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# Soil protein: A key indicator of soil health and nitrogen management

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**Abstract**

Monitoring soil nitrogen (N) dynamics in agroecosystems is foundational to soil health management and is critical for maximizing crop productivity in contrasting management systems. The newly established soil health indicator, autoclaved-citrate extractable (ACE) protein, measures an organically bound pool of N. However, the relationship between ACE protein and other N-related soil health indicators is poorly understood. In this study, ACE protein is investigated in relation to other soil N measures at four timepoints across a single growing season along a 33-year-old replicated eight-system management intensity gradient located in southwest Michigan, USA. On average, polyculture perennial systems that promote soil health had two to four times higher (2–12 g $kg^{-1}$ higher) ACE protein concentrations compared to annual cropping and monoculture perennial systems. In addition, ACE protein fluctuated less than total soil N, $NH_4^+$-N, and $NO_3^-$-N across the growing season, which shows the potential for ACE protein to serve as a reliable indicator of soil health and soil organic N status. Furthermore, ACE protein was positively correlated with total soil N and $NH_4^+$-N and negatively correlated with $NO_3^-$-N at individual sampling timepoints across the management intensity gradient. In addition, ACE protein, measured toward the end of the growing season, showed a consistent and positive trend with yield across different systems. This study highlights the potential for ACE protein as an indicator of sustainable management practices, SOM cycling, and soil health and calls for more studies investigating its relationship with crop productivity.

**Abbreviations:** ACE, autoclaved-citrate extractable; KBS, Kellogg Biological Station; LAP, leucine-aminopeptidase; LTER, Long-Term Ecological Research; MCSE, Main Cropping System Experiment; SOM, soil organic matter; TN, total nitrogen; UAN, urea ammonium nitrate.

## 1 | INTRODUCTION

Nutrient management in agriculture is important for long-term sustainability and resilience of cropping systems in the face of global climate change. While soil organic carbon (C) has been the primary focus of many soil health studies to improve agroecological sustainability, recent studies have shown the importance of considering soil nitrogen (N) within the soil health framework, especially in relation to crop productivity (Wade et al., 2020). In row crop agriculture, N fertilizer applications optimize crop yields; however, less than half of synthetic N fertilizer applications are recovered in crops (Lassaletta et al., 2014; Robertson & Vitousek, 2009). As a result, excess nutrients are lost to the environment, making it increasingly important to assess how regenerative agricultural practices can both enhance crop productivity while retaining N (Bowles et al., 2022; Wade et al., 2020). Establishing reliable N-related soil health metrics is critical to furthering our understanding of soil N dynamics in order to improve the sustainability of management practices and promote optimal crop yield.

Soil N cycling is driven by abiotic and biotic processes that influence the balance between organic N and inorganic plant-available soil N (Robertson & Groffman, 2015). Synthetic N fertilizers provide inorganic forms of N, including ammonium ($NH_4^+$) and nitrate ($NO_3^-$), that are immediately available to plants (Hurisso & Culman, 2021; Robertson & Groffman, 2015). Organic N constitutes over 90% of total soil N (TN) (Kelley & Stevenson, 1995) and comes from plant biomass, plant root exudates, organic fertilizer additions, or via soil microbial immobilization of inorganic soil N (Robertson & Groffman, 2015). The soil microbiome also drives the reverse transformation of organic N into $NH_4^+$ through mineralization and the nitrification of $NH_4^+$ into $NO_3^-$ (Robertson & Groffman, 2015). Nitrate is more prone to losses through leaching as an anion, whereas ammonium is a cation that is retained more by negatively charged soil organic matter and clay particles (Robertson & Groffman, 2015). Nitrate can also be lost through denitrification, or the reduction of $NO_3^-$ into $N_2$ or $N_2O$, which is less energy intensive than the reduction of $NO_3^-$ into $NH_4^+$ (Robertson & Groffman, 2015). While inorganic N fractions reflect immediately available soil N to plants, they are temporally variable across the growing season, which makes it hard to assess how management impacts plant-available soil N (Hurisso & Culman, 2021). Moreover, to further assess soil N status, a metric that holistically assesses the organic fraction of N is also needed (Hurisso et al., 2018). Understanding how organic soil N mineralization contributes to inorganic N availability will further our understanding of how the soil microbiome contributes to N status in the soil which reflects soil health and impacts crop productivity. However, conceptual frameworks of plant–microbial–mineral interactions that influence N bioavailability are based on samples collected from the field are lacking (Daly et al., 2021; Grandy et al., 2022).

**Core Ideas**

- ACE protein was more impacted by system than total N or inorganic soil N concentrations.
- ACE protein fluctuated less across the growing season compared to total and inorganic soil N.
- ACE protein was more correlated with total N and $NH_4^+$-N than $NO_3^-$-N.
- ACE protein represented a legacy effect of sustainable management practices on soil organic N.

Autoclaved-citrate extractable (ACE) protein is a newer metric of soil health that deserves more investigation for its indication of organic N status and responsiveness to sustainable management practices. Soil protein represents the largest fraction of organically bound soil N that is microbially available (Hurisso & Culman, 2021; Rillig et al., 2007). ACE protein has been defined as the primary mineralizable pool of organic soil N (Hurisso & Culman, 2021; Hurisso et al., 2018). Previous studies have reported positive correlations between ACE protein and N mineralization that range from $R^2 = 0.21$ to $R^2 = 0.76$ (Geisseler et al., 2019; Jha et al., 2022; Liptzin et al., 2023). The ACE protein method has been recommended as an indicator of soil N status in many soil health assessments (Hurisso & Culman, 2021; Moebius-Clune et al., 2016; Stott, 2019). The ACE protein method co-extracts glycoproteins and other proteins, in addition to humic acids, lipids, carboxylic acids, polyphenols, and heterocyclic N-containing compounds (Agnihotri et al., 2022; Gillespie et al., 2011; Schindler et al., 2007).

Recent studies are uncovering how ACE protein may be a reliable indicator of soil health. Sustainable management practices including reduced tillage, cover cropping, and rotations with perennial legumes promote higher levels of ACE protein compared to conventional row-crop systems (Liptzin et al., 2023; Martin & Sprunger, 2021; Roper et al., 2017; Nichols & Millar, 2013; Wright et al., 2007). ACE protein contributes to soil health by inducing soil aggregate formation and soil organic matter (SOM) stabilization (Agnihotri et al., 2022; Hurisso et al., 2018). Previous studies have reported significant positive correlations between ACE protein and soil aggregate stability ($0.32 < R^2 < 0.59$) (Fine et al., 2017; Nichols & Millar, 2013; Rillig et al., 2002; Wright & Anderson, 2000; Wright & Upadhyaya, 1998). ACE protein directly contributes to soil C sequestration as the glycoproteins that constitute a substantial fraction of ACE protein are produced by arbuscular mycorrhizal fungi with hyphae that bind aggregates together and stimulate plant growth, belowground root biomass, and rhizodeposition (Agnihotri et al., 2022; Miller & Jastrow, 2000). The ACE protein and hyphae-facilitated occlusion of organic compounds in soil aggregates protect them from extracellular enzyme activity and indirectly contributes to soil C sequestration (Agnihotri et al., 2022; Rillig et al., 2007).

Soil N mineralization is a multi-step process that involves the depolymerization of proteins in SOM into amino acids, which is the rate-limiting step, followed by the deamination of amino acids into $NH_3$ (Mooshammer et al., 2012; Schimel & Bennett, 2004). The rate-limiting step in N mineralization is driven by extracellular enzymes such as leucine-aminopeptidase (LAP) that catalyze the hydrolysis of peptides to produce leucine (Geisseler et al., 2010). While LAP does not drive the complete N mineralization process, previous research has shown that N additions impact LAP activity and subsequent N cycling. For example, in a wheat–corn cropping system, Ferrosols that were intensively managed with manure and urea had higher LAP activity, net N mineralization, and microbial biomass compared to less intensively managed soils (Ali et al., 2021). Conversely, urea applications in a long-term temperate grassland experiment resulted in lower LAP activity and higher concentrations of $NH_4^+$ in a calcic-orthic Aridisol (Shi et al., 2016). Combined applications of urea and manure in Ali et al. (2021) provided labile, organic N that may have stimulated LAP activity than provided through urea alone. While long-term effects of N addition on LAP activity have been studied, the short-term variability of LAP over the course of a single growing season in relation to different soil N fractions has not been investigated. Moreover, understanding how agroecosystem management practices impact temporal dynamics in bioavailable organic N and plant-available inorganic N will allow for more accurate N management strategies that improve crop yield and agricultural sustainability.

