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Bone char effects on soil: sequential fractionations and XANES spectroscopy

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22 **Abstract.** Acceptability of novel bone char fertilizers depends on their P release but
23 reactions at bone char surfaces and impacts to soil P speciation are insufficiently known. By
24 sequential fractionation and synchrotron-based X-ray absorption near edge structure
25 (XANES) spectroscopy we investigated whether and how the chemical composition of bone
26 char particles has been altered in soil and, consequently, has affected the P speciation of
27 amended soils. Therefore, two different kinds of bone char particles (BC, produced by
28 pyrolysis of degreased animal bone chips at 800 °C and BC^{plus}, a BC enriched with reduced
29 sulfur compounds) were manually separated from the soil at the end of two different
30 experiments: “incubation-leaching” and “ryegrass cultivation”. Sequential P-fractionation of
31 amended soils showed P-enrichment in all fractions as compared to the control. The most P
32 increase between all treatments significantly occurred in the NaOH-P and resin-P fractions in
33 response to BC^{plus} application in both, incubation-leaching and ryegrass cultivation
34 experiments. This increase of the readily available P fraction in BC^{plus} treated soils was
35 confirmed by linear combination fitting (LCF) analysis on P K-edge XANES spectra of BCs
36 particles and amended soils. The proportion of Ca-hydroxyapatite decreased, whereas the
37 proportion of CaHPO₄ increased in BC^{plus} particles after amended soils had been incubated
38 and leached and cropped by ryegrass. Based on P XANES speciation as determined by LCF
39 analysis, the proportion of inorganic Ca(H₂PO₄)₂ increased in amended soils after BC^{plus}
40 application. These results indicated that soil amendment with BC^{plus} particles leads to elevated
41 P concentration and maintains more soluble P species than BC particles even after 230-days
42 of ryegrass cultivation.

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45

46 **1 Introduction**

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
51 (Rubaek et al., 2013). Further problems are the quick formation of stable and inaccessible
52 compounds that limit plant P uptake (Shen et al., 2011) and the low agronomic efficiency of
53 not more than 15% of fertilizer P in the first year of application (Schnug et al., 2003). Many
54 recent studies have targeted sustainable agriculture through improving P-availability from
55 applied fertilizers (Delgado et al., 2002; Schröder et al., 2011), increasing P-uptake efficiency
56 from organic and inorganic P-pools in the soil (Kaur and Reddy, 2014) and developing new
57 technologies for P-recycling from human and animal waste (Siebers and Leinweber, 2013;
58 Herzel et al., 2016). Particular attention has been paid to the oxidation process, e.g. by
59 thiobacilli of elemental sulfur to sulfuric acid, in order to enhance the solubility of non water-
60 soluble P from rock phosphates (Powers, 1923; Lee et al., 1987; Fan et al., 2002) or meat and
61 bone ashes (Schnug et al., 2003).

62 As an economically and environmentally attractive example, pyrolyzed animal bone chips
63 branded as “bone char” (BC), a slow-release apatite-based P-fertilizer, have been surface
64 modified by sulfur (S) compounds to enhance its solubility in neutral to alkaline soils.
65 Incubation-leaching and pot experiments confirmed that surface-modification was an effective
66 approach in P-release promotion from BC fertilizer (Morshedizad et al., 2016; Zimmer, D.
67 and Panten, K., personal communication). Such an "in situ" digestion of an apatitic phosphate
68 with elemental S was first described by Fan et al. (2002; 2012).

69 Despite these attempts to raise dissolution and use-efficiency of BC in supplying P for crop
70 requirements, a considerable fraction of applied BC-P to the soil remains insoluble in the

71 short term and is not taken up by plants over the entire cropping period. A detailed P
72 speciation can clarify the fate of insoluble P from BC which has not been done before.

73 Chemical speciation is described as analytical identification of chemical species of defined
74 elements and measuring their quantities in the system (Templeton et al., 2000). The precise
75 characterization of various P species in the soil as a dynamic response to non-equilibrium
76 conditions imposed by human activities such as fertilization can support a better
77 understanding of reactivity, stability and particularly plant-accessibility of different P-forms
78 and provide a basis for best management practices. Several techniques such as sequential
79 fractionation (Dieter et al., 2010; Condrón and Newman, 2011), nuclear magnetic resonance
80 (NMR) spectroscopy (Liu et al., 2009; Vestergren et al., 2012; Ahlgren et al., 2013), Raman
81 spectroscopy (Lanfranco, 2003; Vogel et al., 2013) and chromatography coupled to mass
82 spectroscopy (De Brabandere et al., 2008; Paraskova et al., 2015) have been developed for P
83 speciation analysis in soil and sediments. Each one of these techniques can offer specific
84 advantages and disadvantages depending on phase and complexity of sample matrixes (Kruse
85 et al., 2015). Complementarily, X-ray absorption near edge structure (XANES) spectroscopy
86 is well-suited for identification of various P species through fingerprinting of molecular
87 structures in solid and heterogeneous mediums based on fine feature and position of
88 absorbing edges (Kelly et al., 2008; Kizewski et al., 2011). The advantages of XANES
89 spectroscopy for soil samples make it a promising technique for direct and *in situ* P-speciation
90 with no pretreatment and minimum sample manipulation (Toor et al., 2006; Kelly et al.,
91 2008).

92 According to the best of our knowledge, no studies have characterized P-speciation changes
93 in BC particles over non-equilibrium conditions in the soil system, and only few
94 investigations have been reported on the P-release from BC and alteration in P-species of the
95 soil. Siebers et al. (2013) investigated *K*-edge XANES spectroscopy on BC-incubated soil

96 samples and provided evidence that the increase in extractable Ca- and Mg-phosphate
97 fractions were related to the contribution of hydroxyapatite (HAP) increase after BC
98 application. Accordingly, the objective of this study was to provide practical information on
99 the fate and alteration of P-species in BC and novel surface modified BC (BC^{plus}) particles
100 and their treated soils under incubation-leaching and ryegrass cultivation practices, using
101 sequential P-fractionation and P-XANES spectroscopy.

