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1 *Article*

# 2 Sorption to biochar impacts $\beta$-glucosidase and 3 phosphatase enzyme activities

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11 **Abstract:** Extracellular enzymes catalyze biogeochemical reactions in soil, cycling carbon and
12 nutrients in agricultural systems. Enzymes respond quickly to soil management, including organic
13 amendment inputs, such as biochar, a charcoal-like solid byproduct of bioenergy production. In a
14 previous agricultural field trial, a pine biochar amendment caused a 40% decrease in the enzyme
15 activities of $\beta$-glucosidase (BG) and phosphatase (PHOS). The large surface area of the pine biochar
16 has the potential to sorb nutrients and other organic molecules. To test if sorption caused decreased
17 enzyme activity, we used a laboratory assay to quantify the activity of two sorbed enzymes: BG and
18 acid PHOS, involved in the cycling of carbon and phosphorous. The enzymes were incubated with
19 three solid phases: (1) the high surface area pine biochar, (2) the agricultural soil and (3) a low
20 surface area grass biochar, for an additional comparison. We quantified the sorbed enzymes at pH
21 6, 7, and 8, using a Bradford protein assay and measured the immobilized enzyme activities via
22 high-throughput fluorometric analysis. After sorption onto pine biochar, detectable BG and PHOS
23 activity levels dropped by over 95% relative to the soil, supporting direct sorption as one mechanism
24 reducing enzyme activity in biochar amended soil. This laboratory assay demonstrated sorption
25 could account for lack of priming of native soil organic matter and changes in soil P cycling after
26 pine biochar addition.

27 **Keywords:** biochar; surface area; enzyme activity; sorption; protein assay
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## 29 1. Introduction

30 Agricultural amendment of soils with biochar, the solid co-product of bioenergy production, can
31 have variable impacts on soil productivity and health [1]–[4]. Differences in pyrolysis times and
32 temperatures create a wide variability in biochar surface area and charge, pore size distribution, pH,
33 and chemical composition [3], [5], [6]. Due to these physiochemical differences, the impact of biochar
34 on soil structure and biogeochemistry also varies widely. To assess the effect of biochar on soil
35 function in agriculture, researchers can analyze the activity of microbial extracellular enzymes [7]–
36 [9]. The extracellular proteins excreted by microbes break down macromolecules for uptake,
37 catalyzing the rate-limiting steps of biochemical reactions [10]–[12]. The enzyme conformation, i.e.
38 the specific structure, maintains the active site, shaped precisely to break down a specific substrate.
39 Each enzyme acts most efficiently within a narrow optimal range of pH, temperature, and moisture
40 levels. When the soil environment changes, the enzyme catalytic efficiency changes [13], [14]. Due to
41 this sensitivity, enzymes provide rapid indication of changes to soil quality, microbial activity, and
42 nutrient cycling [11], [15].
43 Biochar surfaces have a high potential to sorb organic molecules, including enzymes and
44 substrate, thus altering enzyme activities [16], [17]. Due to the varied nature of enzyme-solid phase
45 interactions, impacts of biochar on enzyme activities remain nebulous. For example, in a single

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agricultural field trial, a 2.5% (w/w) addition of pine wood biochar increased activities of α-1,4-glucosidase, β-D-cellobiohydrolase, and β-1,4-N-acetylglucosaminidase and significantly decreased β-1,4-glucosidase and phosphatase activities [18]. Of particular concern are the observed decreases in enzyme activities, which may reduce soil carbon and nutrient cycling. Specifically, the agricultural field trial found a 41% decrease in a glucose cycling hydrolytic enzyme, β-glucosidase (BG), and a 43% decrease in an organic phosphorus cycling enzyme, phosphatase (PHOS). Decreased enzyme activity in soil may relate to changes in soil structure and nutrient diffusion rates, to sorption of substrate, or to the direct sorption of the enzymes. These interactions require in-depth laboratory analysis before further interpretation of field results, especially if the enzymes sorb to biochar directly, resulting in loss of activity and cycling of critical crop nutrients. Management requires an understanding of the impact of biochar on extracellular enzymes before designing amendments that maximize, rather than impede, nutrient release.

Numerous studies on enzyme-soil mineral interactions reveal that sorption frequently occurs and can alter activity rates [19], [20]. The sorption of enzymes, either adsorption to the solid phase surface or absorption into pore spaces, involves potential electrostatic, pH-controlled, hydrophobic, and physical interactions [21], [22]. Depending on the mechanism, sorption to solid phases can either maintain the protein structure and functional active site [17], [23], or alter the shape of the active site, reducing activity levels [21], [24], [25]. Certain biochars can stabilize [17] and increase activity for select enzymes [18], [26]. However, more frequently, biochar exposure reduces enzyme activity, due to sorption of substrate [16], [27], [28], or direct interaction of the enzyme with biochar hydrophobicity and surface area [28]–[30]. Despite this growing research field, to our knowledge, no experiment directly quantifies the sorption and activity of biochar-immobilized enzymes in response to biochar surface properties.

This laboratory experiment tested the mechanisms behind previously reported field results: Does pine biochar directly sorb enzymes and thus account for the ~40% reduction in BG and PHOS activities? The assay explicitly measured enzyme sorption and subsequent activity after immobilization onto solid phases at three pH levels. This experiment tested the enzymes interacting with three solid phases: (1) the original sandy clay loam soil, (2) the pine biochar amended in the initial field experiment, and (2) a grass biochar, for further insight on different biochar physical properties. We quantified sorption via Bradford protein assay, followed by a fluorescence-based analysis of immobilized enzyme activities. The sorption of substrate was also tested with a similar activity assay. Based on previous in situ results from Foster et al., 2016, we predicted that extracellular BG and PHOS would sorb onto pine biochar at similar rates, resulting in reduced activities relative to the soil and grass biochar. We predicted a higher percentage of PHOS sorption due to its smaller size, leading to lower PHOS activity than BG. Finally, we predicted higher sorption and lower activity would occur at the lowest assay pH, as the solid phases and enzymes approach their isoelectric points of zero charge.

## 2. Materials and Methods

### *2.1. Concentrated enzymes*

Two commercially available enzymes were selected to match the previous field study: β-glucosidase (BG) (from Aspergillus niger, Sigma-Aldrich 49291) and acid phosphatase (PHOS) (from sweet potato, Sigma-Aldrich P1435) (Table 1). The acid PHOS was selected rather than alkaline PHOS, to provide a comparison between enzymes with similar optimal pHs and maintain assay consistency. The enzymes were dissolved into 50 mM Tris at a concentration of 2 mg $mL^{-1}$ for BG and 4 mg $mL^{-1}$ for PHOS. These different concentrations were specifically chosen to meet detection limits of the Bradford protein assay (ThermoFisher Scientific; Waltham, MA, USA) and to help equalize the units of activity (U) present in each well per mg of enzyme (Table 2). The enzyme solutions were buffered to a pH of 6, 7, and 8. The highest pH was closest to the initial pH of the alkaline field soil, and the lower pH levels were closer to the optimum conditions for the two enzymes.