A current knowledge gap in the soil health literature is how long-term sustainable management practices, including lower synthetic inputs, crop diversification, and increased perenniality, impact ACE protein and other N-related soil health indicators, including total and inorganic N, and activity of N depolymerizing enzymes, across a single growing season. In this study, we explore ACE protein in relation to other soil N measures to better understand how ACE protein can inform soil N management strategies and improve soil health. This study investigates a suite of soil N measures over a single growing season to explore seasonal fluctuations using a 33-year-old cropping system experiment with consistent management histories. Our specific objectives are to (1) determine how ACE protein varies across a management intensity gradient that varies in perenniality and diversity, (2) measure ACE protein over the course of a single growing season to assess short-term fluctuations, (3) assess the relationship of ACE protein with and other soil N fractions and LAP activity across a single growing season to better understand how to incorporate ACE protein into the soil health framework, and (4) determine which soil N measures best correlate with crop yield and identify at what point this occurs during the growing season. We hypothesize that (1) ACE protein will be higher in management systems with greater perenniality and crop diversity, (2) ACE protein will increase over the course of the growing season because of increased plant inputs of organic N, (3) ACE protein will be more stable across the growing season compared to other N-related soil health indicators, and (4) correlations between yield and the N-related soil health metrics will vary across the growing season with stronger positive correlations toward the end of the growing season due to greater respective plant N demand and supply of inorganic and organic soil N.

## 2 | MATERIALS AND METHODS

### 2.1 | Site description

This study was based at the Michigan State University W.K. Kellogg Biological Station's (KBS) Long-Term Ecological Research site (LTER) in southwest Michigan, located at 85°22′38″W, 42°24′40″N. The two primary soil series at the LTER are the Kalamazoo and Oshtemo soil series, both of which are mixed mesic Typic Hapludalfs. Weather data were obtained from the KBS National Weather Station, which is located at 85°22‘25″W, 42°24′30″N with an elevation of 277 m. The average annual temperature is 9.7°C, and the average annual precipitation is 1005 mm (Robertson & Hamilton, 2015). The cumulative precipitation and growing degree days for the 2021 and the previous 30-year average (1990–2020) for the growing season (March through August) are shown in Figure 1. From March through the middle of June, cumulative precipitation for the growing season in 2021 was below the 30-year average (Figure 1a). Prior to the July sampling date, there was a major precipitation event that occurred at the end of June that raised cumulative precipitation closer to the 30-year average (Figure 1a). On the other hand, cumulative growing degree days in 2021 were slightly above the 30-year average for the entire growing season (Figure 1b).

### 2.2 | Experimental design

The systems at the KBS LTER Main Cropping System Experiment (MCSE) include four annual cropping systems: two perennial systems and two unmanaged systems. All systems besides the mown grassland (an unmanaged system) are at the main LTER MCSE in a randomized complete block design, each with six replicates of 90 × 110 m plots. The mown

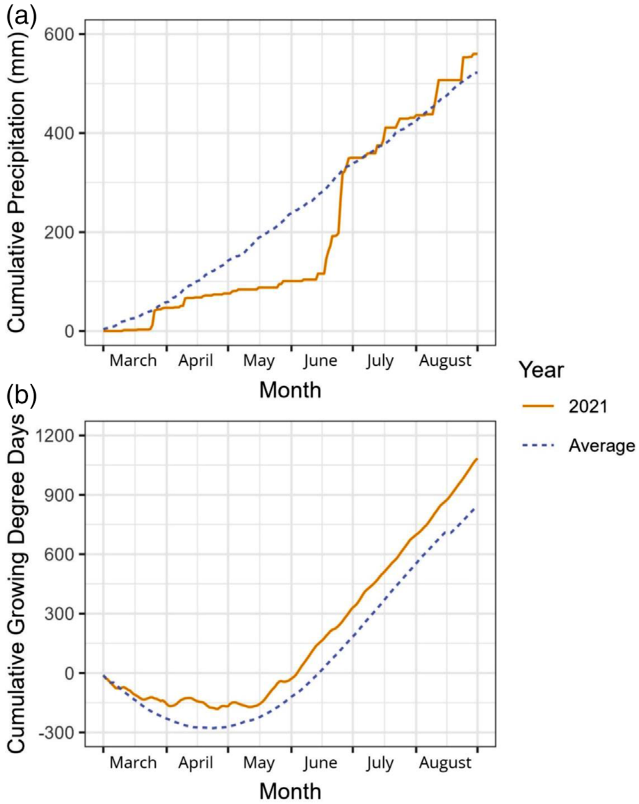


**FIGURE 1** Cumulative (a) precipitation and (b) growing degree days for 2021 and over a 30-year average (1990–2020).

grassland system is ∼200 m south of the other systems and is replicated four times in 15 × 30 m plots.

The four annual cropping systems were established in 1989 and follow a corn (*Zea mays*)-soybean (*Glycine max*)-wheat (*Triticum aestivum*) rotation. The annual cropping systems range in management including conventional, no-till, reduced input, and biologically based. All annual cropping systems besides the no-till system were chisel plowed. The reduced input system typically receives one third less of the fertilizer applied in the conventional and no-till systems. There are no external inputs to the biologically based system. Potash (K) fertilizer was applied to the no-till soybean system on April 27, 2021, and the conventional and reduced input soybean system was applied on April 30, 2021, at the rates given in Table S1. Then, the conventional soybean, no-till soybean, and reduced input soybean systems received phosphorus (P) fertilizer on May 10, 2021, at the rates given in Table S1. The conventional, no-till, and reduced input systems were planted on May 14, 11, and 22 of 2021 with Roundup Ready soybeans at 67 kg ha$^{-1}$ of Pioneer P22T86E Soybean Seed. The reduced input system was replanted on June 17, 2021, due to poor stands in all reps from seed corn maggot. The biologically based system was planted on May 27, 2021, with Viking Organic Soybean Seed Variety O.2188AT12N at 175,862 seeds per acre. Winter cover crops are incorporated in the reduced input and biologically based annual cropping systems following corn and soybean harvest and follow a corn–ryegrass (*Lolium multiflorum*)–soybean–winter wheat–red clover (*Trifolium pratense*) rotation. The conventional and no-till systems were harvested on September 30, 2021. The reduced input and biologically based systems were harvested on October 19 and 20 of 2021, respectively. Soybean plants were harvested within a 1-m$^2$ quadrat (1.5 m by 0.65 m) at five sampling stations per plot in each of the six replicate plots per system.

The two perennial systems included switchgrass (*Panicum virgatum*) and hybrid poplar (*Populus nigra* × *Prunus maximowiczii*). Switchgrass was established in 2019, and previously the system had alfalfa from 1989 to 2019. The switchgrass system was fertilized with 28% urea ammonium nitrate (UAN) at 50 lbs N per acre on May 27, 2021, and mowed for aboveground biomass collection on August 11, 2021. Aboveground switchgrass biomass was harvested in 0.5 × 2 m quadrats at five sampling stations in each of the six replicated plots. The poplar system was established in 1989 and is harvested every 10 years with the last harvest in 2018 and new crop planted in 2019.

The two unmanaged systems included an early successional community and a mown grassland system. The early successional system was converted from row-crop agriculture with tillage in 1989 and is burned every spring to control for woody species. The main plant species (top 10) in the early successional system in 2021 included *Solidago canadensis* L., *Robinia pseudoacacia* L., *Aster sagittifolius*, *T. pratense* L., *Bromus inermis* Leyss., *Dactylis glomerata* L., *Rubus allegheniensis* T.C. Porter, *Elymus repens* (L.) Gould, *Solidago juncea* Aiton, and *Lotus corniculatus* L. The mown grassland system was established on a cleared woodlot in 1959 and has no historical tillage. The main plant species in the mown grassland system in 2021 included *Sassafras albidum* (Nutt.) Nees, *S. canadensis* L., *B. inermis* Leyss., *Vitis* sp., *R. allegheniensis* T.C. Porter, *Crataegus* spp., *T. pratense* L., *Asclepias tuberosa* L., *Desmodium paniculatum* (L.) DC., and *Monarda fistulosa* L. Additional details on long-term management practices for each system can be found in Robertson and Hamilton (2015).

## 2.3 | Field sampling

Soil sampling in each system occurred over the course of the 2021 growing season (May–August) at four different time periods at the end of each month to account for monthly variability of N-related soil health indicators in the primary growing season. More specifically, soils were collected in May (May 26–28), June (June 29–July 1), July (July 26–28), and August (August 30–September 2) of 2021. Soil cores were collected from 0–10 cm using a 1.9-cm diameter hand probe five times from each of five flagged locations in each of the replicated plots, and 25 soil cores were composited per plot.

## 2.4 | Soil health indicators

### 2.4.1 | Soil N measures

Soils were stored at 4˚C prior to drying for 48 h at 60˚C in a dehydrator prior to TN, soil inorganic N, and ACE protein analyses. Soil samples were analyzed for total soil N via a CHNS elemental analyzer. Soil inorganic N for each soil sample was determined colorimetrically using the methods of Doane and Horwath (2003) and Sinsabaugh et al. (2000) for nitrate ($NO_3^-$-N) and ammonium ($NH_4^+$-N), respectively. Soil inorganic N was extracted with 2 M KCl (40 mL per 5 g dry soil ground to 2 mm), shaken for 30 min, and then centrifuged at 2000 rpm for 3 min. Sample extracts were transferred to a microplate and read on a spectrophotometric plate reader at 540 and 630 nm for $NO_3^-$-N and $NH_4^+$-N, respectively.