102

103 **2 Materials and methods**

104 **2.1 Incubation-leaching experiment**

105 Two particle size fractions (1-2 and 2-4 mm) of bone chars (BC, produced by pyrolysis of
106 degreased animal bone chips at 800°C and BC^{plus} as a surface modified BC obtained by
107 blending with reduced S-containing compounds composed of 60% elemental S, 30% calcium
108 sulfate dehydrate and 10% methansulfonate (Zimmer et al. unpublished results of S X-ray
109 absorption near-edge fine structure spectroscopy) in a commercial biogas desulfurization
110 process; patent application DE 212012000046U1; [www.google.com/patents](http://www.google.com/patents/DE212012000046U1?cl=en&hl=de)
111 [/DE212012000046U1?cl=en&hl=de](http://www.google.com/patents/DE212012000046U1?cl=en&hl=de)) were incubated with a silt loam soil. The soil was
112 classified as *Dystric Cambisol* (FAO) with pH of 4.7 (measured in 0.01 mol L⁻¹ CaCl₂
113 solution) and total (digestion with HNO₃ and analyzed using ICP-OES; USEPA, 1997) and
114 available (extracted by 1 mol L⁻¹ NH₄NO₃ and analyzed using ICP-OES; He and Singh, 1993)
115 P-contents of 1.6 g P kg⁻¹ and 14 mg P kg⁻¹, respectively. The BC and BC^{plus} contained total P
116 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
117 average of pH_{CaCl2} values 7.8 and 4.9, respectively.

118 The BCs were added to 30 g of air dry soil (< 2 mm) at the levels of 0 mg P kg⁻¹ soil (control)
119 and 500 mg P kg⁻¹ soil in five replicates. The soil and BCs mixture was homogenized and
120 packed into glass columns with 10 cm length and inner diameter of 2 cm. A P-free filter (MN

121 616 G; Macherey-Nagel GmbH & Co., KG Düren, Germany) was placed at the bottom of
122 each column to avoid any particle losses. The amended soils were incubated for 70 days at 20
123 °C in the dark and constant soil moisture between 60-70% of soil water holding capacity.
124 During the incubation period, the soil columns were leached with three pore volumes of
125 deionized water added by a droplet irrigation simulator system. The leaching process was
126 repeated in five steps, each one after 1, 5, 13, 34 and 70 days. The P-concentrations in
127 collected leachates were measured using inductively coupled plasma-optical emission
128 spectrometry (ICP-OES). Outcomes of the leaching experiment were described in
129 Morshedizad and Leinweber (2017). After the incubation-leaching experiment, the treated soil
130 samples were carefully removed from the glass columns, air dried and BCs particles were
131 manually separated from the soils very gently. The BCs particles were delicately washed with
132 deionized water to remove adhered soil particles, allowed to dry completely at ambient
133 conditions and finely ground for further analyses.

134

135 **2.2 Pot experiment with annual ryegrass**

136 The same BC and BC^{plus} as described for the incubation-leaching experiment were used in
137 original sizes (mostly between 1 to 5 mm) for P fertilization of annual ryegrass in a pot
138 experiment. The experiment was set-up using an acidic sandy silt soil with available P-content
139 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
140 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
141 each pot and in four replicates arranged in a complete randomized block. After four weeks of
142 incubation at field capacity water content and ambient temperature conditions, 30 seeds of
143 annual ryegrass per each pot were sown on 13th of May 2016. The experiment was conducted
144 in a glasshouse under ambient air and temperature conditions and the soil moisture was
145 maintained at field capacity during the whole experiment. All other essential nutrients were

146 sequentially added at sufficient levels before seeding and after each six cuts of ryegrass
147 between 23rd of June and 3rd of November 2016. Finally, after the last harvest (7th), plant parts
148 (shoots and roots) were dried at 60 °C and BCs particles were manually separated from the
149 soils as they could be detected visually by their size and dark color very gently, using
150 tweezers. Then these particles were washed delicately with deionized water to remove
151 attached soil particles, allowed to dry completely at ambient conditions and finely ground to
152 fine powders for further analyses.

153

154 **2.3 Sequential phosphorus fractionation**

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

159 1) The mobile and readily available P fraction was extracted with resin strips (saturated in 0.5
160 M NaHCO₃) after 18 hours end-over-end shaking in 30 mL deionized water. The resin strips
161 were separated from solids/solution and washed using 50 mL of 1 M HCl to remove absorbed
162 P. The soil suspension was centrifuged at 2500 × g for 20 minutes and the supernatant was
163 decanted.

164 2) Next, the labile inorganic and organic fractions weakly absorbed to mineral surfaces and
165 some microbial P were extracted by 30 mL of 0.5 M NaHCO₃, 18 hours end-over-end shaking
166 and centrifugation at 2500 × g for 20 minutes. The supernatant was filtered (Whatman no. 42
167 filter) and collected for measurements.

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

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

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

176

177 **2.4 Phosphorus K-edge XANES analysis**

178 The XANES data collection for characterizing P-species in all soil samples and BCs particles
179 was acquired at the Synchrotron Light Research Institute (SLRI) in Nakhon Ratchasima,
180 Thailand, on the beamline 8 (BL8) of the electron storage ring with a covering photon energy
181 from 1.25 to 10 KeV, electron energy operated at 1.2 GeV and beam current of 80-150 mA
182 (Klysubun et al., 2012). The P K-edge XANES spectra were collected from dried and very
183 finely ground treated soils and particulate BCs samples which had been diluted to P
184 concentrations $< 10 \text{ mg P kg}^{-1}$ with SiO_2 powder (to eliminate self absorption effects; Prietzel
185 et al., 2013), again ground in agate stone mini-mortar and spread uniformly as a thin layer on
186 P-free kapton tape (Lanmar Inc., Northbrook, IL, USA). Data collection was operated in
187 standard conditions comprising the energy calibration by standard pure elemental P and
188 allocating the reference energy (E_0) at 2145.5 eV using the maximum peak of spectrum. All
189 spectra were recorded at photon energies between 2045.5 and 2495.5 eV in step sizes of 5 eV
190 (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
191 2245.5 eV) and 0.25 eV (2135.5 to 2195.5 eV) with a 13-channel germanium detector in
192 fluorescence mode. At least three scans were collected and averaged for each sample.