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95 **Table 1.** Characteristics of the two commercial enzymes.

| Enzyme | Abbrev. | Source | Expected Activity (U/mg)[c] | Optimal pH | Isoelectric Point pH | Atomic Weight (kDa) |
|---|---|---|---|---|---|---|
| ß-Glucosidase | BG | Aspergillus niger | ≥ 0.75 | 4.0 | 4 [a] | 240 [a] |
| Acid Phosphatase | PHOS | Sweet potato | 0.5-3.0 | 4.8 | 5.2 [b] | 110-112 [b] |

96 [a] Watanabe et al. (1992) examined an isoform of BG from Aspergillus niger. [b] Durmus et al. (1999) examined an
97 isoform of acid PHOS from sweet potato. The commercial enzymes used in this experiment may have contained
98 multiple isoforms of the enzyme. [c] Each unit (U) of enzyme degrades 1.0 μmol of P-Nitrophenyl substrate (either
99 Phophate or β-D glucopyranoside) per minute at the optimal pH and 37°C.

100 **Table 2.** Physical properties of three solid phases, with the pH and hydrophobicity measured in
101 triplicate, and BET surface area and isoelectric point measured on a single sample.

| Solid Phase | Description | $pH_{H20}$ | BET Surface Area ($m^2$ $g^{-1}$) | BET Average Pore Width (nm) | Isoelectric Point (pH) | Hydrophobicity (seconds) |
|---|---|---|---|---|---|---|
| Soil | Irrigated sandy clay loam | 8.7 | 27.5 | 6.16 | < 1 | Low (7.7) |
| Pinewood Biochar | Max pyrolysis temperature: 400-700ºC[a] | 9.2 | 232.7 | 4.51 | 1-2 | Medium (22) |
| Grass Biochar | Max pyrolysis temperature: 300ºC | 9.9 | 6.3 | 19.12 | 1 | High (>300) |

102 [a] This range is reported due to variability in the commercial bioenergy and biochar co-production.

103 *2.2. Solid phases*

104 Enzyme activity was quantified after sorption onto three air-dried and ground solid phases
105 (Table 2). The pine biochar from the previous field experiment had a high surface area [18], so for
106 contrast, we tested both the original sandy clay loam and a low surface area grass biochar. The soil
107 was a mesic Aridic Haplustalfs (NRCS, 2005) and reported in a previous field experiment as 51%
108 sand, 20% silt and 29% clay, an organic carbon stock of 11.28 Mg $ha^{-1}$ and a bulk density of 1.3 g $cm^{-3}$
109 [33], [34]. The grass biochar was produced at a lower pyrolysis temperature and had higher
110 hydrophobicity than the pine biochar (Table 2). Hydrophobicity was measured on triplicate samples
111 using water drop penetration time [35] and each sample given a relative rating based on infiltration
112 time of either low (< 15s), medium (15-30s), or high (>30s). The solid phases were characterized by
113 pH (1:1 in deionized water) and then by measuring their isoelectric points, the pH at which the
114 surface charge, or zeta potential, equals zero. Briefly, the protocol of Asadi et al. (2009) was followed,
115 modified by first grinding the solid phases. The solid phase was suspended in a 0.01 M $KNO_3$ solution
116 with pH levels from 1 to 6 with dilute KOH or $HNO_3$, bringing samples to a final concentration of 1.5
117 mg solid phase $mL^{-1}$. Samples were analyzed on a NanoBrook Omni Zeta Potential Analyzer
118 (Brookhaven Instruments Corp, NY, USA) to calculate a final average from 10 runs. The soil
119 possessed a negative zeta potential at pH 1, the lowest limit of the assay.
120

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121 *2.3. Enzyme sorption assay*

122 To prepare the sorption assay, the three solid phases were air-dried and ground on a roller table
123 into a powder. Five replicate 5 mg samples were weighed into microcentrifuge tubes, into which 100
124 µL of BG or PHOS enzyme solution was added. After vortexing, the mixed solution was incubated
125 for one hour at ambient temperature (~25ºC). Incubation times longer than one hour resulted in
126 measurements higher than the protein amount initially added, likely due to interference from other
127 organic molecules coming from the solid phases. This background interference was measured for
128 each solid phase and later subtracted from the final value. After centrifugation at 282 x g for 3
129 minutes, 5 µL of supernatant was pipetted into a microplate with 250 µL of Coomassie Reagent to
130 conduct a colorimetric Bradford Protein Assay (ThermoFisher Scientific; Waltham, MA, USA). After
131 ten minutes of reaction time, the plates were read at 595 nm with an Infinite M200 Microplate Reader
132 (Tecan Trading AG, Switzerland). Each enzyme plate had a standard curve of six points prepared
133 from the enzyme stock solution and the Coomassie Reagent. The supernatant protein concentration
134 measured by the Bradford Assay was subtracted from the total protein added to calculate the
135 quantity of enzyme immobilized according to the following equation:

136 Eqn. 1:

137 Enzyme sorbed (%) =

138
$$\frac{\text{Total protein added (mg)} - [\text{Supernatent protein (mg)} - \text{Background protein in the solid phase (mg)}]}{\text{Total protein added (mg)}} * 100\%$$

