were considerably

348   more abundant in the soil samples after ryegrass cultivation (Table 1). These differences may

349   be due to the microbial activities in the rhizosphere of grasses and transformation of P_i to more

350   stable P_o fractions during longer plant cultivation period (230 days) than in the non-cropped

351   incubation/leaching experiment (70 days).

352   In general, all P fraction concentrations were elevated by adding BC and BC^{plus} particles which

353   appeared to follow the same pattern in both soils under two different experimental conditions.

354   However, significant differences were found only between the control and BC^{plus} treated soils

355   (1-2 mm in the incubation-leaching experiment) for the resin-P and NaOH-P fractions. Since

356   the BCs particles were separated from the soils before chemical analysis, it was expected that

357   partly dissolved BCs would have a limited impact on different P-fractions rather than totally

358   ground and mixed BCs. This is consistent with the study of Siebers et al. (2013) according to

359   which the BC application (<90 μm BC thoroughly mixed to soil) significantly increased the

360   insoluble P proportion (H_2SO_4-P). Additionally, our study confirmed previous findings

361   concerning the effect of particle sizes on the P release from BCs (Morshedizad and Leinweber,

362   2017) and consequently the P status of treated soils (Ma and Matsunaka, 2013). Sequentially

363   extracted P contents increased with decreasing size of BC particles whereby BC^{plus} treatments

364   appeared more dependent on particle size than BC treatments. The results of sequential P

365   fractionation of BC^{plus} treatments indicated that the P increase was more pronounced for P fixed

366   to Al- and Fe-oxides (NaOH-P) than other fractions, whereas for BC treatments the largest

367   increase occurred in P bound to Ca and Mg minerals (HCl-P). It seems that local pH changes

368   in soil associated with BC and BC^{plus} amendments could eventually lead to a different

369   distribution of released P into differently soluble or insoluble P-pools which are generally

370   controlled by pH (Arai and Sparks, 2007).

371

4.2 P speciation of BCs particles by XANES

373 The predominance of Ca-hydroxyapatite in BCs as evidenced by P K-edge XANES analysis is

374 consistent with findings reported by previous studies (Warren et al., 2009; Siebers et al., 2013).

375 The mineral phase of bone consists mainly of hydroxyapatite, and its contribution to bone and

376 bone char compositions depends on species and the age of animals (Wu et al., 2003) and

377 carbonization temperature and residence time (Novotny et al., 2012). Bone crystallinity might

378 be improved through structural modifications on poorly crystalline fresh bone samples (such as

379 mineral maturity over periods of time or intensive carbonization) which can result in increased

380 proportions of hydroxyapatite and accordingly a decrease in P solubility (Novotny et al., 2012).

381 Based on LC fittings, the second major component of BCs particles was $CaHPO_4$, in good

382 agreement with the results of Rajendran et al. (2013) who indicated the heated bones at 400 ºC

383 contained some more soluble phosphates such as $CaHPO_4$ and CaH_2PO_4 in addition to the

384 hydroxyapatite fraction. The authors reported that spectra of calcined bone samples at 700 ºC

385 had white-line at 2154 eV and two post-edge peaks at 2162 eV and 2169 eV with no pre-edge

386 peaks and appeared similar to $CaHPO_4$ and CaH_2PO_4 spectra. Our LCF also assigned Ca-

387 phytate in BCs samples which seems to be controversial as a component of animal bone

388 materials. The P K-edge spectrum of Ca-phytate is very similar to other Ca-bound P compounds

389 with a distinct white-line and lack of a pre-edge feature, although it is likely distinguishable due

390 to specific shape of white-line tailing and absence of post-edge signal at 2164 eV (Prietzel et

391 al., 2016). Moreover, some inaccuracies in LCF estimations have to be considered because of

392 1) uncertainty in speciation of organic P forms by K-edge XANES, 2) lack of reference

393 compounds representing all P forms in BCs and 3) smaller Ca-phytate proportions than

394 proposed 10 to 15 % of P_t as detection limit for reliable XANES fittings (Beauchemin et al.,

395 2003).

