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Simultaneous determination of β-glucosidase, β-glucosaminidase, acid phosphomonoesterase, and arylsulfatase activities in a soil sample for a biogeochemical cycling index☆

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ABSTRACT

Four enzymes activities (EAs) have been targeted as soil health indicators for their important role in reactions releasing bioavailable nutrients within C (β-glucosidase), N and C (β-glucosaminidase), P (acid phosphomonoesterase) and S (arylsulfatase) cycling. Traditionally these EAs are assayed independently on air-dried soil by measuring the release of *p*-nitrophenol from a substrate analog. Previously, we suggested a novel approach to assess multiple EAs simultaneously in the same soil sample by adding two or three substrates to obtain a comparable index. Our current study provides a combined assay for simultaneous determination of these four EAs in the same soil sample. For the incubation step of our combined assay, we tested modified universal buffer (MUB) because it is used for assaying β-glucosidase and acid phosphomonoesterase activities and tested acetate buffer as it is used for assaying β-glucosaminidase and arylsulfatase activities. Using the acetate buffer (pH 5.8) for the combined assay showed the lowest percent of difference (average of −14%) compared with the sum of individual EAs, and showed positive significant correlations ($p < 0.001$) with the sum of the individual EAs ($r = 0.97$) and with soil organic C ($r = 0.94$) and total nitrogen (TN) ($r = 0.93$). This combined acetate buffer assay (*"CNPS activity"*) differentiated among agroecosystems similarly to the sum of the individual EAs in Texas soils (cotton < grass < Conservation Reserve Program) and both approaches showed a tendency for higher activities in a diversified rotation compared to corn-soybean and a grass system for Minnesota soils. Use of the novel *CNPS activity* will provide a uniform biogeochemical cycling index while reducing time and resources compared to assaying the EAs individually.

1. Introduction

Enzyme activities (EAs) were identified as key soil biological indicators by soil health initiatives for being sensitive to changes related to biogeochemical cycling and soil organic matter (SOM) dynamics. Assays for determining a given enzyme activity are performed in a known amount of soil by adding the required substrate in excess to fully saturate all enzymes present under appropriate buffer and pH conditions and then measuring the product released. Most assays have been designed since the 1960s and provided the first data to compare EAs across soil types and management (Tabatabai, 1994; Dick and Burns, 2011). Previous studies using these assays have shown that EAs are affected by soil depth and the inherent soil physical and chemical properties across soil types, and can distinguish across management including crop rotation, fertilization, tillage and amendments applied to the same soil (Dick, 1984; Acosta-Martínez and Klose, 2008; Dick, 2011; Lehman et al., 2015). Other studies have shown that EAs can be sensitive indicators of the complex interactions of management and climatic variability (Sardans and Peñuelas, 2005; Acosta-Martínez et al., 2014a, 2014b).

From the approximately 15 EAs most commonly used as soil health indicators, the four enzymes β-glucosidase, β-glucosaminidase, acid (or alkaline) phosphomonoesterase and arylsulfatase were chosen for their important roles in releasing nutrients for plants and soil organisms within C, N and C, P and S cycling, respectively (Tabatabai, 1994). β-glucosidase activity provides information on cellulose degradation, which is the most common polysaccharide in nature. This EA has been widely evaluated and shown to reflect soil management effects

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(Bandick and Dick, 1999). Thus, it is the first EA included in a soil quality equation as an indicator of C-cycling by the Soil Management Assessment Framework (SMAF) (Stott et al., 2009). β-Glucosaminidase plays a major role in C and N cycling by hydrolyzing chitin and releasing amino-sugars, which are major sources of mineralized N in soils, and has been identified as an index of N mineralization in soils (Ekenler and Tabatabai, 2004). Phosphomonoesterases are involved in P cycling, which after N is another limiting nutrient in agricultural production worldwide. Phosphorous is needed for energy transfer and as a backbone for DNA. Lastly, arylsulfatase is involved in sulfur mineralization. This process provides inorganic S, which is often limiting for plants and microorganisms for enzyme synthesis and for some amino acids.

Current protocols are to measure each EA independently, which increases time and resource consumption equally for each assay performed. Thus, a novel approach was developed to assess multiple EAs simultaneously in the same soil sample to obtain a comparable index (Acosta-Martínez et al., 2018). For two substrates, we developed a combined assay to assess β-glucosidase and acid phosphomonoesterase (C and P cycling), β-glucosaminidase and arylsulfatase (C, N and S cycling) or β-glucosidase and β-glucosaminidase (C and N cycling). Another combined assay for evaluating β-glucosidase, acid phosphomonoesterase and β-glucosaminidase in the same soil sample was developed. However, arylsulfatase activity was not included in that combined assay. Therefore, the first objective of this study was to develop a combined assay that simultaneously determines the activities of β-glucosidase, β-glucosaminidase, acid phosphomonoesterase, and arylsulfatase that will be referred to as "*CNPS activity*". Soil samples with a wide range in soil organic C (SOC, 4–44 g C kg^{-1} soil) were used to provide an approach that can be used across regions. Our second objective was to test the sensitivity of the combined assay developed here by evaluating another set of soil samples that differed in their management history and comparing trends obtained from our combined assay with individual enzyme assays.

2. Materials and methods

2.1. Soil types, management description and selected chemical properties

To develop our combined assay of four EAs, we used the same soil samples (0–10 cm) as in our previous study (Acosta-Martínez et al., 2018). These soil samples were taken from Minnesota and Texas during summer 2016 and are diverse in inherent properties such as SOM, texture and pH (Table 1). For example, the lowest SOC was 3.68 g kg^{-1} in a soil from Texas and the highest was 43.6 g kg^{-1} in a soil from Minnesota. The soils from Texas have been under similar cotton (*Gossypium hirsutum*)-based cropping systems since the 1990's. The soils from Minnesota included different farm practices (e.g., perennial grasses, cover crops) as previously described by Johnson and Barbour (2016).