139 *2.3. Enzyme activity assay*

140 After completing the sorption experiment, we conducted a high-throughput fluorometric
141 activity assay on the immobilized enzymes. Fluorometric assays are more accurate than colorimetric
142 methods for enzyme activity to account for quenching with biochar [16]. Negative controls of the
143 solid phases alone were run without added enzyme to determine the background activity. We also
144 ran three positive controls, with enzyme only, to quantify the maximum activity of the free enzymes
145 at pH 6, 7, and 8. This allowed analysis of activity at an alkaline pH as in the previous agricultural
146 field experiment [18], as well as pH levels closer to the enzyme optimum (Table 1). Standard curves
147 specific to each solid phase, enzyme, and pH were created to account for differences in fluorescence
148 due to the assay conditions, including adsorption of reaction product to the solid phase. The grass
149 biochar was only analyzed at pH 7 due to space constraints on the plate, thus we could ensure the
150 assay was completed on all samples with the exact experimental conditions.
151 To measure the immobilized enzyme activity, we first used 405 µL of buffer to dilute the
152 remaining 95 µL enzyme and solid phase. This suspension was vortexed and pipetted into a deep-
153 well plate. We added an additional 500 µL to the microcentrifuge tube to ensure all of the solid phase
154 was transferred, confirmed visually. This rinse solution was also pipetted into the deep well plate to
155 capture any remaining solid phase, bringing each well to a 1000 µL final volume. The deep-well plate
156 was then centrifuged for three minutes at 2,900 x g and the supernatant was aspirated, leaving the
157 solid phase and immobilized enzyme. Additional tests of number and volume of rinses demonstrated
158 that the initial dilution effectively removed unbound enzyme and further rinses started to remove
159 the solid phase. Before the activity assay, another 800 µL of buffer was added to suspend the solid
160 phase.
161 The assay measured maximum potential activity via release of fluorescent moieties from
162 enzyme-specific substrate. Following the procedure of Bell et al. 2013, 200 µL of a 200 µM fluorescent
163 substrate was added to each well (4-Methylumbelliferyl β-D-glucopyranoside for BG and 4-
164 Methylumbelliferyl phosphate for PHOS). For each combination of pH, enzyme, and solid phase on
165 the sample plate, there was a unique corresponding standard curve. The standard plates contained
166 the sample suspension and the fluorogenic moiety 4-Methylumbelliferone only, without the attached
167 substrate. The standard curves were made at concentrations ranging from 0 to 200 mM and corrected
168 for quenching due to the solid phase.

Both the sample and standard plates were allowed to react at ambient temperature (~25 ºC) for 24 hours. The longer assay time more closely simulated field conditions, accounting for any desorption or stabilization of the enzyme as might occur naturally. Controls showed that no uncatalyzed hydrolysis of the reactant or degradation of the product occurred over this time period. Fluorescence was read at 365 nm excitation and 450 nm emission on an Infinite M200 Microplate Reader (Tecan Trading AG, Switzerland). The gain was optimized and adjusted for each solid phase and standard curve, as the pine biochar quenched the standard curves more than the soil samples.

To analyze the activity data, we calculated catalytic efficiency using the following equation:

Eqn. 2:

Enzyme Activity ($\mu$mols mg enzyme$^{-1}$ 24hr$^{-1}$) =

$$\frac{\text{Substrate Degraded } (\mu\text{mols})}{\text{Added enzyme (mg)} * \%\text{ Sorbed Enzyme}/100\ * \text{hrs}}$$

This equation incorporates the amount of immobilized enzyme present in the well by multiplying the amount of enzyme added and the percent of sorbed enzyme. The rate was calculated per the entire assay period to more accurately reflect the experimental conditions.

### *2.4. Assay of substrate stability and sorption*

We ran an additional assay to test if the substrate sorption to the solid phases impacted activity levels. We allowed the substrate to interact with each solid phase for 24 hours and then conducted a modified activity assay. Separately, we vortexed 5 mg of the three solid phases with 300 µL of the 200 µM BG and PHOS substrate and MUB fluorescent standard in 50 mM Tris buffer at pH 6, 7, and 8, in the same conditions as the previous assays. There also were controls with substrate only and no solid phase. The slurries incubated for 24 hours, as in the previous assays, to test the stability of the compounds through the incubation period. The slurries were then centrifuged for three minutes at 2,900 x g and 100 µL of each supernatant was moved to a deep plate well with an additional 400 µL of buffer and 50 µL of enzyme (2 mg mL$^{-1}$ BG and 4 mg mL$^{-1}$ PHOS). This volume of enzyme matched the ratio of substrate to enzyme in previous activity assays. The standard curves and fluorescence microplate readings were carried out exactly as the initial assays (Section 2.3) with a standard curve for each solid phase, pH, and enzyme combination. The solid phase specific standard curves accounted for the adsorption of reaction products in the assay.

### *2.5. Statistics*

For the enzyme sorption data, a two-way analysis of variance (ANOVA) examined the effect of enzyme type with the three solid phases (soil, pine and grass biochars). Then a subsequent three-way analysis of variance (ANOVA) tested the interactive effects of enzyme type, pH and only two solid phases (soil and pine biochar). Tukey post-hoc adjustments were used to make pairwise comparisons for significant interactions at the $\alpha = 0.05$ level. To achieve normality, the enzyme sorption data were transformed by log (x+1).

A nonparametric Kruskall-Wallis test analyzed the difference in adsorbed enzyme activities between the two enzymes, solid phases, and pH levels. Again the grass biochar only was compared at pH 7, so the effect of pH was only tested with the soil and pine biochar samples. The final activity values are presented without subtraction of the background activity, because this added unnecessary variability after error propagation and did not change interpretation of results.

All statistical analyses were conducted using R software version 3.3.3 (R Core Team, 2017). The sorption data analyses used the lm(), aov(), and TukeyHSD() functions. The nonparametric activity analysis implemented the kruskal.test() package with a Dunn test for multiple comparisons from the FSA package [38], with false discovery rate adjusted p-values.

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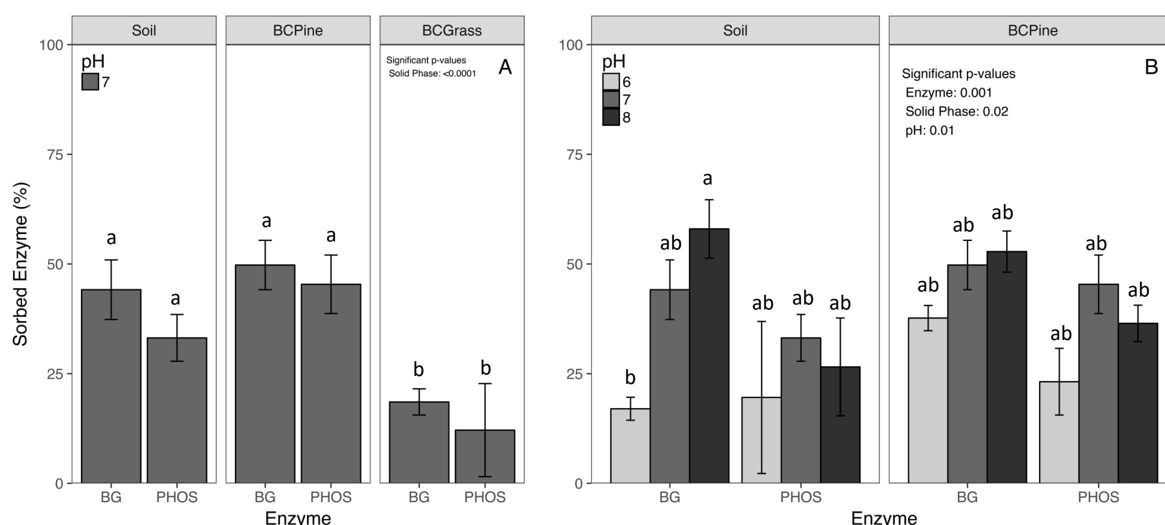
214 **3. Results**