ACE protein was measured using methods adapted from Hurisso et al. (2018). Briefly, 24 mL of sodium citrate was added to 3 g of dry soil ground to 2 mm. The solution was shaken for 5 min, autoclaved at 121˚C for 30 min, cooled for 40 min, shaken for 30 min, and then 1.5 mL of the solution was transferred to a clean centrifuge tube and centrifuged for 3 min. ACE protein was quantified using the colorimetric bicinchoninic-acid assay (Thermo Scientific) in a 96-well spectrophotometric plate reader at 562 nm.

### 2.4.2 | Enzyme activity

Soils for enzyme activity were stored at −20˚C. LAP enzyme activity on each soil sample was measured using a fluorometric assay described in Bell et al. (2013). Briefly, 2.75 g of soil was combined with 91 mL of 50 mM acetate buffer and mixed on a stir plate. For each soil sample, a standard curve was prepared in a column of a 96-well microplate by adding 800 µL of the soil solution to 0, 2.5, 5, 10, 25, 50, and 100 µM 7-amino-4-methylcoumarin. In a separate microplate, 800 µL of the soil solution was added to 200 µL of the substrate, L-leucine-7-amido-4-methylcoumarin hydrochloride. Standard and sample microplates were sealed, inverted several times, incubated for 1.5 h at 35˚C, and then centrifuged at 1500 rpm for 2 min. Then, 250 µL of solution from each well in standard and sample microplates were added to a black microplate. Fluorescence was measured at excitation and emission wavelengths of 365 and 450 nm, respectively, on an H1 synergy BioTek microplate reader.

## 2.5 | Statistical analysis

Data analysis was conducted in RStudio version 4.2.2 (Posit Team, 2023), and figures were produced using ggplot2 (Wickham, 2016). For each soil property, a linear mixed model analysis of variance was conducted where system, sampling timepoint, and their interaction were fixed effects, and replicate was a random effect using the "lmer" function in the "lme4" package (Bates et al., 2015). Model diagnostics were performed using the "resid_panel" function in the "ggResidpanel" package (Goode & Rey, 2022), and assumptions of normality and constant variance were met. The significance of the main fixed effects was subsequently tested using the "Anova" function in the "car" package (Fox & Weisburg, 2019). Tukey pairwise mean comparisons were performed on estimated marginal means using the "emmeans" function in the "multcomp" package (Hothorn et al., 2008). Correlations between ACE protein and other N-related soil health indicators were tested for significance in a linear model using stat_poly_eq() in the "ggpmisc" package (Aphalo, 2022). Correlations of soybean yield and aboveground switchgrass biomass with N-related soil health indicators were investigated using ggpairs() in the "GGally" package (Schloerke et al., 2021).

# 3 | RESULTS

## 3.1 | ACE protein

We found that ACE protein was significantly ($p < 0.001$) impacted by system across a management intensity gradient, sampling timepoint across the growing season, and the interaction between system and time (Figure 2). The unmanaged polyculture perennial systems, including the early successional and the mown grassland systems, had the highest ACE protein, and the annual cropping systems, including conventional, no-till, reduced input, and biologically based soybean systems, had the least ACE protein (Figure 2). The average concentration of ACE protein in each system across all timepoints was progressively higher along the management intensity gradient (from low intensity in unmanaged systems to high intensity in annual cropping systems) except for comparing the poplar system to the switchgrass and early successional systems (Table 1). Averaged across all timepoints, ACE protein concentrations ranged from 3.95 g $kg^{-1}$ ACE protein in the conventional soybean system to 16.3 g $kg^{-1}$ ACE protein in the mown grassland system (Table 1).

Averaged across all timepoints, the mown grassland had significantly ($p_{adj}$ < 0.001) higher ACE protein concentrations than the early successional, switchgrass, poplar, biologically based soybean, reduced input soybean, no-till

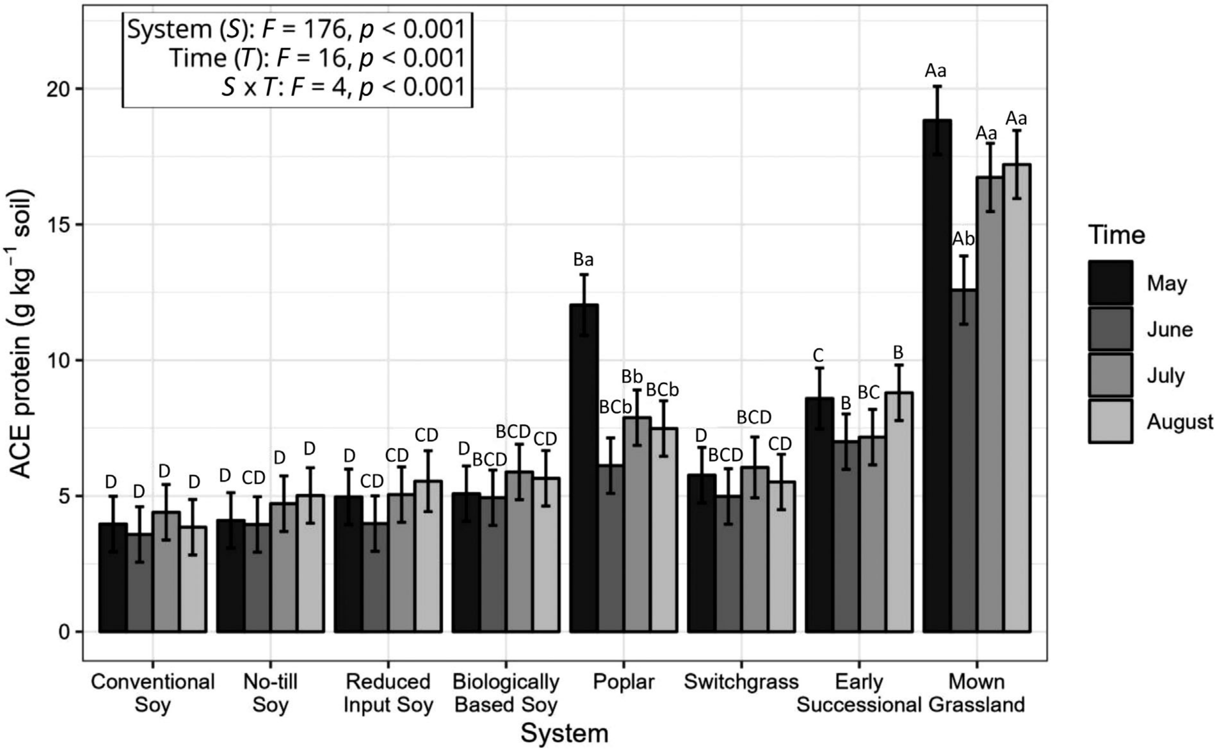


**FIGURE 2** Autoclaved-citrate extractable (ACE) protein concentrations (g $kg^{-1}$) across the growing season under different systems. Bars show estimated marginal means and confidence intervals of four replicates for the mown grassland system and six replicates for all other systems. *F*-statistics and *p*-values are from an analysis of variance on the linear mixed effect modesls for fixed effects of system, time, and their interaction. Uppercase Tukey letters indicate significant ($p_{adj}$ < 0.05) differences in estimated marginal means of systems within individual timepoints. Lowercase Tukey letters indicate significant ($p_{adj}$ < 0.05) differences in estimated marginal means of timepoints for individual systems.

soybean, and conventional soybean systems by 8.45, 10.76, 7.96, 10.95, 11.45, 11.89, and 12.39 g $kg^{-1}$ ACE protein, respectively (Table 1). The early successional system also had significantly ($p_{adj}$ < 0.001) higher ACE protein concentrations compared to the switchgrass, biologically based soybean, reduced input soybean, no-till soybean, and conventional soybean systems averaged across all timepoints by 2.31, 2.50, 3.00, 3.44, and 3.94 g $kg^{-1}$ ACE protein, respectively (Table 1). The poplar system had significantly ($p_{adj}$ < 0.001) higher ACE protein concentrations compared to the switchgrass, biologically based soybean, reduced input soybean, no-till soybean, and conventional soybean systems averaged across all timepoints by 2.80, 2.99, 3.49, 3.93, and 4.43 g $kg^{-1}$ ACE protein, respectively (Table 1). The switchgrass and biologically based soybean systems also had significantly higher ACE protein concentrations averaged across all timepoints compared to the conventional soybean system by 1.63 g $kg^{-1}$ ACE protein ($p_{adj}$ < 0.001) and 1.44 g $kg^{-1}$ ACE protein ($p_{adj}$ = 0.003), respectively (Table 1).

Sampling timepoint significantly impacted ACE protein across the growing season averaged across all systems ($p$ < 0.001) (Figure 2), which coincided with a precipitation event in June after several months of below-average precipitation (Figure 1). Averaged across all systems, ACE protein concentrations peaked in May with 7.92 g $kg^{-1}$ ACE protein and then dropped to 5.89 g $kg^{-1}$ ACE protein in June (Table 2). Soils sampled in June had significantly ($p_{adj}$ < 0.001) lower ACE protein concentrations than those sampled in May, July, and August by 2.03, 1.34, and 1.49 g $kg^{-1}$ ACE protein, respectively (Table 2).