For evaluating the sensitivity of the combined assay to distinguish management practices, another set of soil samples from Texas and Minnesota (n = 18) were used. The soil samples from Texas were under continuous cotton, grass or Conservation Reserve Program (CRP) for over 10 years (e.g., CRP was established in 1991). Minnesota soil samples were under corn (*Zea mays* L)-soybean (*Glycine* max L.) and grass (big bluestem, *Andropogon gerardii*) for > 10 years, while the rotation of alfalfa (*Medicago sativa* L.) -corn-soybean-wheat (*Triticum aestivum*) had been in alfalfa for seven years, then had one complete four-year rotation prior to sampling. Soil parameters of the Texas soils were performed in a private laboratory (Ward Laboratories, Nebraska). Soil pH was measured in air-dried soil (< 5 mm) using a combination glass electrode (soil:water mixture, 1:2.5). Soil organic C (SOC) and total N (TN) were determined in air-dried soil samples by automated dry combustion using a Leco TruSpec® CN (Saint Joseph, MI). Soil texture was determined with the pipette method. For the Minnesota soils, similar methods were used, however, these analyses were conducted at the USDA-ARS unit in Morris, MN. The Minnesota soils are calcareous therefore the soil inorganic carbon was determined (Wagner et al., 1998), and the SOC calculated by subtracting the inorganic C from the total carbon content measured by combustion.

2.2. Individual enzyme assay determination

Individual assays for the soil enzymes β-glucosidase, β-glucosaminidase, acid phosphomonoesterase (also known as acid phosphatase), and arylsulfatase were performed by Acosta-Martínez et al. (2018) following well-known assays (Tabatabai, 1994; Parham and Deng, 2000; Dick, 2011). The volume of solutions and amount of soil were reduced by half while maintaining the soil:solution proportions of the original assays (Acosta-Martínez and Cotton, 2017) as given in Table 2. Briefly, for each assay 0.5 g of air-dried soil (sieved < 5 mm) was incubated at 37 °C in 2 mL of its appropriate buffer and 0.5 mL of substrate at optimal pH for each enzyme for 1 h. Assays were done without toluene as recommended by Acosta-Martínez and Tabatabai (2011) to reduce safety and environmental concerns associated with toluene. Each sample was 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. The product released (*p*-nitrophenol; PNP) was determined colorimetrically at 400 nm in a visible spectrophotometer (Thermo Scientific Evolution 60S). Activity values obtained from the control samples were subtracted from the sample value. The concentration of $CaCl_2$ was increased by Acosta-Martínez et al. (2018) from 0.5 M to 1.0 M to precipitate organic compounds extracted from the high SOM content in Minnesota soils.

2.3. Development of the combined assay for simultaneous determination of four enzyme activities

Developing a combined assay for simultaneous determination of four EAs required testing for the best incubation buffer. MUB was tested because it is used for the acid phosphatase (pH 6.5) and β-glucosidase (pH 6.0) assays, while acetate buffer is used for β-glucosaminidase (pH 5.5) and arylsulfatase (pH 5.8). To maintain a buffer:stop solution ratio comparable to that from the original EA assay, substrates were prepared in same corresponding buffer (i.e., MUB pH 6.0 or acetate buffer pH 5.8). As with the individual EAs, half of the solutions and amount of soil were used. For example, 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 1.0 M $CaCl_2$ was added (instead of 0.5 M) as done in Acosta-Martínez et al. (2018) to have a clear filtrate for the Minnesota soils, which should not interfere with the enzymatic mediated reaction (Margenot et al., 2018). Lastly, the reaction was terminated using 2 mL of 0.1 M THAM pH 12.0 instead of NaOH because NaOH can react with β-glucosidase assay substrate, cause non-enzymatic degradation of PNP (Hayano, 1973; Tabatabai, 1994), and can extract dissolved organic matter, which can contribute to absorbance at 400–415 nm (Klose et al., 2011). The total final volume (5 mL) of soil suspension was filtered through a Whatman No. 2v filter and PNP released was determined colorimetrically at 400 nm. Activity values obtained from the control samples were subtracted from the sample value.

The solutions for the combined assay require simple preparation as described in the enzymes chapter by Tabatabai (1994). In brief, the incubation buffer (0.5 M acetate buffer, pH 5.8) is prepared by dissolving 68 g of sodium acetate trihydrate in 700 mL of deionized (DI) water. The pH is adjusted to 5.8 by adding 99% glacial acetic acid (e.g., 1.70 mL) and then, bringing final volume to 1 L with DI water. The stop

Table 1
Selected properties as determined by Acosta-Martínez et al. (2018) for soil samples used to develop the combined assay.[a]