193 The P-XANES spectra were normalized and after merging replicates, a linear combination
194 fitting (LCF) was performed using the ATHENA software package (Ravel and Newville,
195 2005). All XANES spectral data were baseline corrected in the pre-edge region between

196 2115–2145 eV and normalized in the post-edge region of 2190–2215 eV. The same ranges
197 were used for the reference P *K*-edge XANES spectra to achieve consistency in the following
198 fitting analysis (Prietz et al. 2016). To achieve the best compatible set of references with
199 each specified sample spectrum, LCF analysis was performed in the energy range between -
200 20 eV and +30 eV relative to the E_0 using the combinatorics function of ATHENA software
201 to attain all possible binary, ternary and at most quaternary combinations between all nineteen
202 P reference spectra. The following set of reference P *K*-edge XANES spectra, all recorded in
203 SLRI under the same adjustments by Werner and Prietz (2015) and Prietz et al. (2016),
204 were used for fitting and calculations; Ca-, Al- and Fe-phytate, non-crystalline and crystalline
205 AlPO_4 , non-crystalline and crystalline $\text{FePO}_4 \cdot 2\text{H}_2\text{O}$, Ca-hydroxyapatite ($\text{Ca}_5(\text{OH})(\text{PO}_4)_3$),
206 inositol hexakisphosphate (IHP), ferrihydrite-IHP, montmorillonite-Al-IHP, soil organic
207 matter (SOM)-Al-IHP, ferrihydrite-orthophosphate, montmorillonite-Al-orthophosphate,
208 SOM-Al-orthophosphate, bohemite-IHP, bohemite-orthophosphate, CaHPO_4 , $\text{Ca}(\text{H}_2\text{PO}_4)_2$
209 and MgHPO_4 . To select the best possible combination fit between the sample spectrum and
210 the P-reference spectra, the lowest reduced chi value (χ^2) and *R*-factor were chosen.

211

212 **3 Results**

213 **3.1 Effect of BCs on sequentially extracted P after incubation-leaching**

214 After 70 days of incubation-leaching, the sequential P-fractionation of amended soils showed
215 variations in the amount and distribution of various P-fractions between different treatments
216 (Table 1). For all treatments, NaOH extracted the majority of fractionated P (62.4 to 66.5% of
217 total fractionated P), followed by the labile P fraction (NaHCO_3 , 19.2 to 20.0%), HCl-P (9.5
218 to 13.9%) and the readily available P (resin strips, 4.1 to 4.8%). The BCs addition increased
219 the total soil P pools although the difference was significant only for the $\text{BC}_{1-2\text{mm}}^{\text{plus}}$ and BC_{1-}
220 $_{2\text{mm}}$ treatments. The largest increase in total fractionated P_t ($\text{resin-}P_t + \text{NaHCO}_3\text{-}P_t + \text{NaOH-}P_t$

221 + HCl-P_i) occurred in BC^{plus}_{1-2 mm} (133.8 mg P kg⁻¹ soil) followed by BC_{1-2 mm} (118.6 mg P
222 kg⁻¹ 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
223 the control soil.

224

225

INSERT Table 1 HERE

226

227 The proportion of P-enrichment in each fraction varied between different treatments in the
228 order NaOH-P > HCl-P > NaHCO₃-P > resin-P for BC^{plus}_{1-2 mm} and BC^{plus}_{2-4 mm}. For the BC<sub>1-
229 2 mm</sub> and BC_{2-4 mm} treatment the order was HCl-P > NaOH-P > NaHCO₃-P > resin-P. In all
230 treatments, the P_i proportions in each of the P fractions were greater than the P_o proportions.
231 Compared to the control soil, the most P_i increase was observed in NaOH-P_i and resin-P_i in
232 response to BC^{plus}_{1-2 mm} application (Table 1). Moreover, after 70 days of incubation-leaching,
233 soil pH increased in BC treatments whereas BC^{plus} amendments had an acidifying effect. Soil
234 pH levels of BC_{1-2 mm} and BC_{2-4 mm} increased by 0.07 and 0.05 units and decreased for BC^{plus}<sub>1-
235 2 mm</sub> and BC^{plus}_{2-4 mm} treatments by 0.21 and 0.15 units compared to unamended control soil
236 (pH = 5.06).

237

238 **3.2 Effect of BCs on sequentially extracted P after ryegrass cropping**

239 Sequentially extracted P fractions in soil varied between different treatments after 230 days of
240 ryegrass cropping (Table 1). In all treatments (control, BC and BC^{plus}), NaOH-P was the
241 largest P pool mainly associated with Al- and Fe-oxide minerals and humic substances (65.0
242 to 67.5% of total fractionated P) followed by the NaHCO₃-P (18.2 to 19.0%), HCl-P (11.5 to
243 12.6%) and resin-P (2.2 to 3.4%) fractions. Enrichments of P fractions in BC^{plus} treatments
244 were more pronounced than in treated soils with BC particles. In this treatment the
245 concentrations of readily available and labile inorganic P fractions were insignificantly

246 smaller than in the control. Additionally, a significant increase in P concentration was
247 obtained only in resin-P_i and NaOH-P_i fractions of the BC^{plus} treated soil (Table 1). The
248 maximum increase of total fractionated P was obtained in BC^{plus} treatment (37.6 mg P kg⁻¹
249 soil). In comparison to incubation-leaching results, a similar sequence was observed for the
250 order of increasing magnitude of P fractions in response to BC and BC^{plus} amendments
251 (NaOH-P > NaHCO₃-P > HCl-P > resin-P). However, for BC treatment, the total P extracted
252 by resin strips was lowered in comparison with the control. In the control and BC treatments,
253 P_o was the predominant form in NaOH-P and NaHCO₃-P fractions while for BC^{plus} it was
254 only in the NaOH-P fraction. Each P fraction was highest under BC^{plus} application, except for
255 NaHCO₃-P_o of BC treatment.

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

264

265 **3.3 XANES analysis of BCs particles**

266 All spectra from BCs were characterized by an intense white-line peak, post-edge position
267 and without a distinct pre-edge which corresponded to calcium phosphate compounds
268 including Ca-hydroxyapatite, dicalcium phosphate (CaHPO₄) and Ca-phytate (Fig. 1). The P
269 K-edge XANES results indicated no obvious alterations in spectral features of BCs particles
270 after the incubation-leaching experiment. After 70-days of incubation-leaching, the BC

271 spectra were shifted towards Ca-hydroxyapatite, and this was more pronounced for the 2-4
272 mm than for the 1-2 mm BC-particles. The opposite trend was the case for BC^{plus} particles
273 where the white-line signal intensity decreased after incubation-leaching period and the post-
274 edge of spectra tended more to dicalcium phosphate. This effect was stronger for BC^{plus}-
275 particle size reduction from 2-4 mm to 1-2 mm.