The interaction of system and sampling timepoint on ACE protein was also significant ($p$ < 0.001) (Figure 2). Systems differed in ACE protein concentrations the most during May when they ranged from 18.83 g $kg^{-1}$ ACE protein in the mown grassland system to 3.97 g $kg^{-1}$ ACE protein in the conventional soybean system (Figure 2). System had consistent effects on ACE protein at all individual sampling timepoints with the exception of the poplar and early successional systems. The poplar system had significantly higher ACE protein in May ($p_{adj}$ < 0.01) and July (nonsignificant [n.s.]) but lower ACE protein in June (n.s.) and August (n.s.) compared to the early successional system (Figure 2). The poplar and mown grassland systems each significantly differed in ACE protein concentrations over the growing season, but the other systems did not (Figure 2). In the poplar system, ACE protein concentrations were significantly ($p$ < 0.001) higher in May

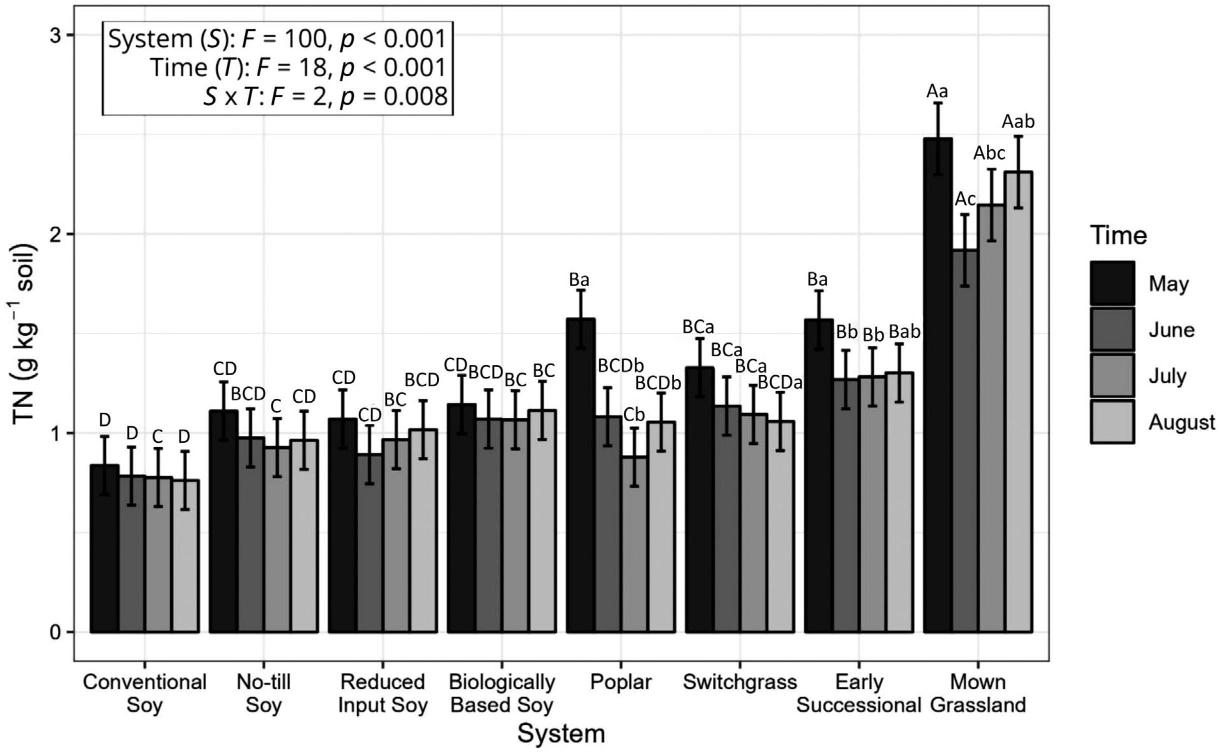


**FIGURE 3** Total soil nitrogen (TN) concentrations (g kg$^{-1}$) across the growing season under different systems. Bars show estimated marginal means and confidence intervals of four replicates for the mown grassland system and six replicates for all other systems. $F$-statistics, and $p$-values are from an analysis of variance on the linear mixed effect models for fixed effects of system, time, and their interaction. Uppercase Tukey letters indicate significant ($p$adj < 0.05) differences in estimated marginal means of systems within individual timepoints. Lowercase Tukey letters indicate significant ($p_{adj}$ < 0.05) differences in estimated marginal means of timepoints for individual systems.

compared to June, July, or August by 5.92, 4.15, and 4.55 g kg$^{-1}$, respectively (Figure 2). In the mown grassland system, ACE protein concentrations were significantly ($p$ < 0.001) higher in May, July, and August compared to June by 6.25, 4.15, and 4.63 g kg$^{-1}$ ACE protein, respectively (Figure 2).

## 3.2 | Total soil nitrogen

TN was significantly impacted by system ($p$ < 0.001), sampling timepoint ($p$ < 0.001), and their interaction ($p$ = 0.008) (Figure 3). Similar to ACE protein, the unmanaged polyculture perennial systems had the highest TN concentrations, and the annual cropping systems had the lowest TN concentrations (Figure 3). The average concentration of TN in each system across all timepoints was progressively higher along the management intensity gradient (Table 1). Averaged across all timepoints, TN concentrations ranged from 0.79 g kg$^{-1}$ TN in the conventional soybean system to 2.21 g kg$^{-1}$ TN in the mown grassland system (Table 1).

The mown grassland system had significantly ($p_{adj}$ < 0.001) higher TN concentrations compared to the early successional, switchgrass, poplar, biologically based soybean, reduced input soybean, no-till soybean, and conventional soybean systems averaged across all timepoints by 0.86, 1.06, 1.07, 1.11, 1.23, 1.22, and 1.42 g kg$^{-1}$ TN, respectively (Table 1). The early successional system had significantly higher TN concentrations compared to the switchgrass and poplar systems (both $p_{adj}$ < 0.01), and the biologically based soybean, reduced input soybean, no-till soybean, and conventional soybean (all $p_{adj}$ < 0.001) systems by 0.20, 0.21, 0.26, 0.37, 0.36, and 0.57 g kg$^{-1}$, respectively (Table 1). Averaged across all timepoints, TN concentrations were also significantly higher in the switchgrass, poplar, biologically based soybean (all $p_{adj}$ < 0.001), reduced input soybean, and biologically based soybean (both $p_{adj}$ < 0.01) systems by 0.36, 0.36, 0.31, 0.20, 0.20 g kg$^{-1}$ TN, respectively (Table 1).

Averaged across all systems, TN concentrations were significantly impacted by sampling time across the growing season ($p$ < 0.001) (Figure 3; Table 2). Soils sampled in May had significantly ($p_{adj}$ < 0.001) higher TN concentrations compared to those sampled in June, July, and August averaged across all systems by 0.25 g kg$^{-1}$, 0.25 g kg$^{-1}$, and 0.19 g kg$^{-1}$ TN, respectively (Table 2).

There were also significant interaction effects between system and sampling timepoint on TN ($p$ < 0.01) (Figure 3).

**TABLE 1** System-level estimated marginal means and standard error (SE) of autoclaved-citrate extractable (ACE) protein, total soil nitrogen (TN), $NO_3^-$-N, and $NH_4^+$-N concentrations, and leucine-aminopeptidase (LAP) activity.

| | ACE protein (g kg$^{-1}$) | | TN (g kg$^{-1}$) | | $NH_4^+$-N (mg kg$^{-1}$) | | $NO_3^-$-N (mg kg$^{-1}$) | | LAP (nmol g$^{-1}$ h$^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|
| **System** | **Mean** | **SE** | **Mean** | **SE** | **Mean** | **SE** | **Mean** | **SE** | **Mean** | **SE** |
| Conventional soy | 3.95D | 0.26 | 0.79E | 0.04 | 3.71D | 0.26 | 4.63A | 0.32 | 88 | 22 |
| No-till soy | 4.45CD | 0.26 | 0.99CD | 0.04 | 3.55D | 0.26 | 3.83A | 0.32 | 83 | 23 |
| Reduced input soy | 4.89CD | 0.27 | 0.99D | 0.04 | 3.73D | 0.26 | 3.87A | 0.32 | 130 | 23 |
| Biologically based soy | 5.38C | 0.26 | 1.98CD | 0.04 | 3.94CD | 0.26 | 4.09A | 0.32 | 116 | 22 |
| Poplar | 8.38B | 0.27 | 1.15CD | 0.04 | 4.89BC | 0.26 | 3.57AB | 0.32 | 95 | 23 |
| Switchgrass | 5.58C | 0.27 | 1.15C | 0.04 | 3.98CD | 0.26 | 2.24BC | 0.32 | 153 | 23 |
| Early successional | 7.89B | 0.27 | 1.36B | 0.04 | 5.84AB | 0.26 | 0.80D | 0.32 | 135 | 24 |
| Mown grassland | 16.34A | 0.32 | 2.21A | 0.05 | 6.94A | 0.32 | 1.23CD | 0.40 | 145 | 28 |

*Note*: Uppercase Tukey letters indicate significant ($p_{adj}$ < 0.05) differences in estimated marginal means of systems averaged across all timepoints.