Soil ID	Soil series	Subgroup	pH	SOC	TN	β-glucosidase	β-glucosaminidase	Acid phosphatase	Arylsulfatase
				g kg^{-1} soil		mg PNP kg^{-1} soil h^{-1}			
Texas soils									
TX5	Amarillo	Thermic Aridic Paleustalfs	7.2	3.7	0.46	57	7	67	3
TX4	Amarillo	Thermic Aridic Paleustalfs	7.2	4.2	0.46	47	7	74	2
TX6	Amarillo	Thermic Aridic Paleustalfs	7.4	4.6	0.53	60	9	70	3
TX3	Olton	Thermic Aridic Paleustalfs	7.2	4.8	0.52	22	7	21	1
TX2	Olton	Thermic Aridic Paleustalfs	7.7	4.9	0.52	19	5	31	1
TX1	Olton	Thermic Aridic Paleustalfs	7.7	5.0	0.53	23	5	39	1
TX8	Amarillo	Thermic Aridic Paleustalfs	7.0	5.7	0.65	26	4	75	1
TX9	Amarillo	Thermic Aridic Paleustalfs	7.1	5.8	0.65	34	6	82	1
TX7	Amarillo	Thermic Aridic Paleustalfs	7.1	6.4	0.62	32	7	84	1
TX14	Portales	Thermic Aridic Caliustolls	7.8	10.8	1.11	117	8	69	9
TX15	Portales	Thermic Aridic Caliustolls	7.6	11.3	1.22	90	8	82	7
TX13	Portales	Thermic Aridic Caliustolls	7.9	12.4	1.29	95	8	67	10
TX12	Spur	Thermic Fluventic Haplustolls	7.4	16.4	1.81	150	14	108	19
TX10	Spur	Thermic Fluventic Haplustolls	7.4	17.6	1.72	176	14	105	17
TX11	Spur	Thermic Fluventic Haplustolls	7.7	20.5	2.22	157	16	115	18
Minnesota soils									
MN1	Barnes	Frigid Calcic Hapludolls	7.2	28.3	2.56	257	22	207	167
MN3	Hamerly/Barnes	Frigid Aeric Calciaquolls /Calcic Hapludolls	7.5	32.4	2.75	125	23	214	189
MN9	Barnes	Frigid Calcic Hapludolls	6.9	32.5	2.47	126	46	320	211
MN4	Hamerly	Frigid Aeric Calciaquolls	7.5	33.8	2.97	137	16	156	157
MN6	Barnes	Frigid Calcic Hapludolls	7.5	37.8	3.33	139	19	173	238
MN12	Aastad	Frigid Pachic Argiudolls	7.6	38.7	3.16	152	20	182	222
MN8	Hamerly	Frigid Aeric Calciaquolls	7.4	40.4	3.34	117	35	231	197
MN11	Hamerly	Frigid Aeric Calciaquolls	7.5	40.4	3.34	120	24	177	328
MN10	Vallers	Frigid Typic Calciaquolls	7.7	41.4	3.54	139	20	176	275
MN7	Vallers	Frigid Typic Calciaquolls	7.5	41.8	3.44	119	34	206	210
MN2	Hamerly	Frigid Aeric Calciaquolls	7.0	41.8	3.41	143	22	193	237
MN5	Vallers	Frigid Typic Calciaquolls	7.6	43.6	3.74	120	19	183	203

[a] Included with permission of Applied Soil Ecology for reference of the soil characteristics.

Table 2
Steps and reagents used for determining EAs using original (individual) assays and the combined assay of four EAs simultaneously.

Enzyme ecological role and EC number	Incubation step		After incubation step	
	Buffer	Substrate[a]	$CaCl_2$[b]	Stop solution
Individual assay				
β-glucosidase (C cycling), 3.2.1	MUB pH 6.0; 2 mL	*p*-Nitrophenyl-β-D-glucopyranoside (0.05 M); 0.5 mL; CAS (2492-87-7)	0.5 M; 0.5 mL	0.1 M THAM pH 12; 2 mL
β-glucosaminidase (C and N cycling), 3.2.1.30	0.1 M acetate pH 5.5; 2 mL	*p*-Nitrophenyl-*N*-acetyl-β-D-glucosaminide (0.01 M); 0.5 mL; CAS (3459-18-5)	0.5 M; 0.5 mL	0.5 M NaOH; 2 mL
Acid phosphatase (P cycling), 3.1.3.2	MUB pH 6.5; 2 mL	*p*-Nitrophenyl phosphate (0.05 M); 0.5 mL; CAS (333338–18-4)	0.5 M; 0.5 mL	0.5 M NaOH; 2 mL
Arylsulfatase (S cycling), 3.1.6.1	0.5 M acetate pH 5.8; 2 mL	*p*-Nitrophenyl sulfate (0.05 M); 0.5 mL; CAS (6217-68-1)	0.5 M; 0.5 mL	0.5 M NaOH; 2 mL
Combined assay				
CNPS activity	0.5 M acetate pH 5.8; 0.5 mL	2 mL containing 0.5 mL each substrate prepared in acetate buffer with concentration of individual assay	1.0 M; 0.5 mL	THAM pH 12; 2 mL

[a] Substrates for individual assays were prepared using the corresponding incubation buffer (Tabatabai, 1994).
[b] Original (individual) assays concentration is 0.5 M, however, certain soils with high soil organic matter could require higher concentration (e. g., 1.0 M) without affecting the reaction (Margenot et al., 2018).

buffer [0.1 M Tris(hydroxymethyl)aminomethane (THAM) pH 12] is prepared by dissolving 12.2 g of THAM in about 800 mL of DI water, adjusting the pH to 12 using 0.5 M NaOH and then completing the volume to 1 L with DI water. The substrates for the combined assay developed here are prepared in 0.5 M acetate buffer (pH 5.8) in their appropriate concentration as described in Table 2.