276

277

INSERT Figure 1 HERE

278

279 To quantify the P speciation of BC and BC^{plus} particles, LCF analyses using all possible
280 combinations were performed on all P *K*-edge XANES spectra (Table 2). The fitting results
281 indicated that untreated BC and BC^{plus} particles before the experiment contained on average
282 61 and 60% Ca-hydroxyapatite, 22 and 30% CaHPO₄ and 18 and 10% Ca-phytate. After 70-
283 days of incubation-leaching, the proportion of Ca-hydroxyapatite increased to the average of
284 80% in BC while it remained unchanged in BC^{plus} particles. The CaHPO₄ proportion increased
285 in BC^{plus} particles to the average of 34% whereas the lower content was assigned in the
286 spectra of BC particles accounting for 10% of total P species. Moreover, the Ca-phytate
287 proportion decreased slightly in BC and BC^{plus} particles from about 18 and 10% to averages
288 of 11 and 7%, respectively.

289

290

INSERT Table 2 HERE

291

292 Spectra of BC and BC^{plus} particles, before and after 230-days of ryegrass cultivation, were
293 characterized by a sharp white-line followed by a shoulder and then a post-edge feature
294 between 2160 and 2175 eV which was divided into two peaks (Fig. 2). These features were
295 most similar to P *K*-edge XANES spectra of Ca-hydroxyapatite, CaHPO₄ and Ca-phytate

296 standard compounds. Treated BC particles had a white-line with higher intensity which
297 appeared more similar to the Ca-hydroxyapatite spectrum. In contrast, BC^{plus} particles under
298 ryegrass cultivation showed a weaker white-line exhibiting the shoulder and post-edge feature
299 more comparable to the CaHPO₄ spectrum.

300

301

INSERT Figure 2 HERE

302

303 Some differences in proportions of P species observed between BCs particles before and after
304 the cropping period in the ryegrass pot experiment are presented in Table 3. The LCF results
305 revealed overall contributions of 63 and 70% Ca-hydroxyapatite, 29 and 29% CaHPO₄ and 8
306 and 1% Ca-phytate in the original BC and BC^{plus}, respectively. After the cropping period, the
307 percentage of Ca-hydroxyapatite was increased in BC particles. In the BC^{plus} treatment, the
308 percentage of CaHPO₄ increased from 29 to 43, while the percentage of Ca-hydroxyapatite
309 was reduced from 70 to 49%. The Ca-phytate proportion remained unchanged in BC particles
310 while that of BC^{plus} increased from 1 to 8% after the ryegrass cultivation period.

311

312

INSERT Table 3 HERE

313

314 **3.4 XANES analysis of soil samples**

315 The P *K*-edge XANES spectra of soil samples from the incubation-leaching experiment
316 showed two dominant features including 1) a strong white-line lacking pre-edge and shoulder
317 and 2) a tailed post-edge feature (Fig. 3). The most similarity to these features was seen in
318 XANES spectra of amorphous AlPO₄, FePO₄ and SOM-Al-IHP compounds. Distinct
319 differences appeared between the control and treated soil with BC^{plus}, not with BC treatments.
320 This was reflected by slightly lower intensities of both white-line and post-edge features.

321

322

INSERT Figure 3 HERE

323

324 The P species of treated soils in the incubation-leaching experiment were determined by LCF
325 analysis to select at most four reference compounds in combinatorics of all possible fitting
326 combinations (Table 4). The fitting results indicated that P in the control soil and BC
327 treatments occurred dominantly as AlPO_4 amorphous ($\approx 40\%$), FePO_4 ($\approx 30\%$) and SOM-Al-
328 IHP ($\approx 20\%$) compounds. In BC^{plus} treated soils, the average proportion of amorphous AlPO_4
329 decreased to 26% and instead, $\text{Ca}(\text{H}_2\text{PO}_4)_2$ was identified with an average of 25% which did
330 not appear in the control and BC treatments. The LCF results showed that the soil treated with
331 BC^{plus} had no detectable Ca-hydroxyapatite which was found in the control and BC
332 treatments.

333

334

INSERT Table 4 HERE

335

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

342

343

INSERT Figure 4 HERE

344

345 Amorphous AlPO_4 was identified by LCF analysis as dominant component ($\approx 35\%$) in all
346 treated soil samples from the ryegrass pot experiment (Table 5). The second major P form in
347 the control soil was IHP (29%) followed by Ca-phytate (27%), with the latter also as
348 pronounced as that observed for BC and BC^{plus} treatments. All treated soils varied in
349 proportions of free or bound IHP forms. The Mont-Al-PO_4 and $\text{Ca}(\text{H}_2\text{PO}_4)_2$ compounds were
350 only assigned in the control and BC^{plus} treatments, respectively.

351

352

INSERT Table 5 HERE

353

354 **4 Discussion**

355 **4.1 P availability as revealed by sequential fractionation**

356 The sequence of P distribution between sequentially extracted P-fractions was in accordance
357 with findings by many studies (Cross and Schlesinger, 1995; McDowell and Stewart, 2006;
358 Hashimoto and Watanabe, 2014), reflecting the general status of different P-pools in acidic
359 soils. The results indicated that the largest P proportion was found in the NaOH fraction
360 reflecting P fixed to Fe- and Al-oxides as followed by the NaHCO_3 -P fraction assigned to
361 weakly P absorbed on crystalline Fe- and Al-oxides or surface of minerals. Guo et al. (2000)
362 reported that the NaOH-P fraction may support the labile NaHCO_3 -P fraction as a buffering
363 P-pool in highly weathered and acidic soils. According to soil pH values (4.7 and 5.2), the
364 larger proportions of NaHCO_3 -P even than HCl-P can be explained by the abundance and
365 surface loadings of Fe- and Al-oxides that support the electrostatic binding of phosphate ions
366 and a scarcity of Ca and Mg minerals or soluble ions. As expected, the lowest P proportions
367 were found in the mobile and readily available P fraction extracted by resin strips in
368 agreement to many comparable studies (Cross and Schlesinger, 1995; Bauchemin et al., 2003;
369 Sharpley et al., 2004; Siebers et al., 2013). Among the two soils which were used in the two

370 different experiments, the largest proportions of inorganic P were achieved in the soil after
371 incubation-leaching experiment, while the organic P forms were considerably more abundant
372 in the soil samples after ryegrass cultivation (Table 1). These differences may be due to the
373 microbial activities in the rhizosphere of grasses and transformation of P_i to more stable P_o
374 fractions during longer plant cultivation period (230 days) than in the non-cropped
375 incubation-leaching experiment (70 days).

