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Validation of Soil Enzyme Activity Assay for a Biogeochemical Cycling Index in Biochar Amended Soils

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Abstract

Biochar offers several benefits as a soil amendment, including increased soil fertility, carbon sequestration, and water-holding capacity in nutrient-poor soils. Here, we performed a series of enzyme assays on pine biochar-amended soils, comparing multiple enzyme activities (EAs) simultaneously determined in the same soil sample vs. the sum of individual EAs involved in the C, N, S, and P cycles to provide information of the impacts of biochar on biogeochemical cycling. The combination of these four EAs has been considered an indicator of soil health due to their role in the reactions that release bioavailable nutrients in the cycling of C (β-glucosidase), N and C (β-glucosaminidase), P (acid phosphomonoesterase), and S (arylsulfatase) in soils. Comparisons of the theoretical EAs and the CNPS activity assay approaches in the biochar-modified soil revealed similar activity trends with the different concentrations of added biochar. Two years after adding biochar, study results showed the amended soils did not retain more pNP substrate than the un-amended control soils in three different pH buffers (5.5, 5.8, and 6.5) commonly used in EA reactions. Finally, we performed a third experiment to determine if the biochar previously added to the EAs interfered with the reactions' enzyme or substrate. The results indicated that greater activity was measured using the combined assay, which suggests the CNPS activity method was less affected by biochar than the individual EAs. Our findings indicate that the potential soil biochemical-health index, CNPS activity (combination of four enzymes) assay is more robust than the individual EAs and can be used as an alternative tool to monitor soil functioning.

Keywords

Enzyme Activities, Biochar, Biogeochemical Cycles

1. Introduction

The status of soil physiochemical (aggregates, pH, organic matter, and nutrient levels) and biological (microbial biomass and community composition, enzyme activities, respiration) properties used to assess soil health vary due to regional differences in major soil-forming factors, climate, biota, and time [1]. Biological indicators of soil health offer certain advantages over physiochemical methods. Thus, the success of soil conservation efforts depends significantly on evaluating these soil responses to the amendment [2]. The evaluation of soil health emphasizes the soil's biological components, focusing on the metabolic activities of soil microorganisms [3] [4] [5] via their enzymes that mediate many of the rate-limiting steps in nutrient transformations in soils [6] [7]. Soil enzyme activities (EAs) are used as proxies of ecosystem functions [8] [9]. EAs serve as functional indicators, and variations in production are linked to changes in microbial community structure or activities, which are also impacted by resource inputs [10]. EAs are widely assayed and sensitive enough to reflect changes in soil biogeochemical cycling and soil organic matter (SOM) dynamics due to agricultural management and climate variability [11] [12] [13] [14]. Traditionally four of the 15 most common enzymes used as soil health indicators include β-glucosidase (C cycling), Acid phosphatase (P cycling), β-glucosaminidase (C and N cycling), and Arylsulfatase (S cycling). However, as protocols that measure each EA independently could be laborious and the consumption of resources is costly, there is an option in which multiple EAs are assessed simultaneously in the same soil sample to obtain a comparable potential soil biogeochemical-health index, CNPS activity [15] [16]. EAs have distinguished several agricultural management practices and help with redirection management and assessment. Thus, using the novel CNPS activity approach requires an in-depth evaluation of EAs, particularly when biochar is the organic soil amendment.

Biochar is a C-rich material produced by pyrolyzing organic materials, such as agricultural crop residues, wood, and green waste, under low oxygen pressure and temperature [17] [18]. Also known as a soil conditioner, biochar materials have been proposed to be a compliment or an alternative to the use of fertilizers, as they can improve soil's physical, chemical, and biological properties and processes, regulate nutrient bioavailability and reduce the harmful effects of fertilization on the environment [19] [20] [21]. When used as a soil amendment for orchards, biochar has been found to positively impact productivity, restore soil fertility, sequester C in soil, and reduce atmospheric CO_2 concentrations [22].