2.4. Calibration curve for p-nitrophenol (PNP)

The calibration curve was prepared as indicated for original assays by Tabatabai (1994) using 0, 10, 20, 30, 40 and 50 µg of PNP, from which the content of the PNP of the filtrate was calculated. Briefly, a PNP standard solution was prepared by mixing 1 g of PNP in 1 L of water, and 1 mL of this PNP standard solution was diluted in 100 mL of water. Then, 0, 1, 2, 3, 4, and 5 mL of the diluted PNP standard solution was pipetted into volumetric flasks as used for an assay, and the volume was adjusted to 5 mL by adding water (i.e., 5-, 4-, 3-, 2-, 1-, and 0-mL, respectively). Then, 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.1 M THAM were added as done in an assay to terminate a reaction. The suspension was filtered and measured with a spectrophotometer as with a typical sample. The calibration curve was generated by plotting PNP concentration vs A400. This calibration curve uses solution amounts as the original assays from Tabatabai (1994). However, it is also applicable when the soil and solutions are reduced by half as done in the

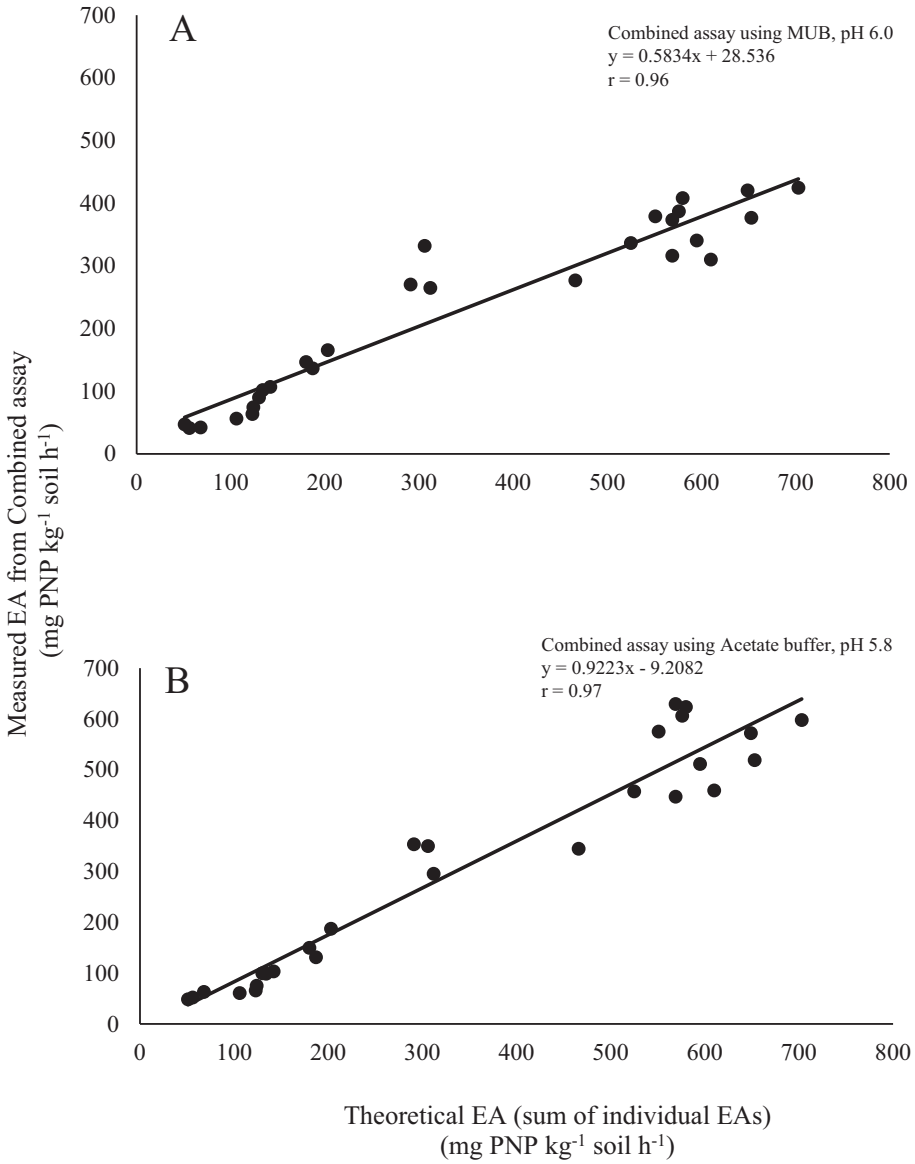

Fig. 1. Linear regression correlation between theoretical sum of the four EAs vs. *measured EAs* using MUB (A) and acetate buffer (B) for the combined assay.

individual and combined assays by Acosta-Martínez et al. (2018) because the concentrations/proportions are maintained.

2.5. Calculations, statistics and validation of combined assay

As done in our previous work for other options of combined assays, we used "*theoretical EA*" (i.e., the sum of each EA assayed individually using the original protocol) as a reference value of the expected combined EAs obtained when different substrates were incubated together simultaneously (Acosta-Martínez et al., 2018). The total EA obtained in the combined assay was referred to as "*measured EA*". For the first set of samples used to develop our combined assay (n = 27), the "*theoretical EA*" and "*measured EA*" were compared according to: 1) Pearson's r coefficient and level of significance obtained from linear regressions; and 2) Percent of difference between these two values (e.g., difference between the "*theoretical EA*" and the "*measured EA*" divided by the "*theoretical EA*" as the expected total EA, then multiplied by 100%). Additionally, correlations between SOC and TN content of these soil samples and the *measured EA* obtained with the combined assay were evaluated. The second set of soil samples (n = 18), also taken from Minnesota and Texas at the same depth (0–10 cm), were used for validation of our combined assay method because they had contrasting management practices (e.g., cropland vs grasses). The mean of the different management systems obtained for the "*theoretical EA*" and "*measured EA*" were compared according to the percent of difference between these values. Additionally, paired t-test was used to compare the *theoretical EA* and *measured EA* values for both sample sets.

3. Results

3.1. Evaluating total enzyme activity from a combined assay using MUB pH 6.0 vs acetate buffer pH 5.8 for the incubation step

The *measured EAs* obtained with the combined assay using MUB pH 6.0 showed a strong positive correlation (r = 0.96, $p < 0.001$) with the *theoretical EAs* (Fig. 1a). The average percent difference between these two values was −30% (Table 3). However, higher percentage differences were observed in Minnesota soils (average of −38%) and for samples 7, 8 and 9 compared to the Texas soils (−40 to −48%). The *measured EAs* showed a significant correlation ($p < 0.001$) with SOC (r = 0.91) (Fig. 2a) and TN (r = 0.92) (Fig. 2c).