376 In general, all P fraction concentrations were elevated by adding BC and BC^{plus} particles
377 which appeared to follow the same pattern in both soils under two different experimental
378 conditions. However, significant differences were found only between the control and BC^{plus}
379 treated soils (1-2 mm in the incubation-leaching experiment) for the resin-P and NaOH-P
380 fractions. Since the BCs particles were separated from the soils before chemical analysis, it
381 was expected that partly dissolved BCs would have a limited impact on different P-fractions
382 rather than totally ground and mixed BCs. This is consistent with the study of Siebers et al.
383 (2013) according to which the BC application ($<90 \mu\text{m}$ BC thoroughly mixed to soil)
384 significantly increased the insoluble P proportion ($\text{H}_2\text{SO}_4\text{-P}$). Additionally, our study
385 confirmed previous findings concerning the effect of particle sizes on the P release from BCs
386 (Morshedizad and Leinweber, 2017) and consequently the P status of treated soils (Ma and
387 Matsunaka, 2013). Sequentially extracted P contents increased with decreasing size of BC
388 particles whereby BC^{plus} treatments appeared more dependent on particle size than BC
389 treatments. The results of sequential P fractionation of BC^{plus} treatments in the incubation-
390 leaching experiment indicated that the P increase was more pronounced for P fixed to Al- and
391 Fe-oxides (NaOH-P) than other fractions, whereas for BC treatments the largest increase
392 occurred in P bound to Ca and Mg minerals (HCl-P). It seems that local pH changes in soil
393 associated with BC and BC^{plus} amendments could eventually lead to a different distribution of
394 released P into differently soluble or insoluble P-pools which are generally controlled by pH

395 (Arai and Sparks, 2007). However, due to lower fertilization level and longer period of
396 experiment in ryegrass cultivation compared to incubation-leaching, it appears that the
397 chemical equilibrium has been established in the soil (no significant change in bulk soil pH)
398 and, accordingly, the soil P fractions were altered minimal.

399

400 **4.2 P speciation of BCs particles by XANES**

401 The predominance of Ca-hydroxyapatite in BCs as evidenced by P *K*-edge XANES analysis
402 is consistent with findings reported by previous studies (Warren et al., 2009; Siebers et al.,
403 2013). The mineral phase of bone consists mainly of hydroxyapatite, and its contribution to
404 bone and bone char compositions depends on species and the age of animals (Wu et al., 2003)
405 and carbonization temperature and residence time (Novotny et al., 2012). Bone crystallinity
406 might be improved through structural modifications on poorly crystalline fresh bone samples
407 (such as mineral maturity over periods of time or intensive carbonization) which can also
408 result in increased proportions of hydroxyapatite and accordingly a decrease in P solubility
409 (Novotny et al., 2012). Based on LC fittings, the second major component of BCs particles
410 was CaHPO₄, in good agreement with the results of Rajendran et al. (2013) who indicated the
411 heated bones at 400 °C contained some more soluble phosphates such as CaHPO₄ and
412 CaH₂PO₄ in addition to the hydroxyapatite fraction. The authors reported that spectra of
413 calcined bone samples at 700 °C had white-line at 2154 eV and two post-edge peaks at 2162
414 eV and 2169 eV with no pre-edge peaks and appeared similar to CaHPO₄ and CaH₂PO₄
415 spectra. Our LCF also assigned Ca-phytate in BCs samples which seems to be controversial
416 as a component of animal bone materials. The P *K*-edge spectrum of Ca-phytate is very
417 similar to other Ca-bound P compounds with a distinct white-line and lack of a pre-edge
418 feature, although it is likely distinguishable due to specific shape of white-line tailing and
419 absence of post-edge signal at 2164 eV (Prietz et al., 2016). Moreover, some inaccuracies in

420 LCF estimations have to be considered because of 1) uncertainty in speciation of organic P
421 forms by *K*-edge XANES, 2) lack of reference compounds representing all P forms in BCs
422 and 3) smaller Ca-phytate proportions than proposed 10 to 15% of P_t as detection limit for
423 reliable XANES fittings (Beauchemin et al., 2003). Therefore, the P proportions assigned to
424 Ca-phytate also could originate from a range of other CaP compounds.

425 In both experiments, incubation-leaching and ryegrass cropping, changes in proportions of
426 Ca-hydroxyapatite and $CaHPO_4$ in BC particles followed an opposite trend than in BC^{plus}
427 particles. After placement of BC particles in the soil, Ca-phosphate seemed to be released
428 gradually over time which provides a locally lime-saturated condition. Due to elevated pH
429 surrounding the BC particles, dissolved P can be resorbed to maintain solubility and the Ca-P
430 equilibrium constant which likely resulted in a decreased proportion of soluble $CaHPO_4$ and
431 possibly the transformation into the relatively insoluble Ca-hydroxyapatite fraction. In
432 contrast, if BC^{plus} particles were applied to soils, larger proportions of $CaHPO_4$ at the expense
433 of Ca-hydroxyapatite could be explained by soil acidification through the microbial oxidation
434 of released S (Lee, et al. 1987; Fan et al., 2002). This effect was more pronounced over the
435 longer time period in the ryegrass cropping pot experiment, favoring a greater $CaHPO_4$ than
436 Ca-hydroxyapatite fraction. This implies that BC^{plus} can actively supply P with predominance
437 of soluble over insoluble P forms in the long-term and, thus, meet crop requirements.

438

439 **4.3 P speciation of treated soils by XANES**

440 Differences between characteristics of two soils, dissimilar mechanisms of incubation-
441 leaching and plant uptake besides different experiment time durations complicate the joint
442 interpretation of the P-XANES data. In unfertilized soil of the incubation-leaching
443 experiment, the proportions of P species followed the order $AlPO_4 > FePO_4 > SOM-Al-IHP >$
444 Ca-hydroxyapatite which did not vary despite partial changes in some proportions after