215 *3.1. Enzyme sorption*

216 Across all three solid phases, the Bradford protein assay revealed average enzyme sorption of
217 33.9% and statistically similar values for BG and PHOS (P = 0.07, S. Table 1, Fig. 1). The pine biochar
218 sorbed significantly more enzyme (47.5%) than the grass biochar at pH 7 (15.3%) (P < 0.01, S. Table 1,
219 Fig. 1A). However, the pine biochar sorbed nearly the same amount of enzyme as the soil (38.6%) (P
220 = 0.20, Fig 1). The pH level significantly impacted sorption (P < 0.05, S. Table 2, Fig. 1B). Sorption was
221 approximately 19% lower at pH 6 than pH 7 and 8 (P <0.01). The highest sorption occurred with BG
222 to soil at pH 8 and the lowest sorption occurred with BG to soil at pH 6 (P = 0.10, Fig. 1B).




223

224 **Figure 1A and 1B.** (A) The enzymes (ß-Glucosidase (BG) or Acid Phosphatase (PHOS)) sorbed to the
225 three solid phases (Soil, Pine Biochar (BC Pine), and Grass Biochar (BC Grass) at pH 7). Percent
226 sorption was calculated as the difference between the total enzyme added and the amount in the
227 supernatant divided by the total enzyme added (Eqn 1). (B) The percent of enzyme sorbed to soil and
228 pine biochar at three pH levels. All results are means ±1 standard error (n=5). Significant differences
229 (P <0.05) are labeled as lowercase letters from the Tukey adjusted comparisons.

230 *3.2. Enzyme activity*

231 In the activity assay, we detected a small amount of background activity (< 2 nmols) from the
232 biochars, and a larger amount in the soil (PHOS = 6.7 nmols and BG = 2.8 nmols) at pH 8. However,
233 subtraction of this background did not alter the interpretation of results; thus initial values are
234 presented in Fig 2. After standard curve calculations were applied, all negative activity rates were set
235 to zero. The exact quantity of substrate degraded (nmols) by the two enzymes adsorbed to solid
236 phases, and free enzymes are presented in Supplemental Table 3.
237 The amount of substrate degraded depended primarily upon the solid phase (P < 0.0001) and
238 their interaction with enzyme and pH level (P < 0.001, Fig 2.). Overall, pine biochar immobilization
239 decreased enzyme activity to less than 1 nmol mg enzyme$^{-1}$ 24hr$^{-1}$ at pH 7. The enzymes maintained
240 activity levels of 48 nmols mg enzyme$^{-1}$ when sorbed to soil and 44 nmols mg enzyme$^{-1}$ 24hr$^{-1}$ when
241 sorbed to grass biochar at pH 7 (P < 0.001, Fig. 2A). The amount of substrate degraded by PHOS
242 averaged over all pH levels was higher than that of BG during the 24-hour assay (+5 nmols mg
243 enzyme$^{-1}$ 24hr-1, P < 0.001, Fig. 2B). When adsorbed to grass biochar there was a decrease in BG
244 activity to 15.0 nmols mg enzyme$^{-1}$ 24hrs$^{-1}$, relative to soil (43.1 nmols mg enzyme$^{-1}$ 24hrs$^{-1}$). In contrast

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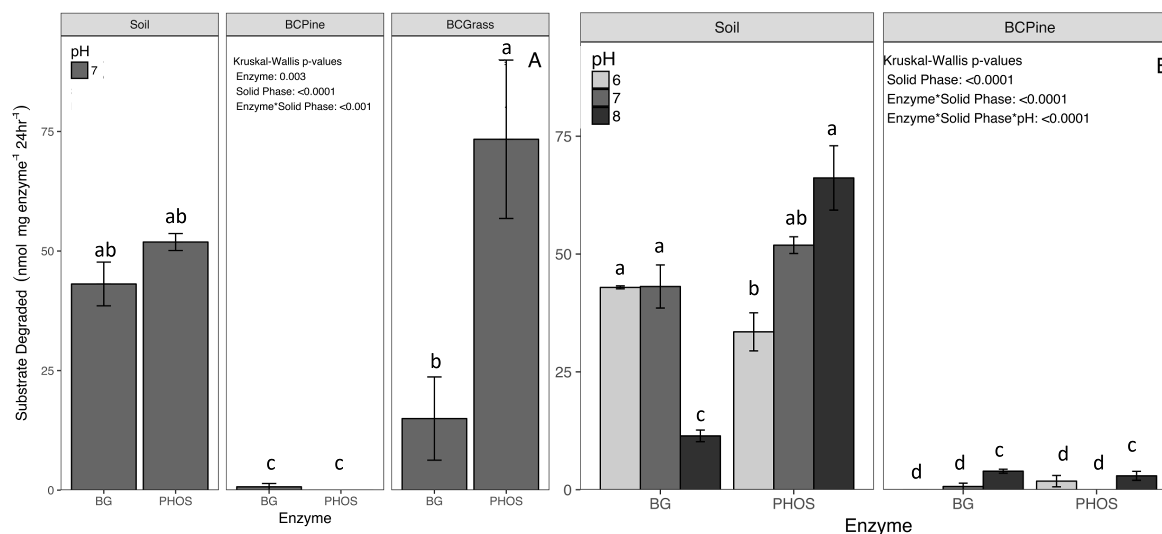
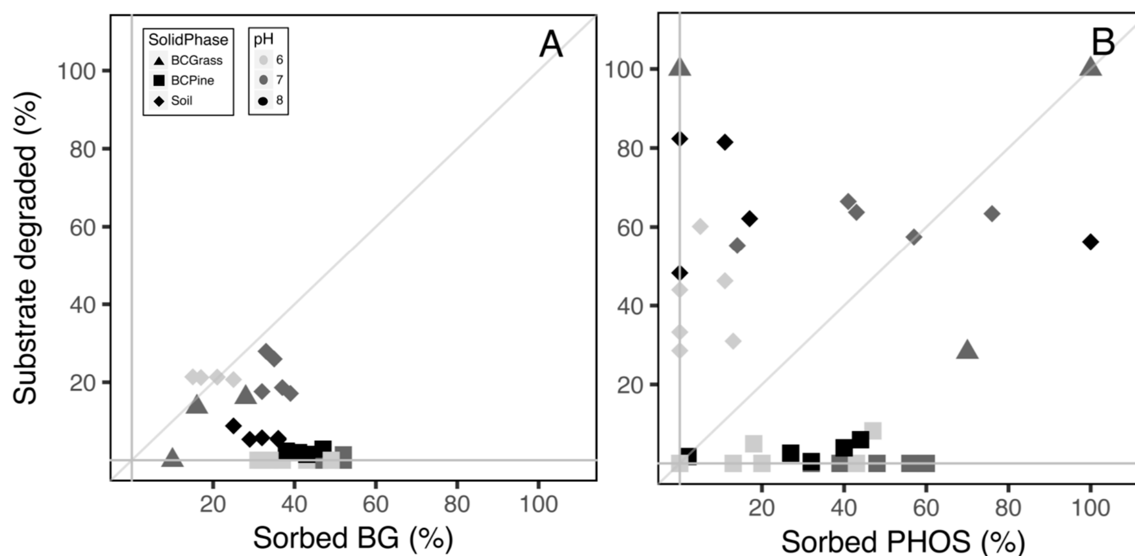
245 the grass biochar adsorbed PHOS activity was (73.4 nmols mg enzyme$^{-1}$ 24hrs$^{-1}$), 41% higher than the
246 soil PHOS activity (51.9 nmols mg enzyme$^{-1}$ 24hrs$^{-1}$) (Fig 2A).