EAs have been used to study the effect of biochar on nutrient cycling in the soil [23] [24] [25] [26] [27] [28]. The influence of biochar on soil enzyme activity mainly depends on the interaction of enzyme and substrate with biochar [27]. Some studies report that extracellular binding enzymes to the biochar surface inhibit enzymatic reactions [28] [29] [30]. Other research suggests that biochar interferes with enzyme substrates such as p-Nitrophenol (pNP), formed as the reaction product of the hydrolysis of different nitrophenyl derivatives, resulting

in significant reductions in substrate concentrations and extractable product in soil enzyme assays [30]. The biochar-substrate interactions vary depending on the type and chemistry of biochar, soil type, and soil organic matter status [31]. For example, Bayley *et al.* [32] found that the interaction between biochar and enzyme-substrate is variable and depends on the enzyme-substrate properties. Additionally, buffer solution pH can influence the interaction of biochar and enzyme substrates [33] [34]. Zhang *et al.* [35] studied the effects of biochar and chemical fertilizer applications on the overall bacterial community in different soil types. Results suggested that the interactions between measured soil parameters, including pH and organic matter, were statistically significant. The enzyme conformation, *i.e.*, the specific structure, maintains the active site, shaped precisely to break down a particular substrate. Each enzyme acts most efficiently within a narrow optimal pH range, temperature, and moisture levels. The enzyme catalytic efficiency changes when the soil environment changes [36] [37]. This study revealed the importance of formulating biochar and fertilizer application schemes based on different soil types.

Numerous studies have assessed the soil enzymes involved in soil C, N, P, and S cycling [38]. Adopting the CNPS activity assay may provide producers with an inexpensive way to assess soil health. However, studies evaluating the effects of pine-biochar on soil enzyme activities in peach-orchard soils are limited [39]. The general objective of the present study was to examine the impact of biochar on the four enzyme activities recommended by soil health initiatives such as β-glucosidase (C cycling), Acid Phosphatase (P cycling), β-glucosaminidase (C and N cycling), and Arylsulfatase (S cycling). More specifically, this study aimed to evaluate the effects of biochar on the sorption of the four recommended enzymes, their substrates, or the products of the reactions, individually in the biochar-treated soils (5% and 10% v/v) compared to non-treated soils. Subsequently, we examined the effect of biochar on pNP readings with three different pH buffers (5.5, 5.8, and 6.5) used for the EAs assayed. Finally, we compared the sum of these EAs (theoretical EA) to the activities obtained with the combined assay for the same four EAs (CNPS activity) to validate the novel assay.

2. Material and Methods

2.1. Site Description

The peach tree orchard was established in 2017 at the Sandhills Research Station in Jackson Springs, NC (35.21˚N, 79.63˚W). The soil is classified as a sandy, Kaolinitic, thermic Grossarenic Kandiuduits with a pH of 5.8. The area is located in a semi-arid region and has an average annual precipitation of 117 cm. Precipitation was highest between September 2018 and April 2019. The yearly average temperature is 16.5˚C. The experimental design at the Research Station was a factorial randomized completed block with six replications. Three evaluated treatments included two rates of biochar incorporated into the soil (5% and 10% v/v) to a depth of 30 cm and untreated control (CT) (without biochar amend-

ment). The biochar used in this experiment was produced at atmospheric pressure from pine tree wood by controlled pyrolysis at 500˚C. The properties of the biochar are as follows: a pH (H_2O) of 5.4, cation exchange capacity (CEC) of 189.3 c·mol·kg^{-1}, organic carbon content of 676.0 g·kg^{-1}, total N content of 3.91 g·kg^{-1}, total phosphorus (P) content of 0.933 g·kg^{-1}, and an ash content of 37.1%.