396   In both experiments, incubation/leaching and ryegrass cropping, changes in proportions of Ca-

397   hydroxyapatite and $CaHPO_4$ in BC particles followed an opposite trend than in BC^{plus} particles.

398   After placement of BC particles in the soil, Ca-phosphate seemed to be released gradually over

399   time which provides a locally lime-saturated condition. Due to elevated pH surrounding the BC

400   particles, dissolved P can be resorbed to maintain solubility and the Ca-P equilibrium constant

401   which likely resulted in a decreased proportion of soluble $CaHPO_4$ and possibly the

402   transformation into the relatively insoluble Ca-hydroxyapatite fraction. In contrast, if BC^{plus}

403   particles were applied to soils, larger proportions of $CaHPO_4$ at the expense of Ca-

404   hydroxyapatite could be explained by soil acidification through the microbial oxidation of

405   released S. This effect was more pronounced over the longer time period in the ryegrass

406   cropping pot experiment, favoring a greater $CaHPO_4$ than Ca-hydroxyapatite fraction. This

407   implies that BC^{plus} can actively supply P with predominance of soluble over insoluble P forms

408   in the long-term and, thus, meet crop requirements. In general, the P composition changes in

409   BCs particles revealed by K-edge XANES can explain overall trends in soils to some extent

410   considering the amount of dissolved and added BCs to the soils.

411

412   4.3 P speciation of treated soils by XANES

413   Differences between characteristics of two soils, dissimilar mechanisms of incubation-leaching

414   and plant uptake besides different experiment time durations complicate the joint interpretation

415   of the P-XANES data. In unfertilized soil of the incubation-leaching experiment, the

416   proportions of P species followed the order $AlPO_4$ > $FePO_4$ > SOM-Al-IHP > Ca-

417   hydroxyapatite which did not vary despite partial changes in some proportions after application

418   of both size fractions of BC particles. In general, these results concur with the findings by

419   Siebers et al. (2013) that Ca-hydroxyapatite proportion was slightly increased by BC

420   application. This could be attributed to irreversibly mixing finely-ground BC to the soil samples

421 whereas in the present experiments the BC particles were separated from the soils before P

422 speciation. Furthermore, these XANES data (Table 4 and Table 5) are in agreement with

423 sequential P fractionation results (Table 1) which indicated the dominance of inorganic over

424 organic P forms and showed the P fractions almost unchanged after BC application.

425 Implications of low solubility of BC particles observed in this work are consistent with previous

426 studies showing a slow release P from BCs (Warren et al., 2009; Siebers et al., 2013;

427 Morshedizad et al., 2016). Besides reducing the $AlPO_4$ and Ca-hydroxyapatite proportions,

428 BC^{plus} particles introduced highly soluble $Ca(H_2PO_4)_2$ to soils in the incubation-leaching

429 experiment. These results imply that considerable changes in P speciation were more attributed

430 to pH reductions and, accordingly, leaching out solubilized P-forms compared with P

431 enrichment by BC^{plus} dissolution. This is in line with Sato et al. (2005) who found that

432 increasing soil pH in a naturally acidic soil (pH = 4.32) was an effective approach to minimize

433 P leaching, while pH decrease resulted in transformation of stable to soluble and more leachable

434 P species. Regarding to the XANES results of ryegrass cultivation experiment (Table 5), the

435 effect of BC^{plus} treatment can be explained better. In control soil, the presence of $AlPO_4$ and

436 increasing abundance of organic P-forms (Ca-phytate and IHP compounds) (Table 5) were

437 consistent with the appearance of NaOH-P and HCl-P fractions by sequential extraction (Table

438 1). In the BC treatment the proportions of $AlPO_4$ and Ca-phytate did not change compared to

439 the control but the contribution of organic P increased by Mont-Al-IHP formation. The stability

440 of different P fractions can be favored by the pH effect (Gustafsson et al., 2012) likewise the

441 dependence of BC particles solubility on the soil pH (Siebers et al., 2013). In agreement with

442 incubation-leaching results (Table 4), $Ca(H_2PO_4)_2$ was detected as a result of BC^{plus} amendment

443 even though similar proportions of $AlPO_4$ and Ca-phytate were observed between the control

444 and BC^{plus} treatment. The results of sequential P fractionation and XANES analyses on two

445 different practices presented here demonstrated that surface modification of BC particles

446 effectively improved soluble P fractions in BC^{plus} particles and, consequently, in amended soils.