247

248 **Figure 2A and 2B.** (A) The calculated activity rates for two enzymes, ß-Glucosidase (BG) and Acid
249 Phosphatase (PHOS) sorbed to the three solid phases (Soil, Pine Biochar (BC Pine), and Grass Biochar
250 (BCGrass)). (B) The activity of the two enzymes at three pH levels after sorption to soil and Pine
251 Biochar. Results are means ± 1 SE (n=5). Significant differences ($P < 0.05$) are labeled as lowercase
252 letters based on Dunn test for multiple comparison.

253 To further examine these patterns and to contrast the two enzymes, we plotted the initial data
254 of percent sorption versus the percent of substrate degraded (Fig. 3A, Fig. 3B). The percent of
255 substrate degraded was calculated by taking the nmols mg enzyme$^{-1}$ 24hrs$^{-1}$ and dividing by the 40
256 nmols of substrate added to each well. Activity of 100% indicates all the substrate was degraded in
257 the well. The diagonal 1:1 line represents when the sorbed enzymes degraded the expected quantity
258 of substrate, related to the quantity of enzymes remaining in the well (% sorption). These figures
259 show the higher efficiency of PHOS, with more points along or above the dotted 1:1 line. There was
260 low activity of BG at pH 8 on soil and pine biochar (black diamonds and squares (Fig. 3A) and low
261 activity of both enzymes immobilized on the pine biochar (squares, Fig. 3A, Fig. 3B).




262

263 **Figure 3A and 3B.** Percent sorption and activity plotted for two enzymes (A) ß-Glucosidase (BG) and
264 (B) Acid Phosphatase (PHOS), at three pH levels pH (6=light gray, 7=dark gray, 8=black) with three
265 solid phases Soil (diamonds), Pine Biochar (BC Pine, squares), and Grass Biochar (BCGrass,
266 triangles)). The percent activity was calculated as nmols mg enzyme$^{-1}$ 24hrs$^{-1}$ divided by the 40 nmols
267 of substrate added. The diagonal 1:1 line represents when the sorbed enzymes degraded the expected
268 amount of substrate. The solid gray lines represent zero sorption or activity level.

269 *3.3. Assay of substrate stability and sorption*

270 The substrate sorption assay with specific standard curves and plate runs showed only slight
271 differences due to the solid phases in interaction with pH ($P = 0.0965$) (data not shown). Pairwise
272 comparisons revealed no significant differences between the controls without solid phase versus the
273 soil, pine or grass biochar. The enzyme activity rates with the solid phase-exposed substrate did vary
274 in response to pH and enzyme. The activity at pH 6 was lower than pH 7 or 8 ($P < 0.015$) and PHOS
275 activity was lower than BG activity rates ($P = 0.0319$).

276 **4. Discussion**

277 *4.1. Enzyme sorption*

278 The enzyme type, solid phase, and pH all influenced the amount of sorption. Both enzymes
279 sorbed to all of the solid phases. The similar sorption between soil and pine biochar could be due the
280 diameter of the biochar pores. One third of the pine biochar surface area occurred within pore spaces
281 with an average width of 4.51 nm (Table 1), smaller than the enzymes tested in this study [25]. This
282 result indicates that enzyme sorption depends on biochar pore size and does not merely correlate
283 with surface area alone [17].
284 The relatively similar enzyme sorption onto the three solid phases (15-43%) could relate to the
285 negative surface charges of the solid phases and enzymes. As the assay was run at pH 6-8, higher
286 than the isoelectric point of the solid phases (Table 2), each surface had a negative charge, resulting
287 in similar sorption capacities. This also helps to explain the relatively similar sorption onto pine
288 biochar, despite its much larger surface area. An enzyme will sorb highly to a negatively charged
289 mineral surface when the enzyme is at its isoelectric point, and possesses no charge [24], [39]. This
290 was observed previously with a wood biochar and BG at a pH of 5, close to its isoelectric point [27].
291 In this experiment, the pH levels were above the isoelectric points of the enzymes to better match the
292 initial field conditions. Above the isoelectric points for BG (4) and PHOS (5.2), both enzymes
293 possessed negative charges [30] and thus similar sorption rates occurred. Surprisingly, the decrease
294 in negative surface charge at low pH was not observed across our range of pH from 6 to 8. Another
295 consideration is that the measured pH of the bulk solution may be higher than the pH precisely at
296 the solid phase surface. This may explain why the lab incubation resulted in a more variable pattern,
297 with the lowest sorption at pH 6, a finding that contradicted our prediction based on enzyme surface
298 charge.
299 Though the grass biochar also possessed a negative charge, the low surface area and high
300 hydrophobicity likely caused lower sorption rates than the soil and pine biochar. Lower temperature
301 biochars are often more hydrophobic [29]; the grass biochar used in this study would repel hydrated
302 amino-acid sidechains (especially for BG), as previously observed with clay minerals [39]. To tease
303 apart the exact mechanism involved in sorption, future research must consider the exact hydrogen
304 ion concentration at the solid phase surface, and both electrostatic and hydrophobic interactions of
305 enzymes and biochar.