445 application of both size fractions of BC particles. In general, these results concur with the
446 findings by Siebers et al. (2013) that Ca-hydroxyapatite proportion was slightly increased by
447 BC application. This could be attributed to irreversibly mixing finely-ground BC to the soil
448 samples whereas in the present experiments the BC particles were separated from the soils
449 before P speciation. Furthermore, these XANES data (Table 4 and Table 5) are in agreement
450 with sequential P fractionation results (Table 1) which indicated the dominance of inorganic
451 over organic P forms and showed the P fractions almost unchanged after BC application.
452 Implications of low solubility of BC particles observed in this work are consistent with
453 previous studies showing a slow release P from BCs (Warren et al., 2009; Siebers et al., 2013;
454 Morshedizad et al., 2016). Besides reducing the AlPO_4 and Ca-hydroxyapatite proportions,
455 BC^{plus} particles introduced highly soluble $\text{Ca}(\text{H}_2\text{PO}_4)_2$ to soils in the incubation-leaching
456 experiment. These results imply that considerable changes in P speciation were more
457 attributed to pH reductions and, accordingly, leaching out solubilized P-forms compared with
458 P enrichment by BC^{plus} dissolution. This is supported by results from a previous publication in
459 which two particle sizes of BC^{plus} gave a significant rise in the leached P-concentration after
460 1, 5, 13, 34 and 70 days of incubation along with reductions in soil pH (Morshedizad and
461 Leinweber, 2017). This is in line with Sato et al. (2005) who found that increasing soil pH in
462 a naturally acidic soil (pH = 4.32) was an effective approach to minimize P leaching, while
463 pH decrease resulted in transformation of stable to soluble and more leachable P species.
464 Regarding the XANES results of the ryegrass cultivation experiment (Table 5), the effect of
465 BC^{plus} treatment can be explained better. In the control soil, the presence of AlPO_4 and
466 increasing abundance of organic P-forms (Ca-phytate and IHP compounds) (Table 5) were
467 consistent with the appearance of NaOH-P and HCl-P fractions by sequential extraction
468 (Table 1). In the BC treatment the proportions of AlPO_4 and Ca-phytate did not change
469 compared to the control but the contribution of organic P increased by Mont-Al-IHP

470 formation. The stability of different P fractions can be favored by the pH effect (Gustafsson et
471 al., 2012) likewise the dependence of BC particles solubility on the soil pH (Siebers et al.,
472 2013). In agreement with incubation-leaching results (Table 4), $\text{Ca}(\text{H}_2\text{PO}_4)_2$ was detected as a
473 result of BC^{plus} amendment even though similar proportions of AlPO_4 and Ca-phytate were
474 observed between the control and BC^{plus} treatment. However, the data in Table 5 on the
475 presence/absence of $\text{Ca}(\text{H}_2\text{PO}_4)_2$ in soils of ryegrass experiment may have been influenced by
476 small proportions (<10-15%; reliable detection limit by XANES, Beauchemin et al., 2003) of
477 other simple calcium phosphates that have a spectrum similar to the one of $\text{Ca}(\text{H}_2\text{PO}_4)_2$ in
478 LCF analysis. The results of sequential P fractionation and XANES analyses on treatments in
479 the two different experiments presented here demonstrated that surface modification of BC
480 particles effectively improved soluble P fractions in BC^{plus} particles and, consequently, in
481 amended soils.

482

483 **5 Conclusions**

484 In the present study, the P speciation by a sequential P fractionation and P K-edge XANES
485 spectroscopy revealed the noticeable alteration in the P-pools of treated soil samples. Results
486 of incubation-leaching and ryegrass cultivation experiments indicated that BC^{plus} produced by
487 surface-modification of BC through addition of S compounds provided more soluble and
488 plant-available P than non-modified BC during the growth season. The S oxidation and
489 thereby the soil pH decrease seems to stimulate the P release from BC^{plus} particles. The P K-
490 edge XANES analyses of BC^{plus} particles revealed more soluble CaHPO_4 than in BC particles
491 at the expense of Ca-hydroxyapatite. This was associated with the addition $\text{Ca}(\text{H}_2\text{PO}_4)_2$ or
492 similar simple Ca-P-compounds to amended soils, as indicated by sequential P fractionation
493 and XANES analyses. Future studies will be directed to validate the beneficial effects of
494 BC^{plus} at the field scale with different soils and to optimize the surface modification of BCs.

495

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

500

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

502

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512

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712 **Table 1.** Distribution of inorganic P (P_i), organic P (P_o) and total P (P_t) concentrations (mg P kg^{-1} soil)
713 of sequentially extracted P-fractions in the soils as affected by different treatments (treated with two
714 particle size fractions (1-2 and 2-4 mm) and original sizes of BC and BC^{plus} or unfertilized soils
715 (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} |

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717 * Significant at $P < 0.05$; ** Significant at $P < 0.01$; ^{NS} Non-significant difference (treatment vs. control; Tukey-Test)
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731 **Table 2.** Results of linear combination fitting (LCF) conducted on P *K*-edge XANES spectra of bone
 732 char (BC) and surface modified bone char (BC^{plus}) particles before and after a 70-days incubation-
 733 leaching period. [These best fits were achieved using all possible combinations with nineteen spectra of](#)
 734 [P reference compounds.](#)

| 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 |
| <i>R</i> -factor | 0.012 | 0.008 | 0.007 | 0.009 | 0.005 | 0.002 | 0.009 | 0.010 |

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753 **Table 3.** Results of linear combination fitting (LCF) conducted on P *K*-edge XANES spectra of bone
 754 char (BC) and surface modified bone char (BC^{plus}) particles before and after 230-days of ryegrass
 755 cultivation in a pot experiment. [These best fits were achieved using all possible combinations with](#)
 756 [nineteen spectra of P reference compounds.](#)

| 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 |
| <i>R</i> -factor | 0.012 | 0.005 | 0.006 | 0.018 |

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775 **Table 4.** Results of linear combination fitting (LCF) conducted on P *K*-edge XANES spectra of
 776 unfertilized (control) and fertilized soils with bone char (BC) and surface modified bone char (BC^{plus})
 777 particles in the 70-days incubation-leaching experiment. *These best fits were achieved using all*
 778 *possible combinations with nineteen spectra of P reference compounds.*

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| 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 |
| <i>R</i> -factor | 0.0003 | 0.0007 | 0.0003 | 0.0005 | 0.0004 |

801 **Table 5.** Results of linear combination fitting (LCF) conducted on P *K*-edge XANES spectra of
 802 unfertilized (control) and fertilized soils with bone char (BC) and surface modified bone char (BC^{plus})
 803 particles under 230-days of ryegrass cultivation in a pot experiment. [These best fits were achieved](#)
 804 [using all possible combinations with nineteen spectra of P reference compounds.](#)

| 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 |
| <i>R</i> -factor | 0.0006 | 0.0008 | 0.0006 |