447

448 **5 Conclusions**

449 In the present study, the P speciation by a sequential P fractionation and P K-edge XANES

450 spectroscopy revealed the noticeable alteration in the P-pools of treated soil samples. Results

451 of incubation-leaching and ryegrass cultivation experiments indicated that BC^{plus} produced by

452 surface-modification of BC through adding S compounds providing more soluble and plant-

453 available P fraction than BCs during the growth season. The S oxidation and thereby the soil

454 pH decrease seems to stimulate the P release from BC^{plus} particles. The P K-edge XANES

455 analysis of BC^{plus} particles revealed more soluble CaHPO$_4$ than BC particles at the expense of

456 Ca-hydroxyapatite. This was associated with Ca(H$_2$PO$_4$)$_2$ addition to amended soils, as

457 identified by sequential P fractionation and XANES analyses. Future studies will be directed to

458 validate the beneficial effects of BC^{plus} at the field scale with different soils and to optimize the

459 surface modification of BCs.

460

461 **Data availability.** All compiled data of this study are published in figures and tables. Detailed

462 primary data including the incubation-leaching and ryegrass cultivation results and the acquired

463 XANES spectra will be saved and published in the BonaRes Data Center

464 (https://www.bonares.de/research-data) and get the BonaRes DOI prefix (10.20387).

465

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

467

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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

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

Reference compound	Before experiment		After 230 days ryegrass cultivation	
	BC	BC^{plus}	BC	BC^{plus}
Ca hydroxy apatite (%)	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

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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$_2$PO$_4$)$_2$ (%)	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$_2$PO$_4$)$_2$ (%)	0	0	14 ± 2
R-factor	0.0006	0.0008	0.0006