The soil samples were collected from two depths (0 - 10 and 10 - 20 cm) at the Sandhills Research Station in July 2019. Samples were immediately sealed in plastic bags, placed on ice in coolers, and transported to the laboratory. Field moist samples were used for the microbiological analyses (<2 mm), and subsamples were air-dried for EA measures.

2.2. Biochar Addition before EAs Assays

Fifty grams (dry weight) of soil subsamples were spiked with biochar in sterile 50 mL centrifuge tubes to yield final concentrations of 5% and 10% (dry weight basis) [40]. Six replicates were prepared for each biochar concentration, and the soil with no added biochar was the control for both the individual EAs and CNPS activity measures.

2.3. pNP Retention on Biochar Assay

To study the retention of pNP during the analysis of the different enzyme activities, the following spiking assay was performed: instead of adding the substrates at the beginning of the procedure, reaction product (pNP) was added (150 mg·L^{-1}) to buffer solution (pH = 5.5, 5.8, and 6.5, corresponding to the EAs assays, respectively). After the incubation, the retention of pNP by the biochar was evaluated. Controls were performed similarly by adding identical amounts of pNP after incubation and before measuring the absorbance in a calibrated spectrophotometer (with an external pNP standard solution).

2.4. Soil Enzymatic Activities

2.4.1. Individual Soil Enzyme Activities

Individual assays for the soil enzymes β-glucosidase (BG), β-glucosaminidase (NAG), acid phosphomonoesterase (also known as acid phosphatase, PME), and arylsulfatase (AS) were conducted according to [12] [41] [42] [43]. For each assay, 0.5 g of air-dried soil (sieved <2 mm) was incubated at 37˚C in 2 mL of appropriate buffer and 0.5 mL of the substrate at optimal pH for each enzyme for 1 h. The samples were assayed in duplicate with one control, to which substrate was added after the incubation. Following the incubation, 0.5 mL of 1.0 M $CaCl_2$ and 2 mL of stop solution (NaOH or THAM buffer depending on the enzyme) were added, and the soil suspension was filtered through a Whatman No. 2v filter. Activity values obtained from the control samples were subtracted from the sample value. The release of p-nitrophenol from the substrate analog (p-nitrophenol; pNP) was determined colorimetrically at 400 nm in a visible spectrophotometer (Thermo Scientific Evolution 60S).

2.4.2. CNPS Activity Assay

The CNPS activity assay used β-glucosidase, β-glucosaminidase, acid phosphomonoesterase, and arylsulfatase in the same reaction. To maintain a buffer: stop solution ratio comparable to the original EA assay methods, substrates were prepared in the same corresponding buffer (acetate buffer pH 5.8). For the CNPS activity assay, 0.5 g of air-dried soil was incubated with 0.5 mL of buffer (acetate buffer or MUB) and 2 mL of the solution with the four substrates (0.5 mL of each substrate prepared in the same buffer used in the assay) without toluene for 1 h at 37˚C. Each sample was assayed in duplicate with one control, to which all substrates were added after the incubation. Following incubation, 0.5 mL of 1 M $CaCl_2$ was added. The reaction was terminated using 2 mL of 0.1 M THAM pH 12 instead of NaOH because NaOH can react with β-glucosidase assay substrate, causing non-enzymatic degradation of pNP [41] [44], and can extract dissolved organic matter, which can contribute to absorbance at 400 - 415 nm. The final total volume (5 mL) of soil suspension was filtered through a Whatman No. 2v filter, and pNP released was determined calorimetrically at 400 nm. Activity values obtained from the control samples were subtracted from the sample value.

2.5. Statistical Analysis

Both the laboratory assay and the field test were performed using six replicates. The "theoretical EA" was used (*i.e.*, the sum of each EA assayed individually using the original protocol) as a reference value of the expected CNPS activity obtained (CNPS activity) when different substrates were incubated concurrently [13]. The difference between treatments (0%, 5%, or 10% biochar) and the difference between the "theoretical EA" and the CNPS activity was compared using paired t-tests, and the significant differences were verified at $p < 0.05$. All statistical analyses and graphics were performed in Infostat [45].