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839 **Figure captions**

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

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

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

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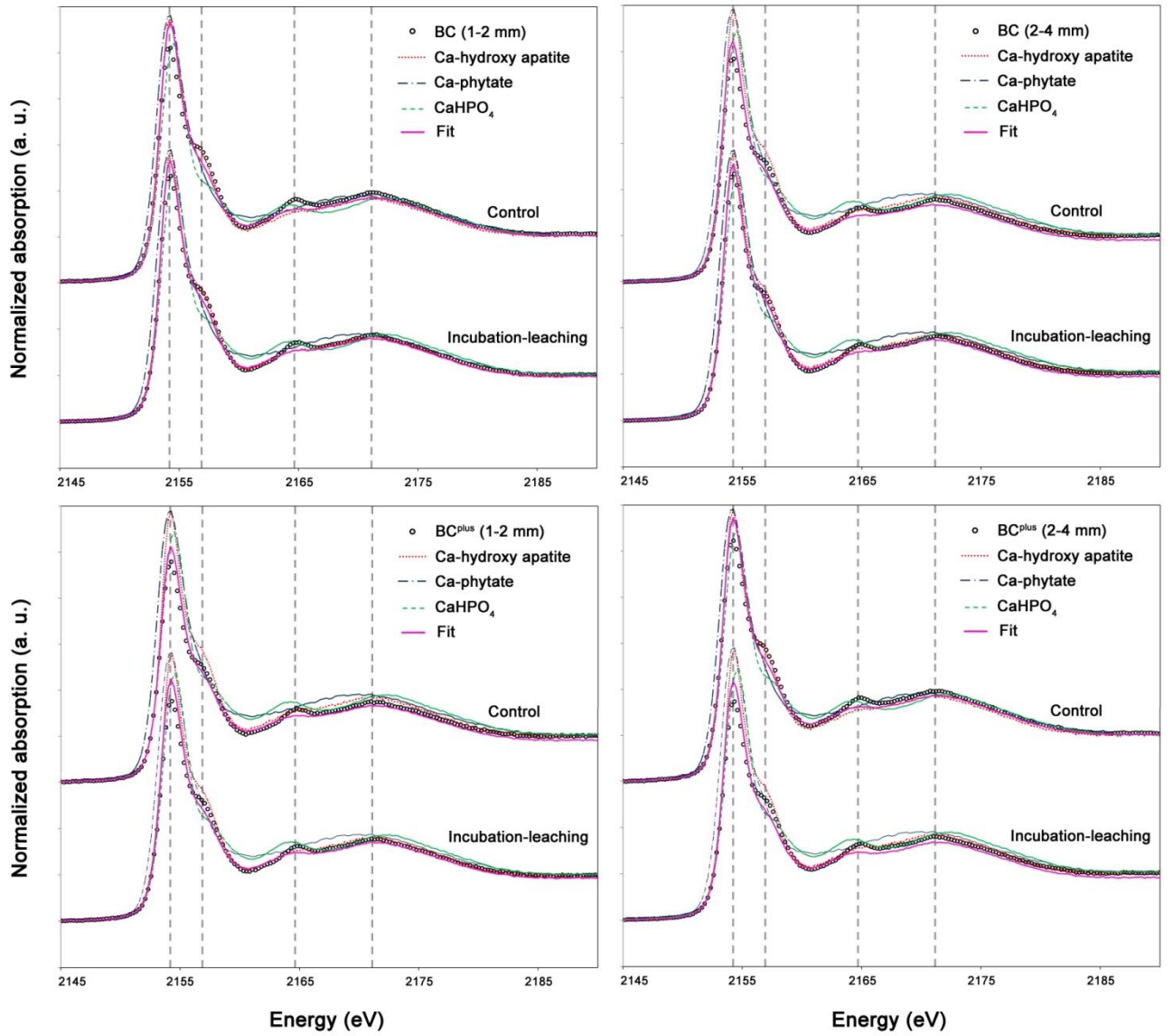
851 **Figure 4.** P *K*-edge XANES spectra of unfertilized (control) and fertilized soils with BC and BC^{plus}
852 particles under 230-days of ryegrass cultivation compared to the reference compounds selected by
853 LCF method.

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859 Morshedizad et al., Figure 1

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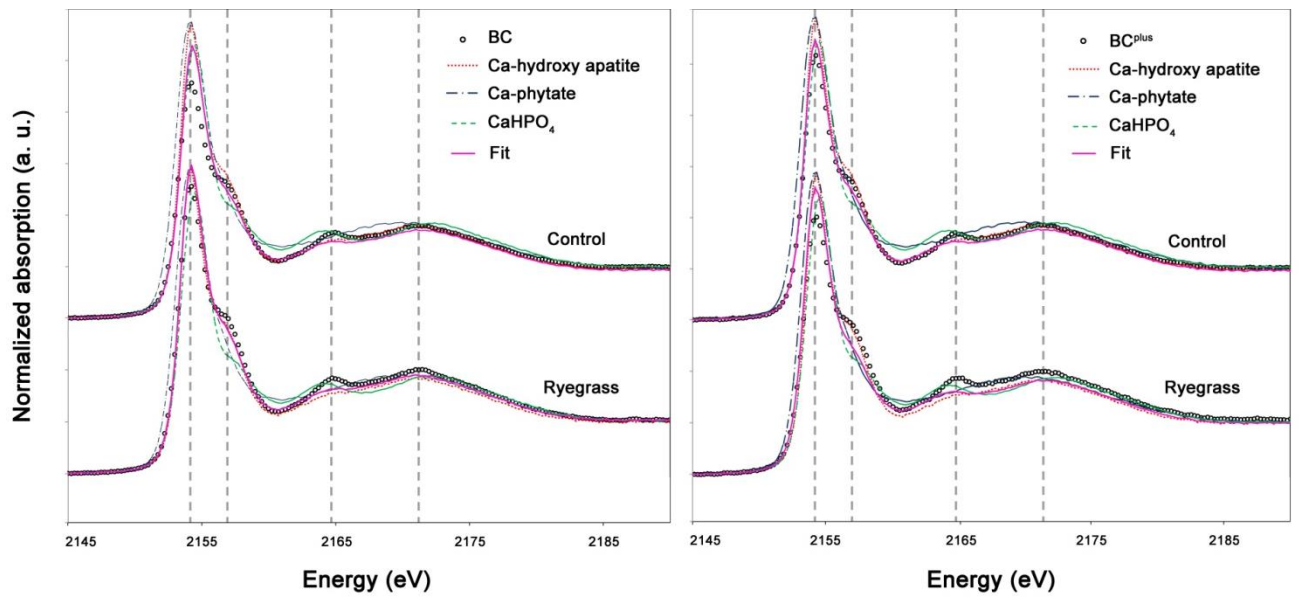
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868 Morshedizad et al., Figure 2