306 *4.2. Enzyme activity*

307 Prior research shows that depending on the environment, biochar-enzyme interactions can
308 increase [40], [41], decrease [15], [18], [30] or have no effect on catalytic capacity [15]. Field results
309 depend on specific enzymes [16]–[18], biochar application rate [40], [42], soil type [16], and indirect
310 effects on soil physio-chemical properties, such as pH of the soil solution [39], sorption of inorganic

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311 and organic chemicals [43], water retention, and pore structure [44]. By simplifying the system, this
312 lab assay quantified the loss of over 95% of BG and 97% of PHOS activity after sorption to pine
313 biochar. This can help to explain the decreased enzyme activities measured in our previous pine
314 biochar agricultural field experiment [18].
315 The decline in enzyme activities resulted primarily from direct sorption. Simultaneously, a
316 small amount of substrate sorption also occurred, particularly onto the pine biochar. Therefore, we
317 do not exclude the possibility of BG substrate adsorption to the pine biochar as one mechanism for
318 reduced activity rates, as the polar glucose substrate may readily adsorb to negatively charged solid
319 phases. The phosphate substrate, in contrast, contains both the hydrophobic fluorescent moiety and
320 the negative phosphate group, so the reduced phosphatase activity is likely only due to direct
321 sorption of the enzyme. Previous laboratory incubations also revealed that biochar can sorb the
322 reaction products of colorimetric enzyme assays [28]. Since we used a fluorometric assay, with solid
323 phase-matched standard curves, this provided a more accurate quantification of enzyme activity with
324 biochar. Although the standard curves for pine biochar were lower than the others, producing
325 slightly negative values (Table 3), each curve fit the data with an $R^2$ between 0.94 - 0.99. Since we
326 detected fluorescence of MUB for the standard curve, the reduced activity on pine biochar in the final
327 assay was not merely due to quenching of the fluorescent product. However, other factors may also
328 have influenced enzyme activity and require further study, including the solid phase interaction with
329 enzyme cofactors, allosteric regulators [16], and inhibiting compounds such as phenols and
330 polyphenols [27] .
331 As the primary mechanism for reduced activity, direct sorption of enzymes could cause either a
332 conformational change in the enzyme active site or rotation of the active site toward the biochar
333 surfaces [45]. Further physical properties of the pine biochar, such as pore size distribution, could
334 have caused reduced enzyme activities due to the substrate diffusing into micropores too small for
335 enzymes to access (4.51nm, Table 1) [25]. In contrast, the grass biochar had larger mean pore size
336 diameter (19.12 nm) that would allow more substrate diffusion and thus enzyme access and activity.
337 The grass biochar also had a higher range of activity, with levels over 100% (Fig. 3B). If the active site
338 faced outwards and maintained its morphology, the enzymes could be stabilized and remain active.
339 This immobilization can serve as protection for enzymes against stress on low-surface area biochar
340 [17], and may explain why samples with low sorption rates maintained high activity (Fig. 3B). We
341 must also consider the high background interference during the Bradford assay could cause
342 artificially low detectable sorption.
343 The patterns of enzyme efficiency at different pH levels did not follow our predictions of higher
344 sorption and thus low activity at lower pH levels. The pattern of lower BG efficiency at higher pH
345 likely relates to increasing distance from the enzyme optimal pH (Fig. 2B, Table 2). With PHOS the
346 increase of activity rate with higher pH matches the increase in substrate degraded by the free
347 enzyme (S. Table 3), which may be a simple outcome of heterogeneity between the small amounts of
348 solid phase used in each well for the assay. To delve further into this question, the surface functional
349 groups of distinct biochars require in-depth characterization.
350 This lab assay explicitly tested if the high surface area pine biochar sorbed enzymes more readily
351 than the other solid phases, finding that this sorption to pine biochar did impact enzyme activity. The
352 observed decrease in activity corroborates patterns observed with biochar priming native soil organic
353 matter: less priming occurs with additions of high temperature wood biochar, and higher C
354 mineralization of native soil organic matter occurs with low temperature grass biochar [46]. With the
355 complexity of these enzyme-solid phase interactions, the exact conditions of the laboratory assay
356 require careful consideration when predicting field responses. This experiment highlights how
357 biochar physical properties interact with specific enzymes to alter activity rates.

358 **5. Conclusions**

359 The decreased activity of pine biochar-immobilized enzymes explains previous in situ findings
360 of reduced BG and PHOS activities in an amended agricultural soil. This lab experiment found that
361 exposure to high pyrolysis temperature pine biochar reduced the activity of BG and PHOS enzymes

362 by over 95% relative to soil. To our knowledge, the activity level of enzymes directly immobilized on
363 biochar surfaces has never before been tested. The enzyme interaction with the pine biochar differed
364 from the low-temperature grass biochar, in agreement with previous research that biochar type, as
365 well as specific enzyme, affects the amount of sorption and activity rates. Comparing two biochars,
366 our results suggest that surface area and pore size distribution are key indicators of potential biochar-
367 enzyme interactions. This experiment explicitly tested the enzyme activity levels after
368 immobilization, rather than enzymes or substrate exposed to biochar; thus, it provides one
369 explanation for the negative priming of native organic matter observed after wood biochar addition.

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384 **References**

385 1. C. J. Atkinson, J. D. Fitzgerald, and N. a. Hipps, "Potential mechanisms for achieving
386 agricultural benefits from biochar application to temperate soils: A review," *Plant Soil*, vol.
387 337, pp. 1–18, 2010.

388 2. L. a. Biederman and W. S. Harpole, "Biochar and its effects on plant productivity and nutrient
389 cycling: a meta-analysis," *GCB Bioenergy*, vol. 5, no. 2, pp. 202–214, 2013.

390 3. J. Lehmann, "Bio-energy in the black," *Front. Ecol. Environ.*, vol. 5, no. 7, pp. 381–387, 2007.

391 4. S. Jeffery, F. G. a. Verheijen, M. van der Velde, and a. C. Bastos, "A quantitative review of
392 the effects of biochar application to soils on crop productivity using meta-analysis," *Agric.*
393 *Ecosyst. Environ.*, vol. 144, no. 1, pp. 175–187, Nov. 2011.

394 5. A. Enders, K. Hanley, T. Whitman, S. Joseph, and J. Lehmann, "Characterization of biochars
395 to evaluate recalcitrance and agronomic performance," *Bioresour. Technol.*, vol. 114, pp. 644–
396 53, Jun. 2012.

397 6. J. Lehmann and S. Joseph, "Biochar for Environmental Management : An Introduction," *Sci.*
398 *Technol.*, vol. 1, pp. 1–12, 2009.

399 7. I. Alkorta, A. Aizpurua, P. Riga, I. Albizu, I. Amézaga, and C. Garbisu, "Soil Enzyme Activities
400 as Biological Indicators of Soil Health," *Rev. Environ. Health*, vol. 18, no. 1, 2003.

401 8. C. W. Bell, B. E. Fricks, J. D. Rocca, J. M. Steinweg, S. K. McMahon, and M. D. Wallenstein,
402 "High-throughput fluorometric measurement of potential soil extracellular enzyme
403 activities.," *J. Vis. Exp.*, no. 81, p. e50961, Jan. 2013.