3. Results

3.1. PNP Retention by Biochar Assay

Figure 1 illustrates the proportion of change in p-nitrophenol (pNP) released from the two different biochar concentrations (5% and 10%) compared to the non-biochar treated control soil (0%), which holds the value of 1 on the y-axis. Values greater than 1 represent available pNP, and values less than 1 represent retained (biochar immobilized) pNP. The buffer with a pH of 6.5 showed a greater interaction between biochar and pNP substrate than pH 5.5 and 5.8 buffers. At 0 - 10 cm soil depth, the 5% biochar sample had 2% - 5% more available pNP than the control following incubation and filtration in the three buffers. The 10% biochar treatment retained 5% more pNP in the buffered samples than the control. While at 10 - 20 cm depth, the amount of available pNP decreased for both concentrations of biochar added. Nevertheless, the comparison of the pNP released between the control and the biochar samples was not significantly

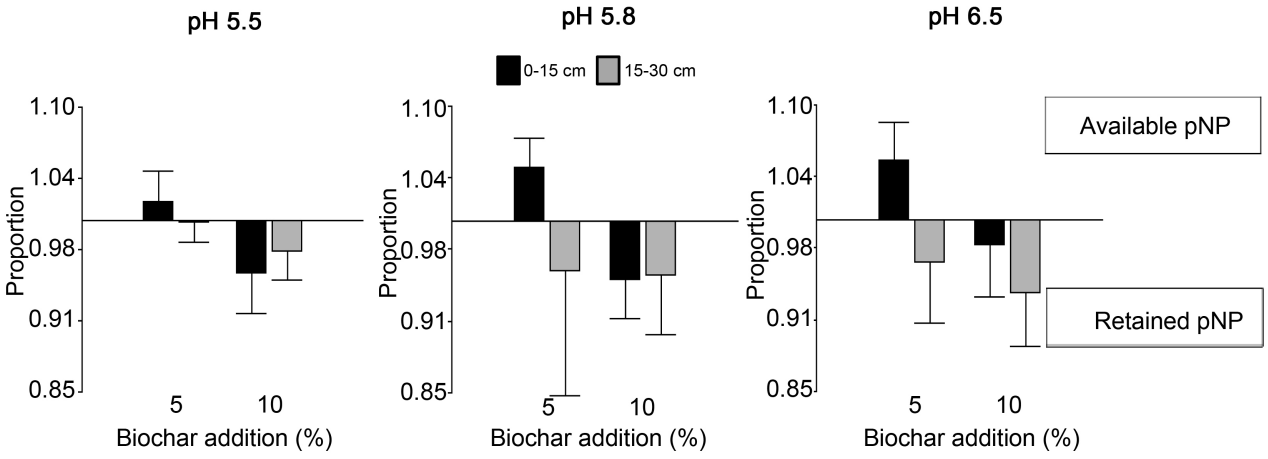

Figure 1. The proportion of change of p-nitrophenol (pNP) retention in the two different concentrations of biochar (5% and 10%) compared to the biochar-free control (0%). Black: soil samples from 0 - 10 cm; Grey: soil samples from 10 - 20 cm.

different among the three buffers ($p > 0.05$).

3.2. Comparison of Theoretical EAs and CNPS Activity Approaches Following *in Vitro* Additions of Biochar

When measuring the EAs using the theoretical EAs and CNPS activity methods following in vitro additions of biochar, comparisons of the 5% and 10% biochar samples and the control revealed a more significant decrease (45%) in the theoretical EAs in the biochar treated samples compared to the CNPS assay (white column), which resulted in a 30% decrease (Figure 2(a)). Evaluation of the theoretical EAs (the sum of BG, AP, NAG, and AS activities) and CNPS activity approaches using the samples without biochar revealed minimal differences (<3%) at both soil depths. While 5% biochar samples resulted in a 27% decrease in theoretical EAs compared to the CNPS activity in the 0 - 10 cm soils, the difference was insignificant ($p > 0.05$, Figure 2(a)).