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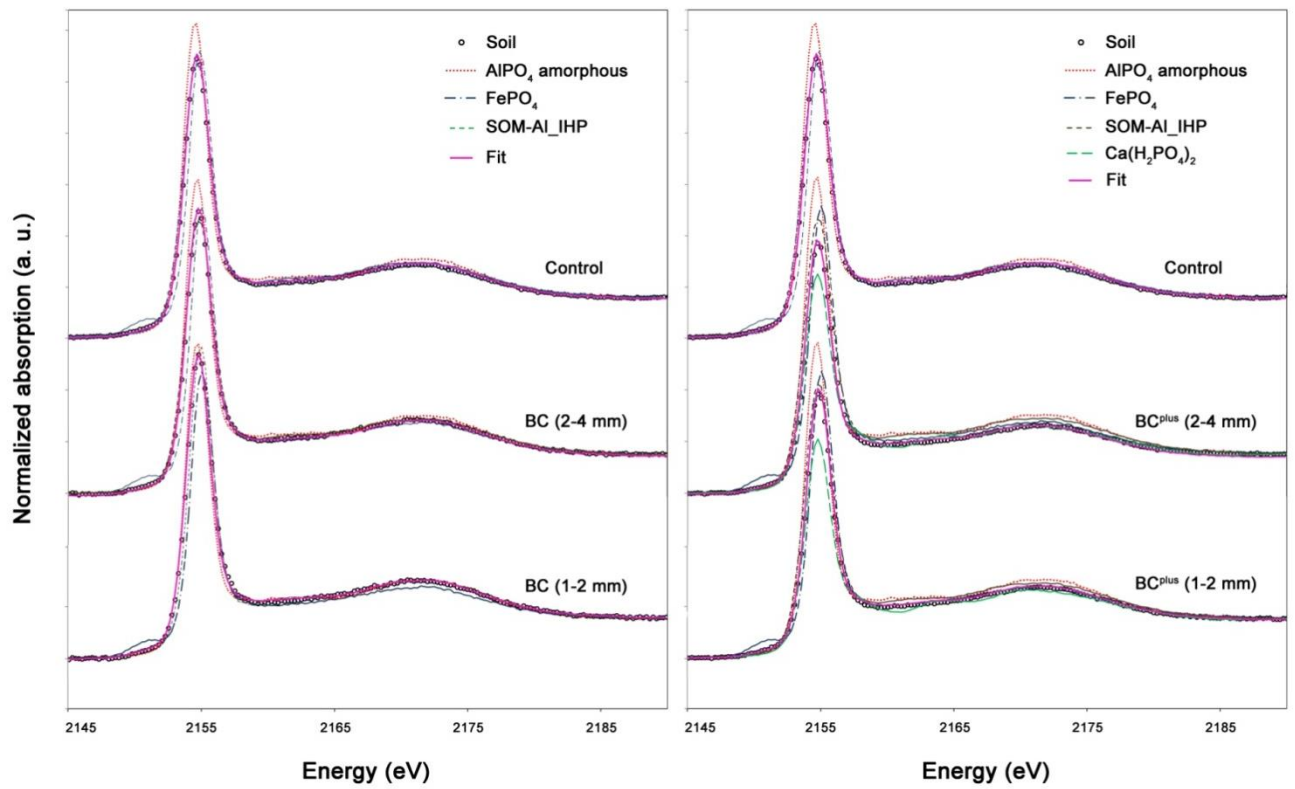
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876 Morshedizad et al., Figure 3

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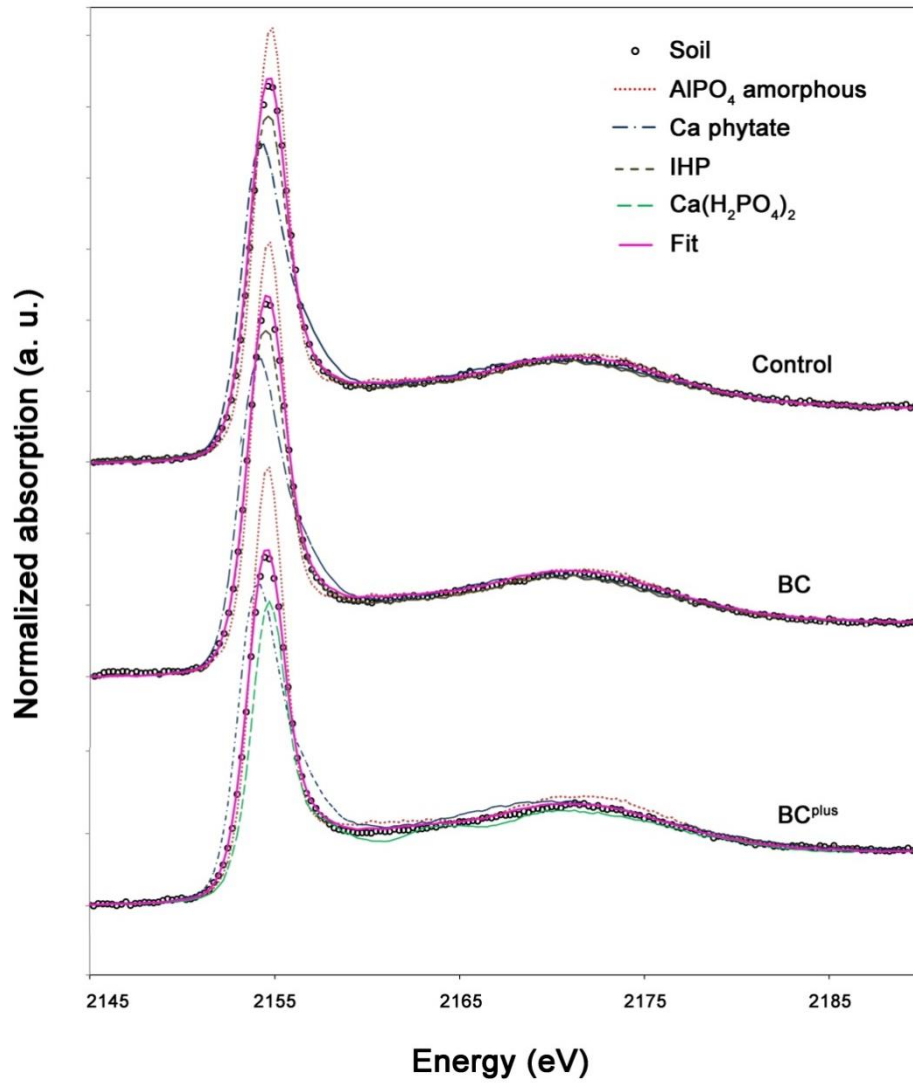
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890 Morshedizad et al., Figure 4

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