**TABLE 2** Time-specific estimated marginal means and standard error (SE) of autoclaved-citrate extractable (ACE) protein, total soil N (TN), $NO_3^-$-N, and $NH_4^+$-N concentrations, and leucine-aminopeptidase (LAP) activity.

| | ACE protein (g kg$^{-1}$) | | TN (g kg$^{-1}$) | | $NH_4^+$-N (mg kg$^{-1}$) | | $NO_3^-$-N (mg kg$^{-1}$) | | LAP (nmol g$^{-1}$ h$^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Time** | **Mean** | **SE** | **Mean** | **SE** | **Mean** | **SE** | **Mean** | **SE** | **Mean** | **SE** |
| May | 7.92A | 0.20 | 1.39A | 0.03 | 5.78A | 0.19 | 5.03A | 0.24 | 97B | 17 |
| June | 5.89B | 0.19 | 1.14B | 0.03 | 3.58C | 0.19 | 2.79B | 0.24 | 231A | 16 |
| July | 7.24A | 0.19 | 1.14B | 0.03 | 4.12BC | 0.19 | 2.67B | 0.24 | 75B | 17 |
| August | 7.38A | 0.19 | 1.20B | 0.03 | 4.80B | 0.19 | 1.63C | 0.24 | 70B | 17 |

*Note*: Uppercase Tukey letters indicate significant ($p_{adj}$ < 0.05) differences in estimated marginal means at each timepoint averaged across all systems.

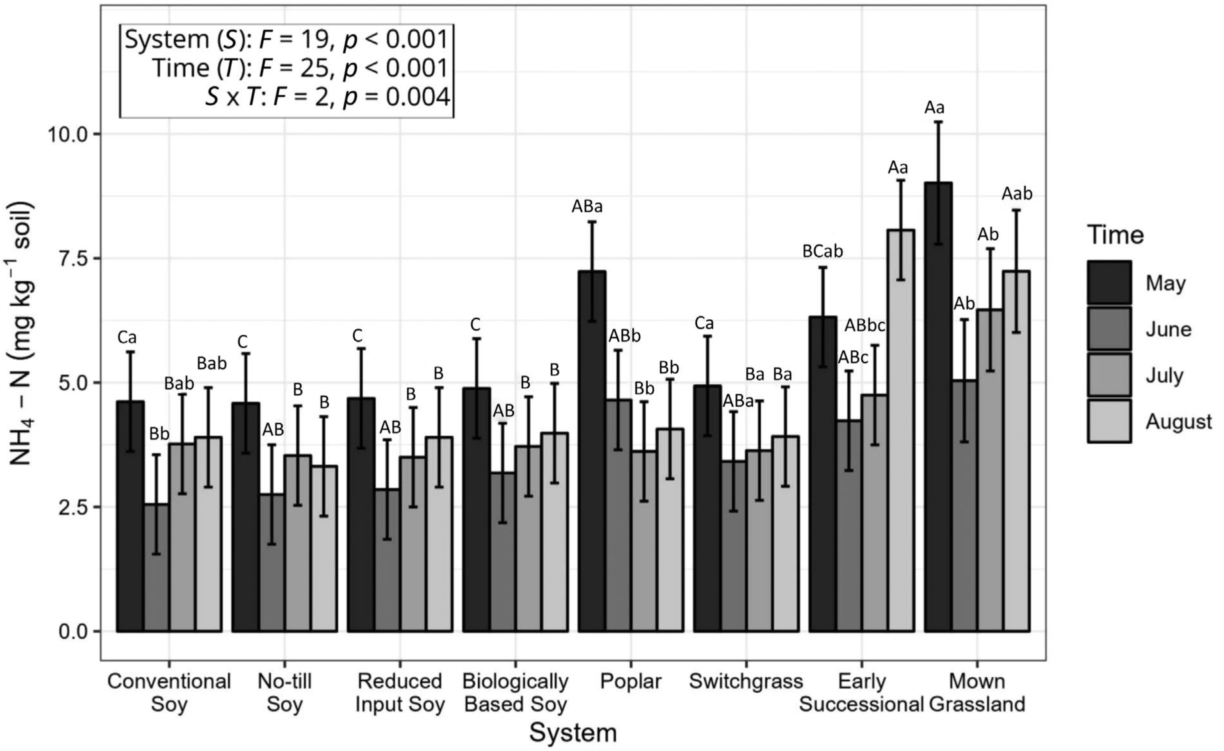


**FIGURE 4** Ammonium-N (NH4+-N) concentrations (mg kg$^{-1}$) across the growing season under different systems. Bars show estimated marginal means and confidence intervals of four replicates for the mown grassland system and six replicates for all other systems. *F*-statistics and *p*-values are from an analysis of variance on the linear mixed effect models for fixed effects of system, time, and their interaction. Uppercase Tukey letters indicate significant ($p_{adj} < 0.05$) differences in estimated marginal means of systems within individual timepoints. Lowercase Tukey letters indicate significant ($p_{adj} < 0.05$) differences in estimated marginal means of timepoints for individual systems.

The systems differed the most in TN in May, which ranged from 2.48 g kg$^{-1}$ TN in the mown grassland system to 0.84 g kg$^{-1}$ TN in the conventional soybean system (Figure 3). The mown grassland and conventional soybean systems had consistently the highest and lowest TN concentrations, respectively, at each individual sampling timepoint. The poplar, switchgrass, and mown grassland systems had larger fluctuations in TN across the growing season than the other systems. In the poplar system, TN concentrations were significantly ($p_{adj} < 0.001$) higher in May compared to June, July, and August by 0.49, 0.69, and 0.52 g kg$^{-1}$ TN, respectively (Figure 3). In the early successional system, TN concentrations were significantly ($p_{adj} < 0.05$) higher in May compared to June and July by 0.30 and 0.29 g kg$^{-1}$ TN, respectively (Figure 3). In the mown grassland system, TN concentrations were significantly ($p_{adj} < 0.001$) higher in May compared to June by 0.56 g kg$^{-1}$ TN (Figure 3).

## 3.3 | Ammonium-N

Ammonium-N ($NH_4^+$-N) was significantly impacted by system ($p < 0.001$), sampling timepoint ($p < 0.001$), and their interaction ($p = 0.004$) (Figure 4). Similar to ACE protein and TN, $NH_4^+$-N was the highest in the unmanaged systems and the lowest in the annual cropping systems (Figure 4). The average concentration of $NH_4^+$-N averaged across all timepoints was progressively higher along the management intensity gradient with the exception of $NH_4^+$-N being higher in the poplar system than the switchgrass system, and also higher in the conventional soybean system than the no-till soybean system (Table 1). Averaged across all timepoints, $NH_4^+$-N concentrations ranged from 3.55 mg kg$^{-1}$ $NH_4^+$-N in the no-till soybean system to 6.94 mg kg$^{-1}$ $NH_4^+$-N in the mown grassland system (Table 1).

The mown grassland system had significantly ($p_{adj} < 0.001$) higher $NH_4^+$-N concentrations compared to the switchgrass, poplar, biologically based soybean, reduced input soybean, no-till soybean, and conventional soybean systems averaged across all sampling timepoints by 2.96, 2.05, 3.00, 3.21, 3.39, and 3.23 mg kg$^{-1}$ $NH_4^+$-N, respectively (Table 1). The early successional system had significantly ($p_{adj} < 0.001$) higher $NH_4^+$-N concentrations averaged across all sampling timepoints compared to the switchgrass, biologically based soybean, reduced input soybean, no-till soybean, and conventional soybean systems by 1.87, 1.90, 2.11, 2.29, and 2.13 mg kg$^{-1}$ $NH_4^+$-N, respectively (Table 1). The poplar system had significantly ($p_{adj} < 0.05$) higher $NH_4^+$-N concentrations compared to the reduced input soybean ($p_{adj} = 0.03$), no-till soybean

($p_{adj} < 0.01$), and conventional soybean ($p_{adj} = 0.03$) systems averaged across all sampling timepoints by 1.16, 1.35, 1.18 mg kg $NH_4^+$-N, respectively (Table 1).

Averaged across all systems, $NH_4^+$-N concentrations significantly changed over the course of the growing season ($p < 0.001$) (Figure 4). Concentrations of $NH_4^+$-N were significantly higher in May compared to June ($p_{adj} < 0.001$), July ($p_{adj} < 0.001$), and August ($p_{adj} = 0.001$) averaged across all systems by 2.20, 1.66, and 0.98 g $kg^{-1}$ $NH_4^+$-N, respectively (Table 2). In addition, $NH_4^+$-N concentrations were significantly ($p_{adj} < 0.001$) higher in August compared to June averaged across all systems (Table 2).

There were also significant interaction effects of system and sampling timepoint on $NH_4^+$-N ($p < 0.01$) (Figure 4). The greatest difference in $NH_4^+$-N concentrations across systems was in May where they ranged from 4.61 g $kg^{-1}$ $NH_4^+$-N in the no-till soybean system to 9.01 mg $kg^{-1}$ $NH_4^+$-N in the mown grassland system (Figure 4). There were also large differences in $NH_4^+$-N concentrations in August compared with the no-till soybean and early successional systems with 3.31 and 8.1 mg $kg^{-1}$ $NH_4^+$-N, respectively (Figure 4). In May and June, system had similar and significant effects on $NH_4^+$-N concentrations where the mown grassland system and the poplar system each had significantly ($p_{adj} < 0.05$) higher $NH_4^+$-N concentrations compared to the other systems (Figure 4). In the poplar system, $NH_4^+$-N concentrations decreased from June to July, which opposed increases in all other systems at this time (Figure 4). In July, the mown grassland system had significantly ($p_{adj} < 0.05$) higher $NH_4^+$-N concentrations compared to all other systems except for the early successional system (Figure 4). In August, the early successional had significantly ($p < 0.001$) higher $NH_4^+$-N concentrations compared to all other systems besides the mown grassland system (Figure 4).