Linear regression comparing the sum of individual EAs and the CNPS activity assay showed an R^2 of 0.56 and a *p-value* of 0.0001. The linear equation for the linear correlation is Sum = 0.791 × CNPS + 7.61. The sample without biochar (white circles) produced a higher sum of individual EA values (Figure 3(a)) than the samples with biochar (5%: grey circles; 10%: black circles).

3.3. Comparison between CNPS Activity Assay and Theoretical EAs with Biochar Amended in the Field

After two years, the 5% biochar field samples revealed a slight increase in measured activity using CNPS activity and theoretical EAs compared to the control treatment at 0 - 10 cm. Still, the increase was insignificant ($p > 0.05$, Figure 2(b)). The theoretical EAs (−5%) and CNPS activity assay (+27%, Figure 2(b)) in the 10% biochar samples trended differently. The theoretical EAs decreased by 15% compared to the CNPS activity assay, but the difference was insignificant ($p > 0.05$, Figure 2(b)). At 10 - 20 cm soil depth, the samples with 10% biochar

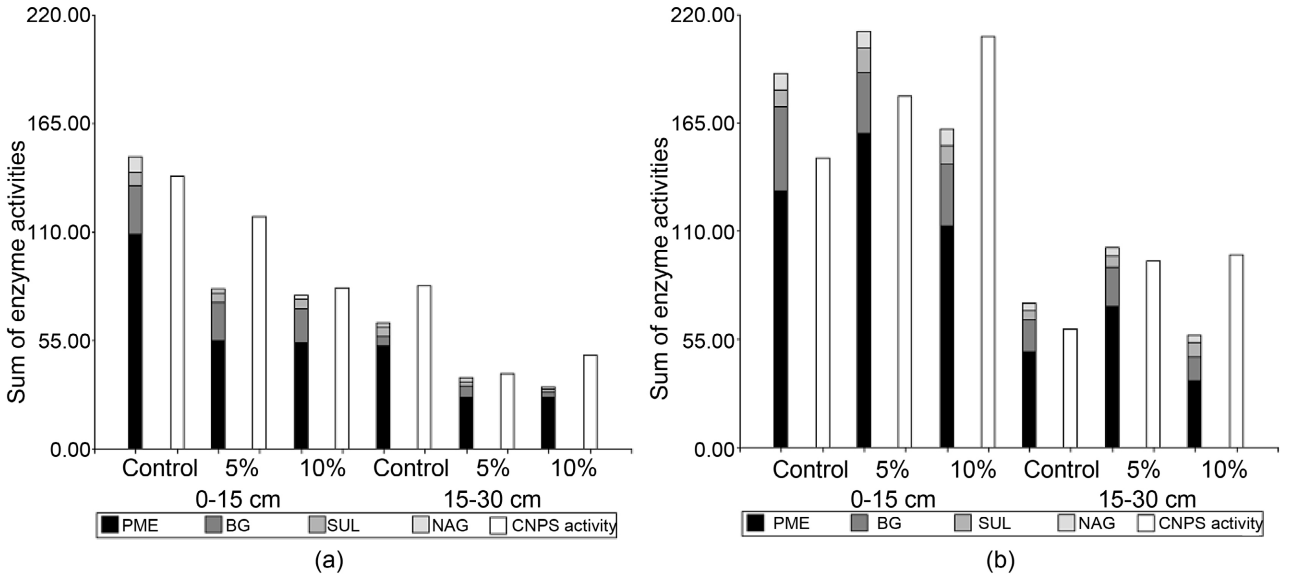

Figure 2. Comparison between the sum of individual enzyme activity (theoretical EAs) and CNPS activity for (a) samples with biochar added in vitro before EAs assay and (b) field applied biochar samples. Black: *β*-glucosidase (BG); dark grey: *β*-glucosaminidase (NAG); grey: acid phosphomonoesterase; light grey: arylsulfatase (AS); and white: CNPS activity.