764 **Figure captions**

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

768

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

771

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

775

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

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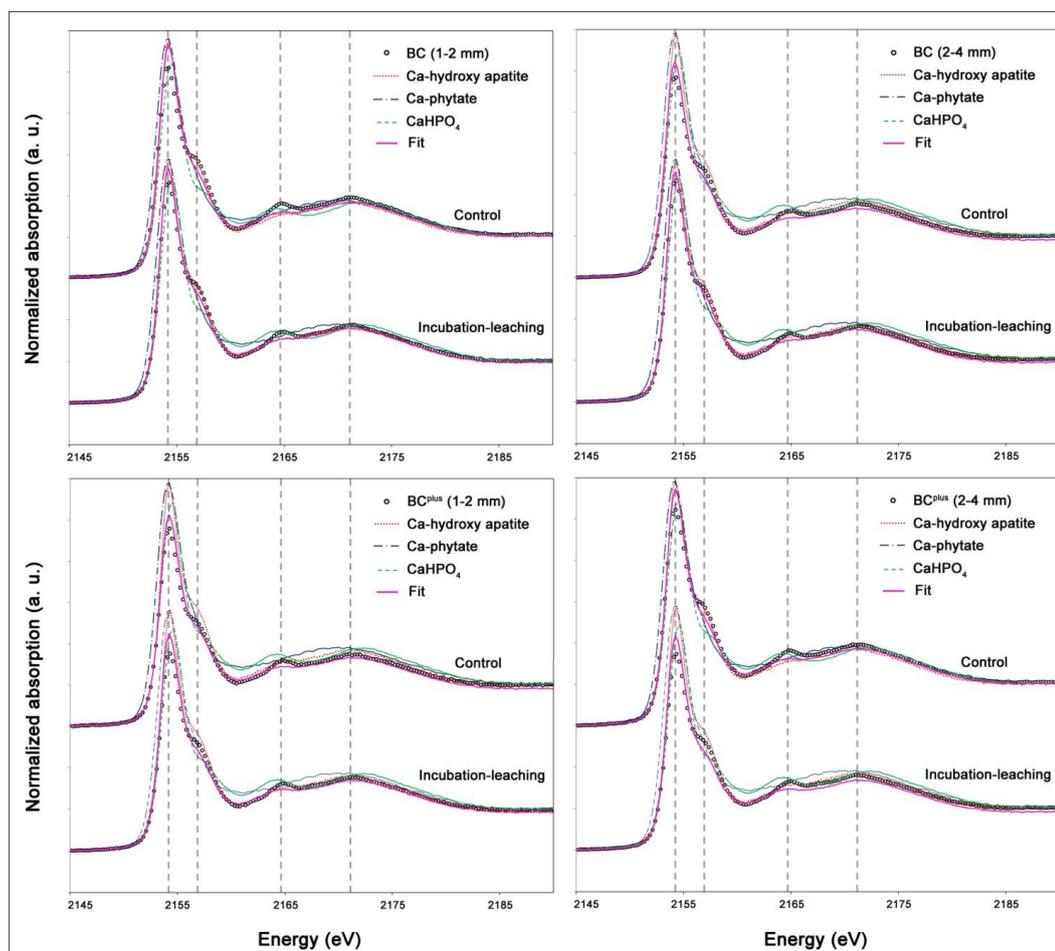
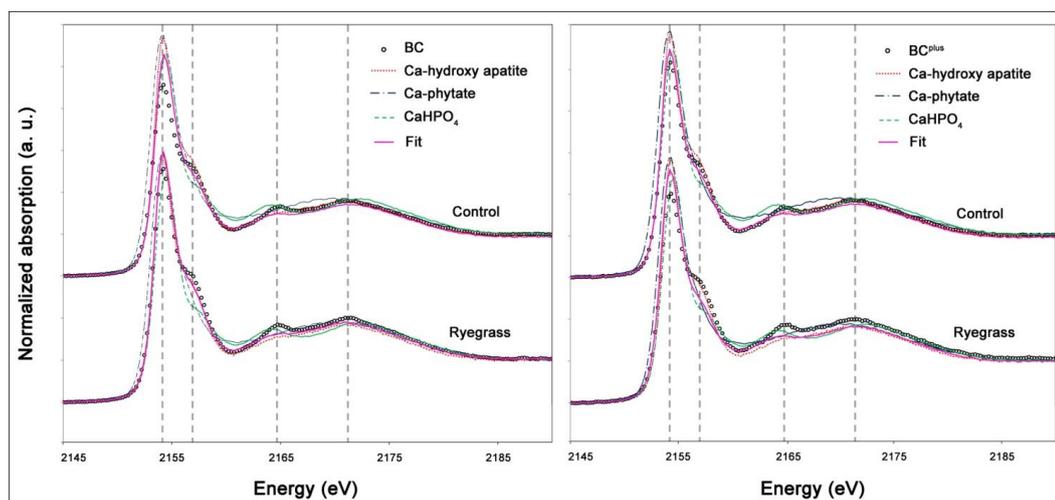