## 3.4 | Nitrate-N

Nitrate ($NO_3^-$-N) was significantly ($p < 0.001$) impacted by system, sampling time, and their interaction (Figure 5). In contrast to ACE protein, TN, and $NH_4^+$-N concentrations, $NO_3^-$-N concentrations were the highest in the annual cropping systems and the lowest in the unmanaged systems (Figure 5). Averaged across all timepoints, the early successional and mown grassland systems had the lowest $NO_3^-$-N concentrations with 0.80 and 1.23 mg $kg^{-1}$ $NO_3^-$-N, respectively, and the conventional soybean system had the highest $NO_3^-$-N concentrations with 4.63 mg $kg^{-1}$ $NO_3^-$-N (Table 1).

The mown grassland system had significantly ($p_{adj} < 0.001$) lower $NO_3^-$-N concentrations averaged across all sampling timepoints compared to the poplar, biologically based soybean, reduced input soybean, no-till soybean, and conventional soybean systems by 2.34, 2.87, 2.64, 2.60, and 3.41 mg $kg^{-1}$ $NO_3^-$-N, respectively (Figure 5; Table 2). The early successional system also had significantly ($p_{adj} < 0.001$) lower $NO_3^-$-N concentrations compared to the poplar, biologically based soybean, reduced input soybean, no-till soybean, and conventional soybean systems averaged across all sampling timepoints by 2.76, 3.28, 3.06, 3.03, and 3.83 mg $kg^{-1}$ $NO_3^-$-N, respectively (Figure 5; Table 2). The switchgrass system also had significantly lower $NO_3^-$-N concentrations compared to the biologically based soybean, reduced input soybean, no-till soybean systems (all $p_{adj} < 0.01$), and the conventional soybean system ($p_{adj} < 0.001$) averaged across all sampling timepoints by 1.85, 1.63, 1.59, and 2.39 mg $kg^{-1}$ $NO_3^-$-N, respectively.

Similar to TN concentrations, $NO_3^-$-N concentrations significantly ($p < 0.001$) decreased across the growing season (Figure 5; Table 2). Averaged across all systems, $NO_3^-$-N concentrations were significantly ($p_{adj} < 0.001$) higher in May compared to June, July, and August by 2.24, 2.36, and 3.40 mg $kg^{-1}$ $NO_3N$, respectively (Table 2). In addition, $NO_3^-$-N concentrations in June and July were significantly ($p_{adj} < 0.01$) higher than in August averaged across all systems by 1.17 and 1.05 mg $kg^{-1}$ $NO_3^-$-N, respectively (Table 2).

There were also significant interaction effects of system and sampling timepoint on $NO_3^-$-N ($p < 0.001$) (Figure 5). The systems differed the most in $NO_3^-$-N concentrations in May when the conventional soybean and mown grassland systems had 11.28and 0.64 mg $kg^{-1}$ $NO_3^-$-N, respectively (Figure 5). In June, the mown grassland system also had the lowest $NO_3^-$-N concentrations compared to all other systems with 0 mg $kg^{-1}$ $NO_3$—N, and the poplar system had the highest $NO_3^-$-N concentrations with 7.28 mg $kg^{-1}$ $NO_3^-$-N (Figure 5). From May to June, the poplar system significantly ($p_{adj} < 0.001$) increased in $NO_3^-$-N concentration by 3.95 mg $kg^{-1}$ $NO_3^-$-N, but the conventional soybean, no-till soybean, and biologically based soybean systems had significantly ($p_{adj} < 0.001$) decreased in $NO_3^-$-N concentrations by 8.28, 6.40, and 3.33 mg $kg^{-1}$ $NO_3^-$-N, respectively (Figure 5). The $NO_3^-$-N concentrations in the conventional soybean and no-till soybean systems significantly ($p < 0.001$) decreased from June to July to August (Figure 5). The mown grassland system had significantly ($p < 0.01$) increased $NO_3^-$-N concentrations from June to July and decreased $NO_3^-$-N concentrations from July to August. The other systems decreased or had little change in $NO_3^-$-N from June to July to August (Figure 5).

## 3.5 | Leucine-aminopeptidase activity

In contrast to the soil N fractions we measured, LAP activity was not significantly impacted by system averaged across all sampling timepoints (Figure 6). However, the mown

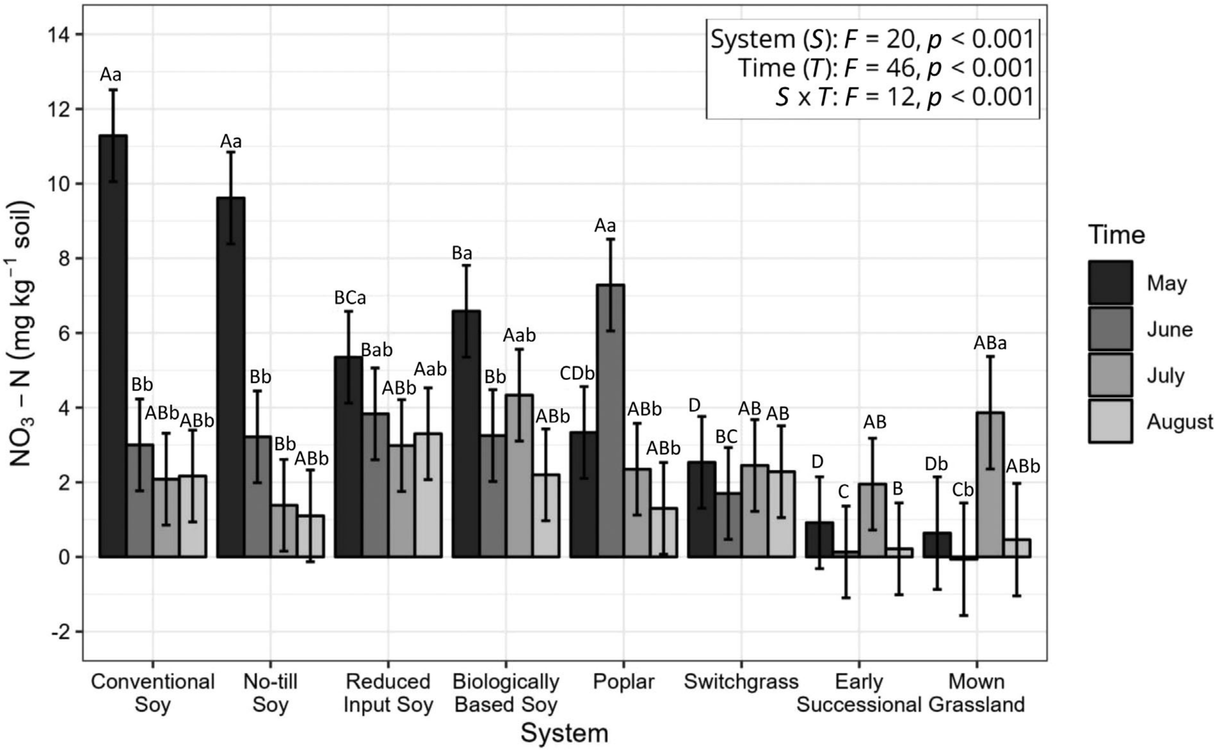


**FIGURE 5** Nitrate-N (NO3⁻-N) concentrations (mg kg$^{-1}$) across the growing season under different systems. Bars show estimated marginal means and confidence intervals of four replicates for the mown grassland system and six replicates for all other systems. *F*-statistics and *p*-values are from an analysis of variance on the linear mixed effect models for fixed effects of system, time, and their interaction. Uppercase Tukey letters indicate significant ($p_{adj} < 0.05$) differences in estimated marginal means of systems within individual timepoints. Lowercase Tukey letters indicate significant ($p_{adj} < 0.05$) differences in estimated marginal means of timepoints for individual systems.

grassland and switchgrass systems generally had higher LAP activity compared to annual cropping systems (Figure 6). Averaged across all timepoints, LAP activity was the highest in the switchgrass system with 153 nmol g$^{-1}$ h$^{-1}$ and the lowest in the no-till soybean system with 83 nmol g$^{-1}$ h$^{-1}$ (Table 1).

Averaged across all systems, LAP activity was significantly impacted by sampling timepoint across the growing season ($p < 0.001$) (Figure 6). In particular, LAP activity was significantly ($p_{adj} < 0.001$) higher in June averaged across all systems compared to May, July, or August by 134, 156, and 162 nmol g$^{-1}$ h$^{-1}$, respectively (Figure 6; Table 2).