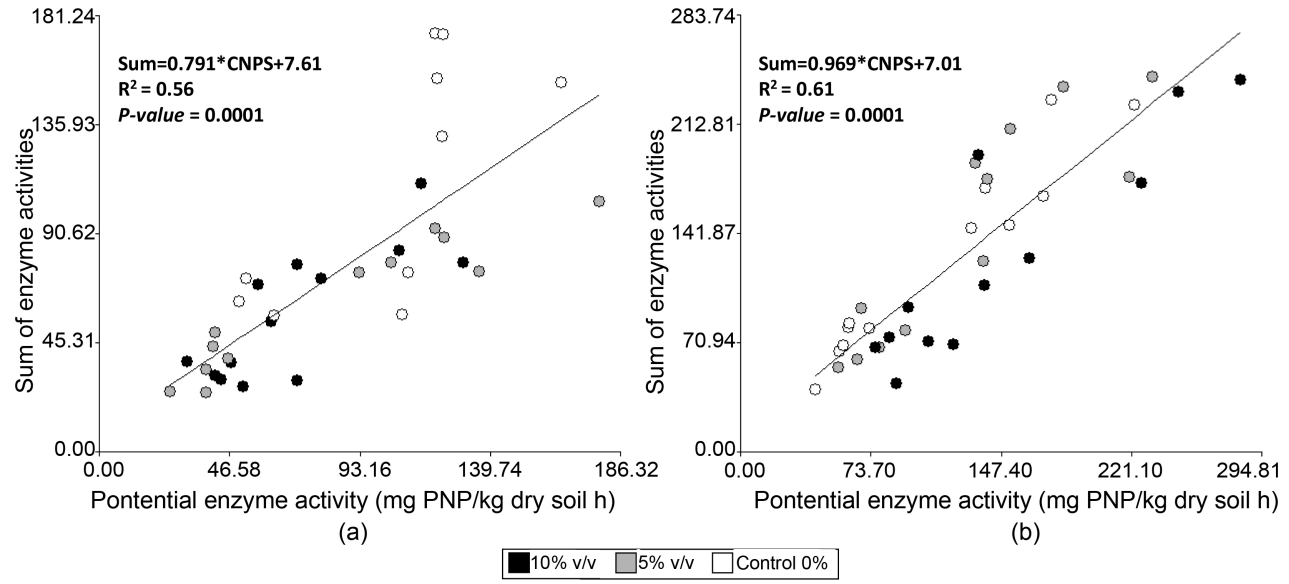

Figure 3. Linear regression between the theoretical EAs (the sum of BG, AP, NAG, and AS activities) and CNPS activity for (a) samples with biochar added in vitro before EAs assay and (b) field applied biochar samples. Both graphics contain Black: samples with 10% biochar, grey: samples with 5% biochar, and biochar free (0%) White: control samples.

revealed a significant decrease in the theoretical EAs compared to the CNPS activity assay ($p = 0.0016$, Figure 2(b)).

Linear regression of individual EAs and the CNPS activity assay resulted in an R^2 of 0.61 and a *p-value* of 0.0001. The linear equation for this linear regression is Sum = 0.969 × CNPS + 7.01. The sample with 10% added biochar (black circles) exhibited higher values from the CNPS activity assay, while the samples without biochar (control; white circles) yielded higher values of activity using

the theoretical EAs approach (**Figure 3(b)**).