The *measured EAs* from the combined assay using acetate buffer

Table 3
Determination of four EAs (β-glucosidase, β-glucosaminidase, acid phosphomonoesterase, and arylsulfatase) testing for MUB and acetate buffer for the incubation step of the combined assay.

Sample	Theoretical sum of EAs[a]	Combined assay (MUB, pH 6.0)	Difference (%)[b]	Combined assay (Acetate, pH 5.8)	Difference (%)[b]
Texas soils					
TX5	134	102	−24	99	−26
TX4	130	90	−31	100	−23
TX6	142	107	−25	103	−27
TX3	51	47	−8	48	−5
TX2	56	41	−26	52	−7
TX1	68	42	−38	63	−7
TX8	106	56	−47	61	−43
TX9	123	64	−48	66	−46
TX7	124	74	−40	75	−40
TX14	203	166	−18	187	−8
TX15	187	137	−27	131	−30
TX13	180	147	−19	150	−17
TX12	291	270	−7	353	21
TX10	312	265	−15	295	−5
TX11	306	332	8	350	14
Minnesota soils					
MN1	653	377	−42	519	−21
MN3	551	379	−31	575	4
MN9	703	425	−40	598	−15
MN4	466	277	−41	345	−26
MN6	569	316	−44	447	−21
MN12	576	387	−33	606	5
MN8	580	408	−30	623	7
MN11	649	420	−35	572	−12
MN10	610	310	−49	459	−25
MN7	569	373	−34	629	11
MN2	595	340	−43	511	−14
MN5	525	336	−36	457	−13
Median			−33		−14
Average			−30		−14

[a] Theoretical sum of individual EAs was determined by Acosta-Martínez et al. (2018).
[b] The percent of difference is the theoretical EA from the sum of individual EAs minus the measured EAs from the combined assay, divided by the theoretical EAs, and multiplied by 100%.

(pH 5.8) showed a significant correlation ($r = 0.97$, $p < 0.001$) with the *theoretical EAs* (Fig. 1b). The percent difference between the *theoretical EAs* and *measured EAs* averaged −14% (Table 3). The *measured EAs* showed significant correlations ($p < 0.001$) with SOC ($r = 0.94$) (Fig. 2b) and TN ($r = 0.93$) (Fig. 2d).

Overall, the *measured EAs* from the combined assay were in average 26% higher when using acetate buffer than with MUB and thus, closer to the *theoretical EAs* (sum of individual EAs ranged from 51 to 703 mg PNP kg^{-1} soil h^{-1}). For example, the combined activity obtained with acetate buffer ranged from 48 to 629 mg PNP kg^{-1} soil h^{-1} while this range was 41 to 425 mg PNP kg^{-1} soil h^{-1} using MUB (Table 3).

3.2. Validation of combined assay by comparing trends obtained across different management

The second set of soil samples ($n = 18$), known to differ in their management history, used for validating the combined assay using acetate buffer (pH 5.8) showed an average percent of difference of −17% between the *measured EA* and *theoretical EA* (Table 4). The *measured EA* and *theoretical EA* were strongly correlated ($r = 0.99$, $p < 0.001$). Paired *t*-test comparison of the means per system showed no statistically difference between *theoretical EA* (sum of individual EAs) and *measured EAs* (CNPS activity from the combined assay) (Fig. 3). Additionally, the combined assay was able to show expected trends associated to different management practices that agreed with those obtained from the sum of individual EAs. For Texas soils, lower CNPS activity was detected under cotton (monoculture) while the highest CNPS activity was found under CRP (Fig. 3b). Neither approach (sum of individual EAs or *CNPS activity*) distinguished among corn-soybean, grass and a rotation for the Minnesota soils (Fig. 3a).

4. Discussion

4.1. Description of the combined assay conditions for determining CNPS activity

Our previous efforts provided different types of combined assays that utilize the same starting buffer and stop solutions under similar assay conditions (Acosta-Martínez et al., 2018). For instance, β-glucosaminidase and arylsulfatase activities were suggested to be assayed together because both require acetate buffer for the incubation step and NaOH is used to stop the reaction. The combined EAs showed the highest correlations (almost 1:1) with the sum of the activities of these two EAs performed individually. However, selecting the most appropriate conditions for the combined assay of the four enzymes evaluated here, required testing the buffer for the incubation step because the original assays require either MUB for acid phosphatase (pH 6.5) and β-glucosidase (pH 6.0) or acetate buffer for β-glucosaminidase (pH 5.5) and arylsulfatase (pH 5.8). Selecting the optimum pH was not a concern as previous research documented that the maximum release of *p*-nitrophenol occurs at or near pH 6.0 for the four enzymes evaluated here (Eivazi and Tabatabai, 1977; Eivazi and Tabatabai, 1988; Tabatabai, 1994). Our experiments demonstrated that although the combined assays with either MUB or acetate buffer showed strong positive correlations with the sum of the individual EAs, the percent of difference obtained when using acetate buffer was much lower than with MUB. This agreed with previous studies showing that the amount of *p*-