404 9. S. D. Allison, "Cheaters, diffusion and nutrients constrain decomposition by microbial
405 enzymes in spatially structured environments," *Ecol. Lett.*, vol. 8, no. 6, pp. 626–635, 2005.

406 10. R. G. Burns *et al.*, "Soil enzymes in a changing environment: Current knowledge and future
407 directions," *Soil Biol. Biochem.*, vol. 58, pp. 216–234, Mar. 2013.

408 11. B. a. Caldwell, "Enzyme activities as a component of soil biodiversity: A review," *Pedobiologia*
409 *(Jena).*, vol. 49, no. 6, pp. 637–644, Nov. 2005.

410 12. J. H. J. R. Makoi and P. a Ndakidemi, "Selected soil enzymes: Examples of their potential roles
411 in the ecosystem," *African J. Biotechnol.*, vol. 7, no. 3, pp. 181–191, 2008.

412 13. C. Alster, A. Koyama, N. G. Johnson, and M. D. Wallenstein, "Temperature sensitivity of soil
413 microbial communities : an application of macromolecular rate theory to microbial respiration
414 ...," *J. Geophys. Res. Biogeosciences*, no. May, 2016.

415 14. R. L. Sinsabaugh *et al.*, "Stoichiometry of soil enzyme activity at global scale," *Ecol. Lett.*, vol.
416 11, pp. 1252–1264, 2008.

417 15. J. Paz-Ferreiro, G. Gascó, B. Gutiérrez, and A. Méndez, "Soil biochemical activities and the
418 geometric mean of enzyme activities after application of sewage sludge and sewage sludge
419 biochar to soil," *Biol. Fertil. Soils*, vol. 48, no. 5, pp. 511–517, 2012.

420 16. V. L. Bailey, S. J. Fansler, J. L. Smith, and H. Bolton, "Reconciling apparent variability in effects
421 of biochar amendment on soil enzyme activities by assay optimization," *Soil Biol. Biochem.*,
422 vol. 43, no. 2, pp. 296–301, Feb. 2011.

423 17. K. a. Elzobair, M. E. Stromberger, and J. a. Ippolito, "Stabilizing effect of biochar on soil
424 extracellular enzymes after a denaturing stress," *Chemosphere*, 2015.

425 18. E. J. Foster, N. Hansen, M. Wallenstein, and M. F. Cotrufo, "Biochar and manure amendments
426 impact soil nutrients and microbial enzymatic activities in a semi-arid irrigated maize
427 cropping system," *Agric. Ecosyst. Environ.*, vol. 233, pp. 404–414, 2016.

428 19. R. G. Burns, "Enzyme activity in soil: location and a possible role in microbial ecology," *Soil*
429 *Biol. Biochem.*, vol. 14, pp. 423–427, 1982.

430 20. P. Nannipieri, E. Kandeler, and P. Ruggiero, *Enzymes in the Environment Activity, Ecology, and*
431 *Applications: Enzyme Activities and Microbiological and Biochemical Processes in Soil*. New York:
432 Marcel Dekker, Inc, 2002.

433 21. H. Quiquampoix and S. Servagent-noinville, "Enzyme Adsorption on Soil Mineral Surfaces
434 and Consequences for Catalytic Activity," in *Enzymes in the Environment: Activity, Ecology, and*
435 *Applications*, R. G. Burns and R. P. Dick, Eds. Mrcel Dekker, Inc, 2002.

436 22. C. Sandhya, K. M. Nampoothiri, and A. Pandey, *Microbial Enzymes and Biotransformations*, vol.
437 17, no. 1. Totowa, New Jersey: Humana Press, 2005.

438 23. J. M. Sarkar, A. Leonowicz, and J. M. Bollag, "Immobilization of enzymes on clays and soils,"
439 *Soil Biol. Biochem.*, vol. 21, no. 2, pp. 223–230, 1989.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 September 2018 doi:10.20944/preprints201809.0372.v1

440 24. R. Datta *et al.*, "How enzymes are adsorbed on soil solid phase and factors limiting its activity : 441 A Review," *Int. Agrophysics*, vol. 31, pp. 287–302, 2017.

442 25. H. Quiquampoix and R. G. Burns, "Interactions between proteins and soil mineral surfaces: 443 Environmental and health consequences," *Elements*, vol. 3, no. 6, pp. 401–406, 2007.

444 26. F. Wu, Z. Jia, S. Wang, S. X. Chang, and A. Startsev, "Contrasting effects of wheat straw and 445 its biochar on greenhouse gas emissions and enzyme activities in a Chernozemic soil," *Biol.* 446 *Fertil. Soils*, vol. 49, no. 5, pp. 555–565, 2013.

447 27. C. Lammirato, A. Miltner, and M. Kaestner, "Effects of wood char and activated carbon on the 448 hydrolysis of cellobiose by β-glucosidase from Aspergillus niger," *Soil Biol. Biochem.*, vol. 43, 449 no. 9, pp. 1936–1942, Sep. 2011.

450 28. M. Swaine, R. Obrike, J. M. Clark, and L. J. Shaw, "Biochar alteration of the sorption of 451 substrates and products in soil enzyme assays," *Appl. Environ. Soil Sci.*, vol. 2013, pp. 1–6, 2013.

452 29. K. M. Keiblinger, D. Liu, A. Mentler, F. Zehetner, and S. Zechmeister-Boltenstern, "Biochar 453 application reduces protein sorption in soil," *Org. Geochem.*, vol. 87, pp. 21–24, 2015.

454 30. C. Lammirato, A. Miltner, L. Y. Wick, and M. Kästner, "Hydrolysis of cellobiose by - 455 glucosidase in the presence of soil minerals - Interactions at solid-liquid interfaces and effects 456 on enzyme activity levels," *Soil Biol. Biochem.*, vol. 42, no. 12, pp. 2203–2210, 2010.

457 31. T. Watanabe, T. Sato, S. Yoshioka, T. Koshijima, and M. Kuwahara, "Purification and 458 properties of Aspergillus niger beta-glucosidase," *Eur. J. Biochem.*, vol. 209, pp. 651–659, 1992.

459 32. A. Durmus *et al.*, "The active site of purple acid phosphatase from sweet potatoes (Ipomoea 460 batatas): Metal content and spectroscopic characterization," *Eur. J. Biochem.*, vol. 260, pp. 709– 461 716, 1999.

462 33. U. S. D. of A. Soil Survey Staff, Natural Resources Conservation Service, "Official Soil Series 463 Descriptions." Soil Survey Staff, Natrual Resources Conservation Service, United States 464 Deaparment of Agriculture.

465 34. M. Abulobaida, "The effect of irrigaiton and cropping systems on soil carbon and nitrogen 466 stocks and organic matter aggregation in semi-arid lands," Colorado State University, 2014.