While the overall interaction effects of system and time on LAP activity were not significant, the largest system effects on LAP activity were observed in June when the early successional system had 311 nmol g$^{-1}$ h$^{-1}$ of LAP activity, and the conventional soybean system had 131 nmol g$^{-1}$ h$^{-1}$ of LAP activity (Figure 6). Examining each system individually, LAP activity significantly increased from May to June in the reduced input soybean ($p_{adj} = 0.02$), biologically based soybean ($p_{adj} = 0.04$), switchgrass ($p_{adj} = 0.02$), and early successional ($p_{adj} < 0.01$) systems by 189, 171, 192, and 226 nmol g$^{-1}$ h$^{-1}$, respectively (Figure 6). Then, from June to July, LAP activity significantly decreased in the reduced input soybean ($p_{adj} = 0.02$), biologically based soybean ($p_{adj} < 0.01$), switchgrass ($p_{adj} = 0.03$), and early successional ($p_{adj} < 0.01$) systems by 199, 219, 175, and 245 nmol g$^{-1}$ h$^{-1}$, respectively (Figure 6).

## 3.6 | Correlations of ACE protein with other N-related soil health indicators

For each sampling timepoint, we assessed how ACE protein correlated with the other N-related soil health metrics across all systems. In general, ACE protein concentrations were both strongly and positively correlated with TN concentrations and also to a lesser extent with $NH_4^+$-N concentrations at individual timepoints (Figure 7). However, the strength and directionality of correlations of ACE protein with $NO_3^-$-N concentrations and LAP activity differed by time (Figure 7). Correlations between ACE protein and $NO_3^-$-N concentrations were slightly positive in July but negative in May, June, and August (Figure 7). Furthermore, ACE protein concentrations were significantly and positively correlated with LAP activity in July and August; however, these correlations were

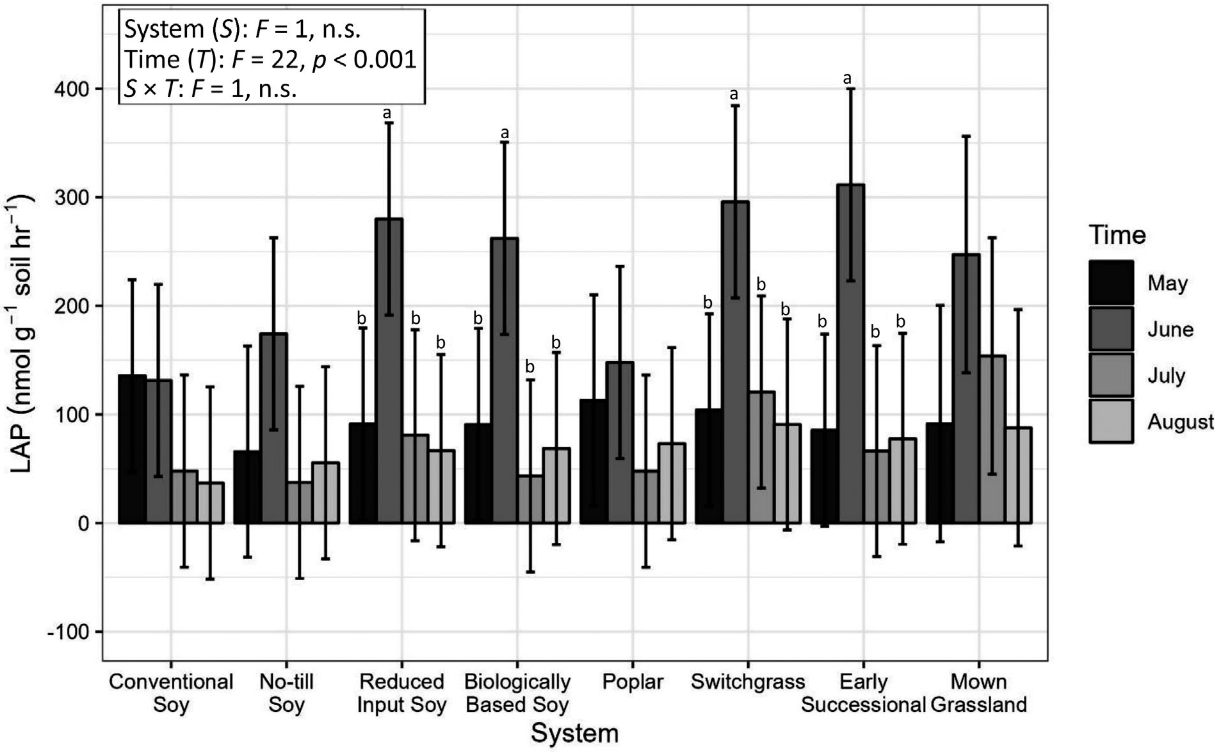


**FIGURE 6** Leucine-aminopeptidase (LAP) activity (nmol $g^{-1}$ $h^{-1}$) across the growing season under different systems. Bars show estimated marginal means and confidence intervals of four replicates for the mown grassland system and six replicates for all other systems. *F*-statistics and *p*-values are from an analysis of variance on the linear mixed effect models for fixed effects of system, time, and their interaction. Lowercase Tukey letters indicate significant ($p_{adj}$ < 0.05) differences in estimated marginal means of timepoints for individual systems. n.s., nonsignificant.

weak (Figure 7). In addition, ACE protein concentrations were not significantly correlated with LAP activity in May or June (Figure 7).

### 3.7 | Correlations of N-related soil health indicators with crop yield

Sampling timepoints across a single growing season impacted correlations between N-related soil health indicators and yield in the annual cropping soybean and switchgrass system (Table S2). Yield and aboveground biomass averages from 2021 for the soybean and switchgrass systems can be found in Table S1. Notably, there was a positive trend between switchgrass aboveground biomass and most N-related soil health indicators in contrast to the soy-based systems (Table S2). In particular, switchgrass aboveground biomass was significantly and positively correlated with ACE protein concentrations in May ($R^2$ = 0.92, $p$ < 0.01) and $NO_3^-$-N concentrations in June ($R^2$ = 0.69, $p$ = 0.04) (Table S2). In contrast, yields of the conventional and no-till soybean systems were not significantly correlated with any N-related soil health indicators (Table S2). However, yields of the reduced input soybean system were significantly and negatively correlated with TN and ACE protein concentrations in June (both $R^2$ = 0.76, $p$ = 0.03) (Table S2). Similarly, yields of the biologically based soybean system were significantly and positively correlated with TN concentrations in June ($R^2$ = 0.85, $p$ = 0.01) (Table S2). At the end of the growing season in August, ACE protein concentrations showed a general positive trend with yield in all systems; however, none of the correlations were significant (Table S2).

## 4 | DISCUSSION

### 4.1 | N-related soil health indicators across a management intensity and perenniality gradient

This study aimed to determine how ACE protein shifts across a management intensity gradient that varies in perenniality and diversity, and how these variations relate to differences in other N-related soil health indicators across a single growing season. Compounding effects of perenniality, crop diversity, tillage, and long-term synthetic N fertilizer applications were observed in N-related soil health indicators when compared across different systems.