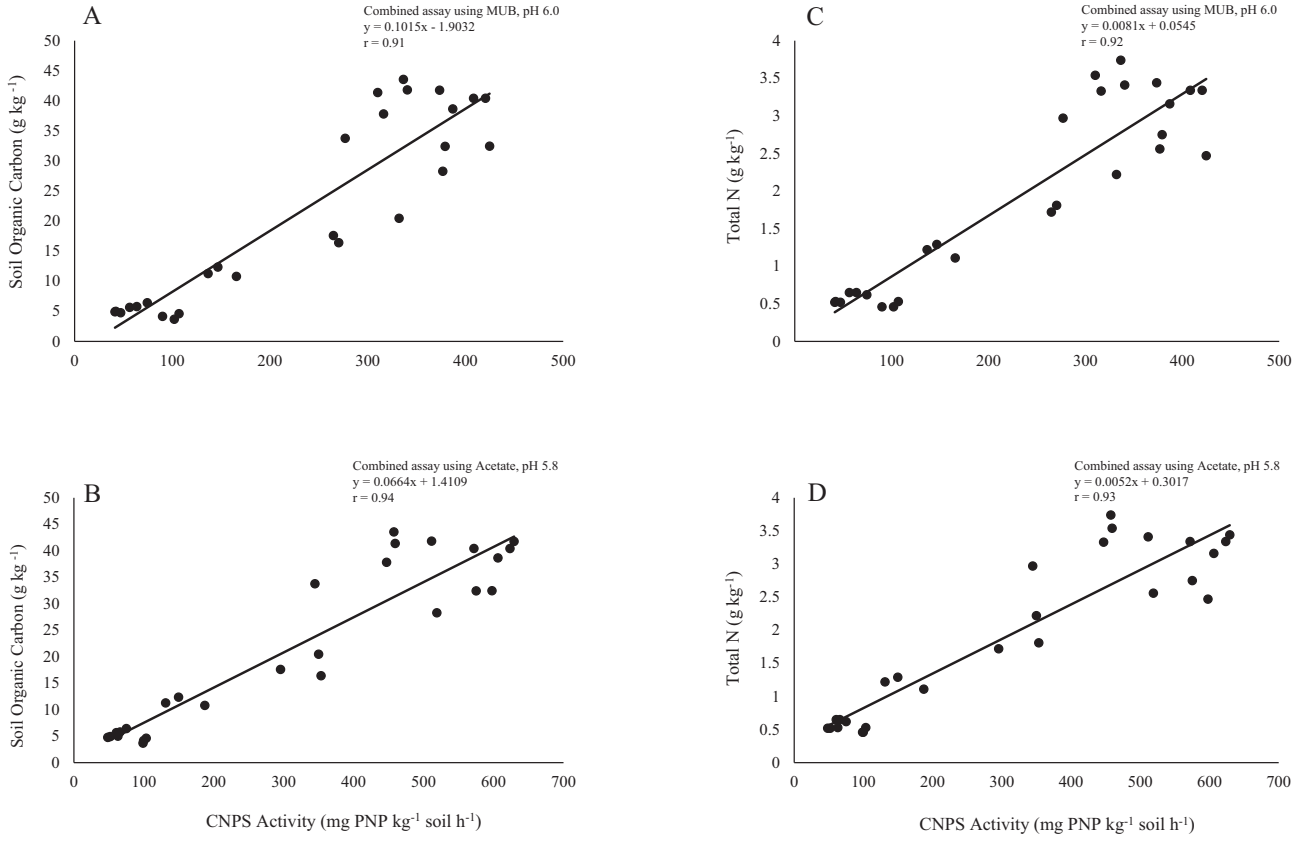

Fig. 2. Linear regression correlation between the measured combined EAs and SOC using MUB (A) or acetate buffer (B), and between EAs and total N (TN) using MUB (C) or acetate buffer (D).

nitrophenol (PNP) released by *p*-nitrophenyl sulfate was higher in acetate than in other buffers (Tabatabai and Bremner, 1970; Klose et al., 2011). The combined assay developed here was possible because these four EAs have common assay conditions when determined individually. Thus, we do not envision additional enzymes to be included in this combined assay as they could require optimum pH > 6.0 (e.g., phosphodiesterase or alkaline phosphatase requires pH 8.0 or 11, respectively).

Generally, when soil's pH is above 7, it is recommended to determine alkaline phosphatase activity rather than acid phosphatase activity (Eivazi and Tabatabai, 1977; Tabatabai, 1994). However, within our study even though the soil pH ranged from 6.9 to 8.4 in the two sets of soil samples used (total of 45 samples), we did not find greater percent of difference between *theoretical* and *measured EAs* using acid phosphatase activity in those soil samples with pH > 7. Nonetheless, since we did not test the protocol on soils with a pH above 8.4, we do not recommend the use of the combined assay for alkaline soils.

The sensitivity of the combined assay relative to individual assays was determined by including very sandy (60%) and very low SOM (< 1%) soils, which originated from Texas. These soils showed much lower arylsulfatase activity (1–19 mg PNP kg^{-1} soil h^{-1}) and β-glucosaminidase activity (4–16 mg PNP kg^{-1} soil h^{-1}) compared to soils from Minnesota used here or other soil types evaluated previously (Johnson et al., 2013; Mbuthia et al., 2015; Bhandari et al., 2018). However, the sandy and low SOM soils from Texas had percent of difference between the *measured EA* and *theoretical EA* that were comparable to the Minnesota soils.

In summary, our combined assay for *CNPS activity* requires incubation of 0.5 g of air-dried soil sample (in duplicates) with 0.5 mL of 0.5 M acetate buffer (pH 5.8) and a 2 mL solution with the four substrates (0.5 mL of each substrate) prepared in the same acetate buffer (Table 2 can be used as a reference). It is possible to prepare a solution that combines the 0.5 mL of acetate buffer with the 2.0 mL substrate solution (per sample) to reduce the pipetting time required to prepare samples for the incubation step. This can be done only to the sample duplicates because controls must be incubated with 0.5 mL of acetate buffer alone, and the 2.0 mL substrate solution is added separately after incubation. Following incubation, the *CNPS activity* is stopped with 0.5 mL of 0.5 M $CaCl_2$ and 2.0 mL of 0.1 M THAM (pH 12.0). The concentration of $CaCl_2$ can be adjusted if needed for soils with high SOM (Margenot et al., 2018) as was done here for the Minnesota soils. The PNP released during the incubation is determined colorimetrically at 400 nm.