467 35. E. S. Vogelmann, J. Prevedello, G. O. Awe, and D. J. Reinert, "Soil hydrophobicity : 468 comparative study of usual determination methods," *Cienc. Rural*, vol. 45, no. 2, pp. 260–266, 469 2015.

470 36. A. Asadi, B. B. K. Huat, M. M. Hanafi, T. A. Mohamed, and N. Shariatmadari, "Role of organic 471 matter on electroosmotic properties and ionic modification of organic soils," *Geosci. J.*, vol. 13, 472 no. 2, pp. 175–181, 2009.

473 37. C. W. Bell, B. E. Fricks, J. D. Rocca, J. M. Steinweg, S. K. Mcmahon, and M. D. Wallenstein, 474 "Supply List: High-throughput Fluorometric Measurement of Potential Soil Extracellular 475 Enzyme Activities," *J. Vis. Exp.*, pp. 1–2, 2013.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 September 2018 doi:10.20944/preprints201809.0372.v1

476 38. D. H. Ogle, “FSA: Fisheries Stock Analysis.” R package verison 0.8.20, 2018.

477 39. M. Baron, M. Revault, S. Servagent-Noinville, J. Abadie, and H. Quiquampoix,
478 “Chymotrypsin Adsorption on Montmorillonite: Enzymatic Activity and Kinetic FTIR
479 Structural Analysis.,” *J. Colloid Interface Sci.*, vol. 214, no. 2, pp. 319–332, 1999.

480 40. P. Oleszczuk, I. Jośko, B. Futa, S. Pasieczna-Patkowska, E. Pałys, and P. Kraska, “Effect of
481 pesticides on microorganisms, enzymatic activity and plant in biochar-amended soil,”
482 *Geoderma*, vol. 214–215, pp. 10–18, Feb. 2014.

483 41. Z. Du *et al.*, “Consecutive Biochar Application Alters Soil Enzyme Activities in the Winter
484 Wheat–Growing Season,” *Soil Sci.*, vol. 179, no. 2, pp. 75–83, 2014.

485 42. X. Wang, D. Song, G. Liang, Q. Zhang, C. Ai, and W. Zhou, “Maize biochar addition rate in fl
486 uences soil enzyme activity and microbial community composition in a fl uvo-aquic soil,”
487 *Appl. Soil Ecol.*, vol. 96, pp. 265–272, 2015.

488 43. H. Shindo, D. Watanabe, T. Onaga, M. Urakawa, O. Nakahara, and Q. Huang, “Adsorption,
489 activity, and kinetics of acid phosphatase as influenced by selected oxides and clay minerals,”
490 *Soil Sci. Plant Nutr.*, vol. 48, no. 5, pp. 763–767, 2002.

491 44. F. Sopeña and G. D. Bending, “Impacts of biochar on bioavailability of the fungicide
492 azoxystrobin: A comparison of the effect on biodegradation rate and toxicity to the fungal
493 community,” *Chemosphere*, vol. 91, no. 11, pp. 1525–1533, 2013.

494 45. F. Leprince and H. Quiquampoix, “Extracellular enzyme activity in soil: effect of pH and ionic
495 strength on the interaction with montmorillonite of two acid phosphatases secreted by the
496 ectomycorrhizal fungus Hebeloma cylindrosporum,” *Eur. J Soil Sci*, vol. 47, no. December, pp.
497 511–522, 1996.

498 46. A. R. Zimmerman, B. Gao, and M. Y. Ahn, “Positive and negative carbon mineralization
499 priming effects among a variety of biochar-amended soils,” *Soil Biol. Biochem.*, vol. 43, no. 6,
500 pp. 1169–1179, 2011.

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Supplemental Tables

**S Table 1.** A two-way ANOVA examined the interaction of solid phase and enzyme on the sorption on soil, pine and grass biochars at pH 7.

| | | Sorption (%) | |
|---|---|---|---|
| Treatment | df | F-stat | p-value |
| Enzyme | 1 | 3.396 | 0.0678 |
| Solid Phase | 2 | 11.256 | <0.0001 |
| Enzyme*Solid Phase | 2 | 0.168 | 0.8452 |

**S Table 2.** A three way ANOVA examined the interactive effects of solid phase, enzyme, and pH level for sorption and activity on the soil and pine biochar. interaction at pH 7 for the sorption to soil and pine biochar.

| | | Sorption (%) | |
|---|---|---|---|
| Treatment | df | F-stat | p-value |
| Enzyme | 1 | 1.151 | 0.287 |
| Solid Phase | 1 | 24.098 | <0.0001 |
| pH | 2 | 3.840 | 0.027 |
| Enzyme*Solid Phase | 1 | 0.523 | 0.472 |
| Enzyme*pH | 2 | 15.865 | <0.0001 |
| Solid Phase*pH | 2 | 1.910 | 0.157 |
| Enzyme*Solid Phase*pH | 2 | 1.779 | 0.177 |

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**S Table 3.** The amount of substrate degraded (nmols) by two enzymes (ß-Glucosidase (BG) and Acid Phosphatase (PHOS)) sorbed to the three solid phases (Soil, Pine Biochar (BC Pine), and Grass Biochar (BC Grass)) compared to the free enzyme in solution. Results are presented as means and 1 SE (n=5). Lower case letters represent significant differences (p-values < 0.05) from the from Dunn multiple comparison tests. Two Dunn tests were used to separate the enzymes, accounting for the different amount of enzyme included in each well (BG = 2 mg/mL , PHOS = 4mg/mL).

| | | Substrate degraded (nmols) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ` | Soil | | BC Pine | | BC Grass | | Free Enzyme | |
| Enzyme | pH | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| BG | 6 | 8.59 a | 0.06 | -1.17 b | 0.22 | nd | nd | 40.59 | 1.1 |
| BG | 7 | 8.62 a | 0.91 | -0.56 b † | 0.40 | -0.60 b † | 4.11 | 40.2 | 0.34 |
| BG | 8 | 2.29 ab | 0.25 | 0.79 b | 0.08 | nd | nd | 37.1 | 0.41 |
| PHOS | 6 | 13.4 a | 1.62 | -0.71 b † | 1.07 | nd | nd | 33.05 | 2.24 |
| PHOS | 7 | 20.75 a | 0.71 | -2.78 b † | 0.88 | 29.42 a | 5.32 | 33.87 | 2.73 |
| PHOS | 8 | 26.46 a | 2.73 | 1.18 | 0.38 | nd | nd | 40.03 | 1.1 |

† Negative values occurred when the standard curve overestimated the slope or intercept; thus, activity was set to zero before calculation of rates per mg of enzyme. No data (nd) was collected for pH 6 and 8 for the grass biochar.

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