Morshedizad et al., Figure 1

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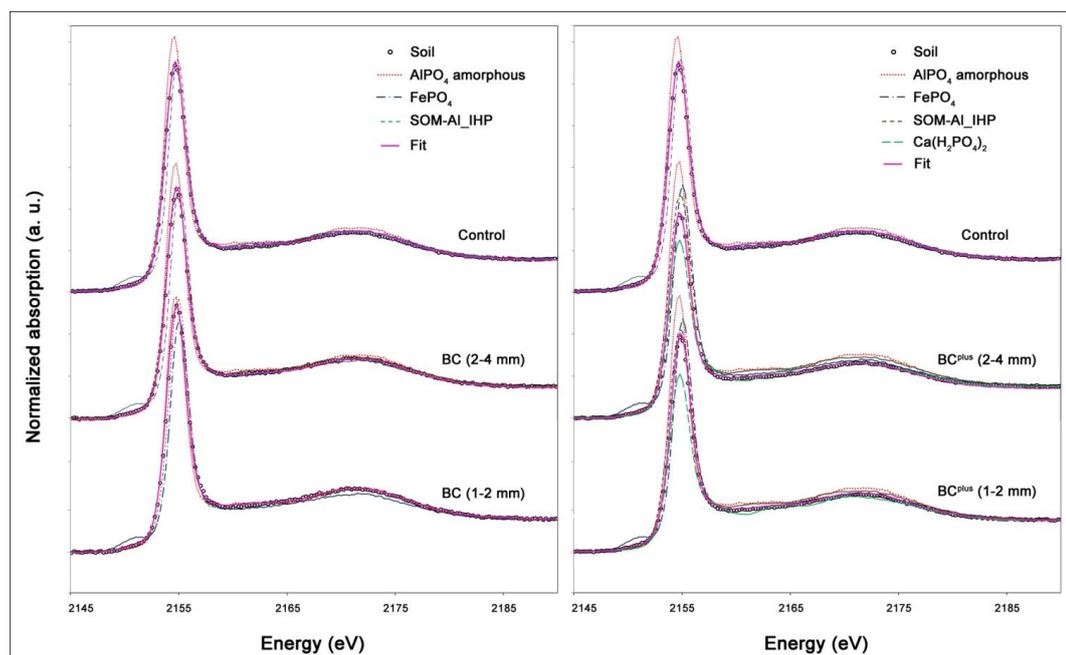
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800   Morshedizad et al., Figure 3

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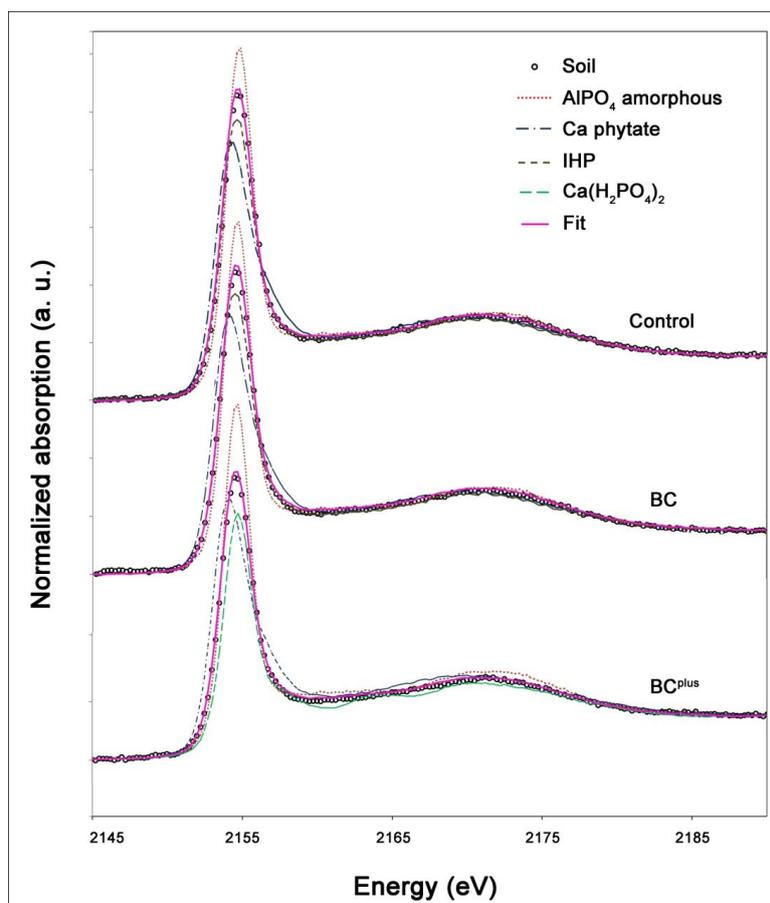
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814   Morshedizad et al., Figure 4

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