4.2. Combined assay for four enzyme activities will provide a soil biogeochemical–health index

Our novel combined assay for *CNPS activity* provides a simple approach to determine simultaneously the four EAs suggested as key soil health indicators for nationwide soil health assessments. This combined assay will allow describing the ability of a soil to perform enzymatic catalyzed reactions involved in the mineralization of important nutrients required by other organisms or plants from cellulose degradation (β-glucosidase), chitin degradation (β-glucosaminidase), P transformations of organic and inorganic P-moieties (acid phosphatase) and S mineralization (arylsulfatase). Assaying *CNPS cycling* with our combined assay strongly correlated with *theoretical EA* (sum of individually determined EAs). Additionally, the *CNPS activity* was strongly correlated with SOC and TN as observed in individual EAs. Thus, the *CNPS activity* can facilitate comparisons among diverse soil types with different characteristics. Since this method has been developed from the original EA individual assays and have the same proportions of the assays, the original methods should be cited (e.g., Tabatabai, 1994; Parham and Deng, 2000) along with our citation. Compared to

Table 4
Validation of combined assay to use CNPS activity as a biogeochemical cycling index from soil samples with different management history.

Management[a]	Texture	pH	SOC	TN	Individual enzyme activities				Theoretical EA	Measured EA	Difference[b]
					β-glucosidase	β-glucosaminidase	Acid phosphatase	Arylsulfatase	(sum individual EAs)	(Combined assay)	
			g kg^{-1}		mg PNP kg^{-1} soil h^{-1}						(%)
Minnesota soils											
Corn-Soybean	Clay loam	8.2	36.9	3.1	80	16	145	183	424	372	−12
Corn-Soybean	Clay loam	8.3	47.8	3.8	120	12	167	214	512	429	−16
Corn-Soybean	Clay loam	8.3	37.4	3.1	87	21	171	209	488	381	−22
Grass	Clay loam	7.7	43.7	3.2	70	23	152	200	446	331	−26
Grass	Clay loam	7.9	46.3	3.1	93	25	155	230	504	409	−19
Grass	Clay loam	8.0	38.4	3.1	102	27	167	241	537	438	−18
Rotation	Clay loam	8.1	30.3	2.5	127	24	163	175	490	445	−9
Rotation	Clay loam	8.4	44.3	3.4	116	19	188	210	532	415	−22
Rotation	Clay loam	8.1	46.1	3.5	163	24	190	276	654	578	−12
Texas soils											
Cotton monoculture	Sandy clay loam	7.7	6.0	0.6	79	17	63	11	171	143	−16
Cotton monoculture	Sandy clay loam	7.6	5.5	0.6	78	16	65	13	172	152	−12
Cotton monoculture	Sandy clay loam	8.0	5.6	0.6	83	10	60	8	162	130	−20
Grass	Sandy clay loam	7.6	8.4	0.9	127	15	70	19	231	195	−16
Grass	Sandy clay loam	7.8	8.4	0.9	124	16	70	19	229	210	−8
Grass	Sandy clay loam	7.8	8.2	0.9	128	14	76	18	236	190	−19
CRP	Loam	6.9	17.9	1.6	180	46	376	64	666	587	−12
CRP	Loam	6.9	21.6	1.9	201	53	476	75	805	664	−18
CRP	Loam	6.9	18.5	1.6	194	63	370	79	706	551	−22
Average											−17

[a] Grass for TX is lovegrass (*Eragrostis*) and for MN is big bluestem (*Andropogon gerardii*); Rotation = Alfalfa-corn-soybean-wheat; CRP = Conservation reserve program. Typical practice in MN is corn-soybean and for TX is cotton monoculture.
[b] The percent of difference is the theoretical EA from the sum of individual EAs minus the measured EAs from the combined assay, divided by the theoretical EAs, and multiplied by 100%.

determining a biogeochemical cycling index that corresponds to SOM dynamics using the EAs individually, the *CNPS activity* generates a direct index without complex calculations, while reducing time, resources and chemical wastes.

A disadvantage of combined assays is their inability to distinguish the contribution of each EA and its corresponding reaction and nutrient cycling (Acosta-Martínez et al., 2018). However, a combined assay for four EAs that represent C, N, P and S cycling could facilitate its adoption in commercial laboratories to help producers consider the health of their soils in their management decisions based on a direct index of biogeochemical cycling. Addition of EAs to commercial laboratories will add a sensitive indicator of early changes in SOM dynamics and biogeochemical cycling to strengthen the assessments performed to soil samples (e.g., SOM, SOC, nutrient contents, pH, CEC). Additionally, the scientific community could benefit from having a simple approach that simultaneously determines several EAs representing a biogeochemical cycling index, allowing the use of remaining resources and budget for other soil health indicators. This will provide a better picture of soil health and functions, and it will be especially helpful, where thousands of samples are analyzed for new efforts to provide national soil health assessments.

4.3. CNPS combined assay showed significant distinctions among management practices

Enzyme activities have shown to be sensitive indicators of soil management practices effects on biogeochemical cycling and overall SOM dynamics (structure and distribution). However, the need to evaluate more than one EA to represent different processes always leads to the question on best calculations/approaches to combine their trends or obtain an index of management effects. The *CNPS activity* index through a combined assay developed here showed the same distinctions and trends of EAs determined with individual assays for comparison among monocultures, grasses, crop rotations and CRP systems (CRP > rotation > grass > continuous cotton). These activities were also strongly correlated with SOC and TN among the sets of soils evaluated. Additionally, *theoretical EA* and *measured EA* (from this combined assay) were not significantly different.