4. Discussion

Recent studies have shown that EAs can be sensitive indicators of changes in biogeochemical nutrient cycling due to soil management practices. Several studies compared the functional differences in biochar-treated soil samples, and the reports varied, reflecting no biochar effects [46], positive biochar effects [23] [26], and adverse biochar effects [24] [47]. Additionally, the consequences of biochar varied with the enzymes evaluated. For example, Foster *et al.* [25] showed that β-glucosidase and Acid Phosphatase activities decreased by nearly 50% by adding biochar to soils, while β-glucosaminidase (C and N cycling) and β-cellobiohydrolase increased over 100%.

One of the objectives of this research was to determine if the effect of biochar on the CNPS activity approach was similar to the impact of biochar on the individual soil EA methods. Our results showed an average difference between theoretical EAs and CNPS activity of 5% (CV = 0.2). Both experiments illustrated similar variations within the different treatments; the first experiment displayed a CV of 0.17 and the second one a CV of 0.19. Previously, Acosta-Martinez *et al.* [15] demonstrated that CNPS activity behaves similarly to the theoretical EA measured separately, having a difference between measurements of 15%. The linear correlation based on theoretical EAs and CNPS activity showed a smaller value (0.791) for the slope of the fit line when the biochar was added before the EAs assay (**Figure 3(b)**).

During the initial assay, the different pHs used for the biochar's colorimetric determination of pNP retention did not significantly differ based on the amount of biochar added into the soil. However, the lowest biochar concentration produced a 2% - 5% increase in available pNP, while the 10% addition had the opposite effect in the three different buffers. These results agree with other studies that reported a similar impact when pine-wood biochar was incorporated at a concentration of 3%, but a decrease occurred with soils amended at 15% [31]. Trigo *et al.* [33] suggest that the pH around biochar particles would initially increase in soil solution due to the Lewis basicity law at lower concentrations of biochar, and, when higher concentrations of biochar are added, the high sorption capacity decrease due to the possible formation of acidic functional groups on biochar surfaces. However, pH did not significantly affect the retention of pNP, and the results are differentiated by [34], who compared two more contrasting pHs such as 5 and 11.

The biochar structurally has surface porosity with a high potential to sorb organic molecules, including enzymes and substrates, thus altering enzyme activities [32]. When the biochar was added before measuring EAs, the values of EAs decreased by approximately 50%. This result implies a more significant impact of biochar on enzyme activities. In another study, Frene *et al.* [48] reported that the soil EAs increased in soil samples after two years of biochar and showed that the biochar promotes the activity of EAs. The increase in EAs may be attributed

to higher SOC, increased co-location, and stabilization of the enzymes [36]. Moreover, due to biochar additions, higher microbial biomass also releases more enzymes than the other treatments [49].

The combined assay ("CNPS activity") differentiated among the different concentrations of biochar in the treated soils, similar to the sum of the individual EAs [15]. Therefore, this study agrees with previous studies that found the CNPS activity assay is sensitive and capable of differentiation between the amount and type of biochar applied compared to assaying the EAs individually, in addition to providing a uniform biogeochemical cycling index while reducing time and resources [15] [47].

5. Conclusion

Soil health assessments need simple, fast, and low cost-effectivity assays to provide a better overview of soil geochemical cycle and soil functions. CNPS assay has achieved these objectives since it simultaneously evaluates four different enzyme activities (β-glucosidase, β-glucosaminidase, acid phosphomonoesterase, and arylsulfatase), reducing time and resources. Biochar has shown several inconsistencies concerning its interaction with soil functions, mainly soil enzyme activities. Our study showed that CNPS assays are a robust method to evaluate soil biogeochemical cycling and soil health in the presence of biochar. Additionally, we observed that amended soils did not retain more pNP substrate than the un-amended control soils in three different pH buffers (5.5, 5.8, and 6.5) commonly used in EA reactions. At the same time, the comparisons of the theoretical EAs and the CNPS activity assay approaches in the biochar-modified soil revealed similar activity trends with different concentrations of added biochar. Furthermore, this work facilitates the future adoption of a uniform biogeochemical cycling index (CNPS activity) for producer-oriented soil management decisions that require informative testing.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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