For the Texas soils, the lowest *CNPS activity* under continuous cotton is in agreement with the low residues produced under cotton compared to other crops (Lal, 2004). Additionally, cotton monoculture has shown the lowest levels of SOM and EAs when compared to systems under conservative management such as rotations or pastures (Acosta-Martínez et al., 2003, 2010, 2011; Dou et al., 2016). On the other hand, CRP, which has been under this practice for 27 years, showed the greatest biogeochemical cycling index than soils under grasses or monocultures as found in a previous study (Acosta-Martínez et al., 2003). The higher biogeochemical cycling index obtained under the CRP could be attributed to the elimination of tillage, reduction of soil erosion, greater root turnover rates and accumulation of surface litter, which have shown to significantly increase microbial biomass C and C sequestration (Gebhart et al., 1994; Sánchez-de León and Johnson-Maynard, 2013; Li et al., 2017).

The *CNPS activity* did not distinguish among the three management systems from Minnesota soils as it was found for the sum of the individual EAs. However, there was a trend with both approaches for higher EAs in the rotation compared to corn-soybean and grass. Although not significantly higher, this trend reflects how this rotation is

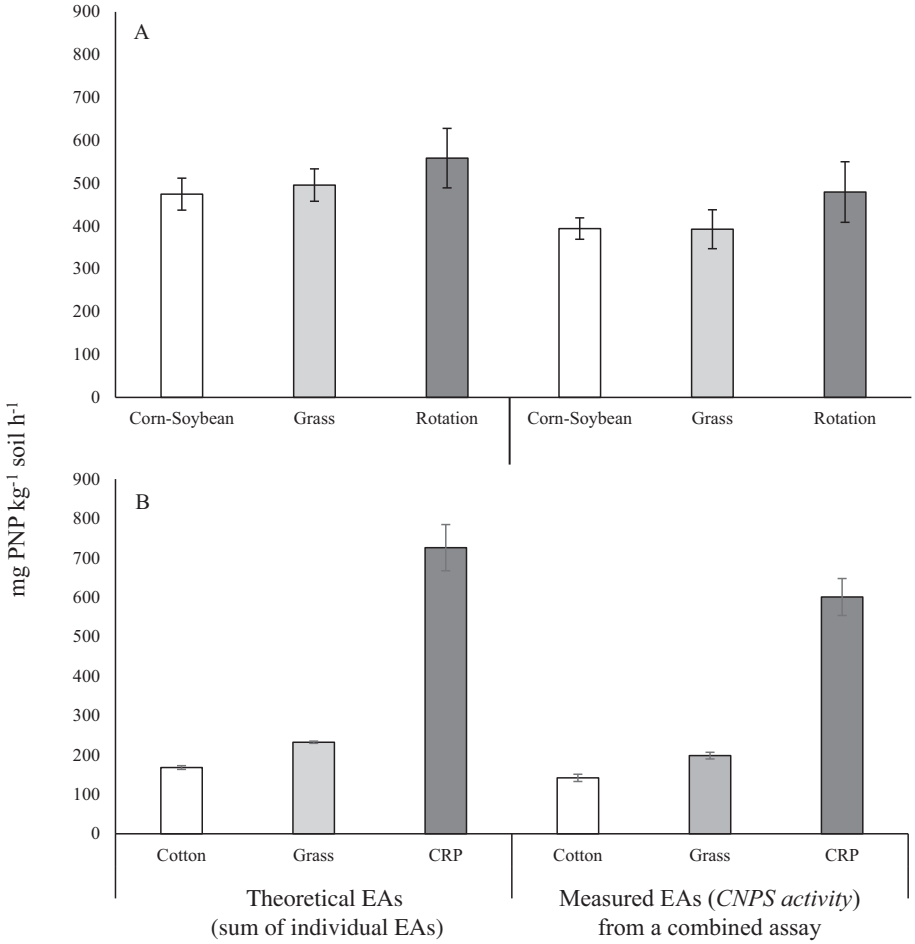

Fig. 3. Evaluation of different systems in Minnesota soils (A) and Texas soils (B). Trends obtained with the *measured EAs* with the combined assay (right side) are compared with trends from the sum of individual EAs (left side). Bars represents mean per system with standard error (SE).

more diverse than the other systems as it has included wheat, alfalfa, corn and soybean. Previous studies have suggested that the large pool of SOM in these northern Mollisols affords considerable resilience to management perturbations making it challenging to detect edaphic changes (Johnson et al., 2013; Johnson and Barbour, 2016). The SOC ranged from 36.9 to 47.8 g kg^{-1} soil among these soils, and did not vary among the different management practices (avg. 40.7, 42.8, and 40.2 for corn-soybean, grass and rotation, respectively).

5. Conclusions

Information on EAs for soil health assessments across a range of agroecosystems will require evaluation of hundreds of samples to provide a better overview of biogeochemical cycling. This could be expensive and time consuming with the individual evaluation of the activities of β-glucosidase, β-glucosaminidase, acid phosphatase and arylsulfatase as important indicators of C, N and C, P and S cycling, respectively. Here we provide a combined assay to evaluate the four EAs simultaneously. This alternative approach will reduce the cost, time, labor, and consumables required and thus, increase the efficiency of achieving this goal. The main limitation to the *CNPS activity* is that it is not possible to distinguish the contribution of each individual EA. However, its sensitivity to reflect changes in diverse soil types as demonstrated in this study, makes it ideal for comparisons across soil types and managements for commercial labs and/or large sets of soil samples taken for soil health assessments.

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