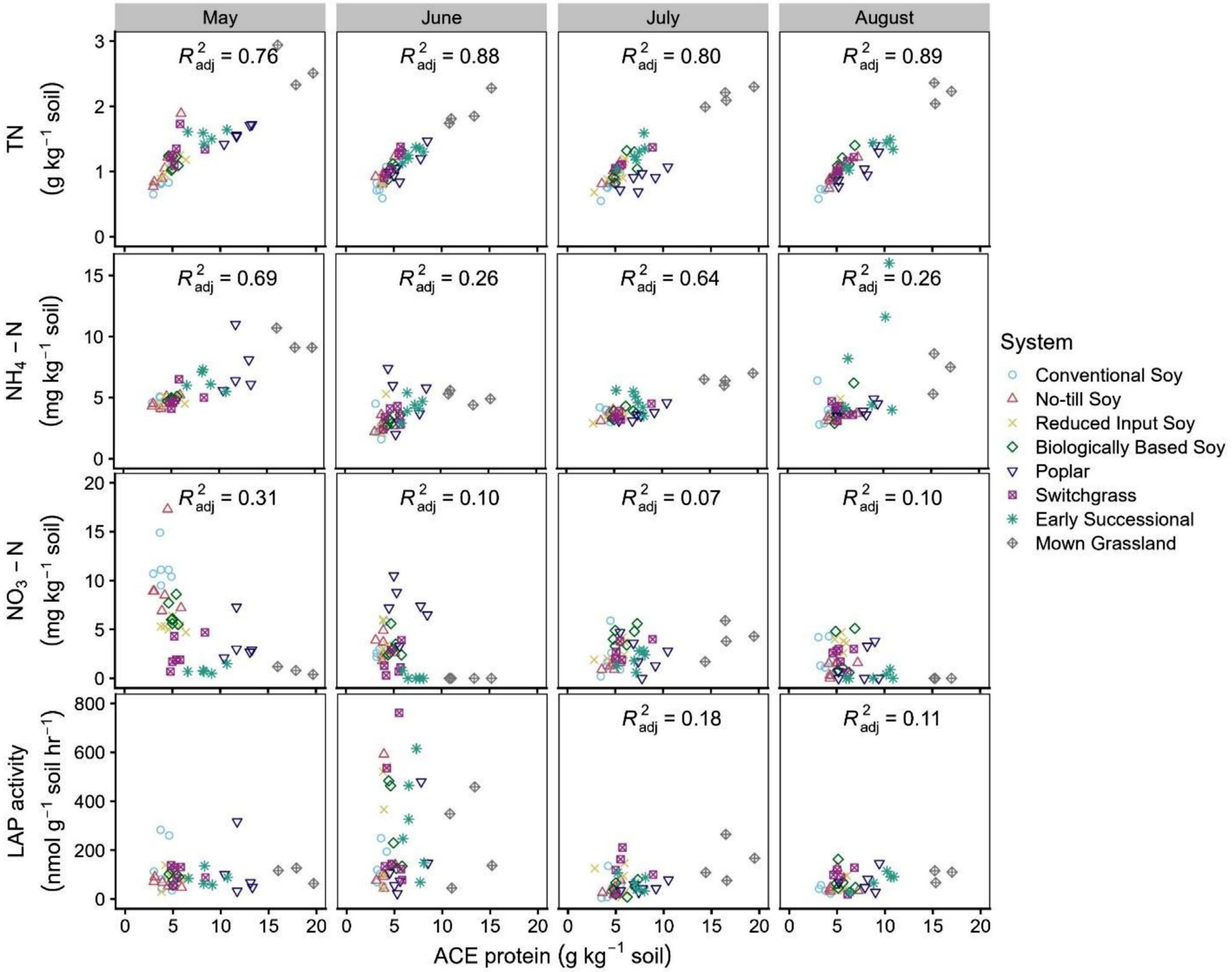


**FIGURE 7** Correlations of autoclaved-citrate extractable (ACE) protein with total soil nitrogen (TN), NH4$^+$-N, and NO3$^-$-N concentrations, and leucine-aminopeptidase (LAP) activity at each timepoint across all systems. Significant correlations are shown based on a threshold of $p < 0.05$. ACE protein was significantly correlated with all N-related soil health indicators at individual sampling timepoints except for LAP activity in May and June.

Perenniality and crop diversity in the early successional and mown grassland systems promoted the accumulation of ACE protein, TN, and $NH_4^+$-N. Fine roots of perennial systems have the ability to build soil N over time (Sprunger et al., 2018), and perennial polycultures often have greater fine root production than monoculture perennial systems (Martin & Sprunger, 2021; Sprunger et al., 2017). Some perennial grasses such as switchgrass harbor N-fixing bacteria that directly provide plant-available N and therefore do not rely on organic N mineralization for their N supply (Roley et al., 2020); this likely promotes the accumulation of organic N in perennial systems. Additionally, the early successional system was burned at the end of the previous growing season, which can increase soil N content in grasslands (Úbeda et al., 2005) and may have contributed to higher ACE protein, TN, and $NH_4^+$-N concentrations when compared to the annual cropping and perennial monoculture systems.

The mown grassland and early successional systems are not tilled, nor do they receive synthetic N fertilizer. Tillage in the conventional, reduced input, and biologically based soybean systems may have caused lower soil organic N relative to unmanaged systems as tillage disrupts soil aggregates and increases SOM oxidation in the plow layer (Yu et al., 2020). Previous studies have shown that ACE protein positively correlates with soil aggregate stability and SOM (Fine et al., 2017; Nichols & Millar, 2013; Rillig et al., 2002; Wright & Anderson, 2000; Wright & Upadhyaya, 1998). While we did not measure aggregate stability as a part of this study, soil aggregates have been extensively studied in the KBS LTER (Ananyeva et al., 2013; Kravchenko et al., 2011, 2015). For example, Kravchenko et al. (2011) reported that soil aggregates (4–6 mm) in the conventional system had more medium pores (39–90 µm) and less small (<15 µm) and large (>100 µm) pores than the no-till and early successional systems. Furthermore, lower ACE protein and TN in the conventional, no-till, and reduced input cropping systems compared to the perennial and unmanaged systems could be an effect of long-term synthetic N fertilizer applications of UAN during corn and wheat years of the rotation. Overapplication of synthetic N fertilizers can stimulate SOM mineralization and N loss (Singh, 2018).

Systems with increased perenniality and crop diversity also had lower $NO_3^-$-N compared to the annual cropping systems. Perennial systems have enhanced N use efficiency and extensive root systems that can retain and uptake $NO_3^-$-N to a greater extent than annual row crops (Sprunger et al., 2018; Syswerda et al., 2012). This increased N use efficiency and N uptake in perennial systems compared to annual cropping systems likely resulted in negative correlations between $NO_3^-$-N and the other N-related soil health metrics across the management intensity gradient. The disparities of perenniality and crop diversity on inorganic soil N emphasize the importance of considering $NH_4^+$-N and $NO_3^-$N as functionally distinct fractions since higher TN, ACE protein, and plant-available $NH_4^+$-N did not correspond to plant-available $NO_3^-$-N.

## 4.2 | Diversity drives differences in N-related soil health indicators among perennial systems

In addition to differences in ACE protein and other N-related soil health indicators across the management intensity and perenniality gradient, there were also differences among the perennial systems. Crop diversification can likely explain the substantial differences in soil N between monoculture and polyculture perennial systems. For example, the early successional, polyculture perennial system had higher concentrations of ACE protein, TN, and $NH_4^+$-N compared to the monoculture perennial switchgrass system. Compared to monoculture perennial systems, polyculture perennial systems in the KBS LTER and other locations have been found to improve soil health through increased aggregate stability (Grandy & Robertson, 2007) and the accumulation of soil organic C (Fornarna et al., 2008; Martin & Sprunger, 2022; Sprunger & Robertson, 2018), which has implications for enhanced organic N pools as soil organic C and organic N often increase together as the primary components of SOM (Murphy et al., 2015). Higher ACE protein in the polyculture perennial systems than the monoculture perennial systems could reflect greater mineral-associated organic matter, which protects organic N fractions (Cotrufo & Lavallee, 2022; Jillig et al., 2018). Additionally, diverse polyculture systems often have greater fine root production relative to their monoculture counterparts, which has substantial effects on soil C and N (Sprunger & Robertson, 2018; Sprunger et al., 2017). Fine roots can lead to increases in belowground nutrient availability from frequent turnover and rhizodeposition (Martin & Sprunger, 2021; Villarino et al., 2021). Our results suggest that perennial diversity increased plant rhizodeposition, fine root production, and organic deposits, which likely influenced ACE protein, given that it represents an organically bound fraction of N (Hurisso & Culman, 2021; Rillig et al., 2007).

## 4.3 | N-related soil health indicators among annual cropping systems

Management differences in tillage, cover cropping, and long-term N fertilizer inputs in the annual cropping systems also were evident in ACE protein and other N-related soil health indicators. These management differences in the annual cropping systems impacted ACE protein and TN. The conventional soybean system had lower ACE protein concentrations compared to the biologically based soybean system and lower TN concentrations compared to the reduced input system. The reduced input and biologically based soybean systems had ryegrass as a cover crop following the corn stage of the rotation. Ryegrass is shown to retain organic soil N following corn more so than other winter cover crops (Kuo et al., 1997) and increases potential availability for the following crop. The residue from winter cover crops protects the soil surface and lowers the potential for SOM mineralization, which could have promoted the accumulation of ACE protein and TN. Interestingly, the biologically based system did not differ in $NH_4^+$-N concentrations from the other soybean systems even though N fertilizers were not applied to the biologically based system, and yields were less than half of the other annual cropping systems. Previously, the biologically based system was reported to have lower C:N in the top 20 cm of the soil profile compared to the conventional system (Syswerda et al., 2011), which can stimulate a more rapid release of inorganic N for crop uptake (Robertson & Groffman, 2015). The lower yields of the biologically based system have been linked to P limitation that is evident in the nutrient test reports for the KBS LTER MCSE (KBS LTER, 2023). There has been a drawdown of P in the biologically based system since the establishment of the trial because no external inputs have been added since 1989. Soybean yields in the biologically based system were on par with the conventional system from 1989 to 2012; however, since then P limitation in the biologically based system has caused a decline in yields (Robertson et al., 2014). Ultimately, ACE protein and TN were more responsive than $NH_4^+$-N and $NO_3^-$-N to differences in management intensity with respect to tillage, cover crops, and fertilizer applications in the annual cropping systems.

## 4.4 | Nitrogen-related soil health indicators shift through time

In this study, ACE protein was the most stable N-related soil health metric across the growing season as it was least impacted by sampling timepoint compared to the other indicators. In general, $NH_4^+$-N and $NO_3^-$-N concentrations were more responsive to interactions between system and sampling timepoint compared to ACE protein, TN, and LAP activity. This suggests that ACE protein may be a more stable measure of soil N that is less sensitive to changes in soil N across a single growing season. However, the stability of ACE protein across a single growing season in systems under different management intensities shows the potential for ACE protein to serve as a metric for stable soil organic N accumulation in long-term studies, especially those that include perennial and plant diversity gradients. At each individual timepoint, systems had similar differences in ACE protein, TN, $NH_4^+$-N, and $NO_3^-$-N concentrations with the greatest differences between unmanaged systems and annual cropping systems in May. However, LAP activity was most differentiated between systems in July. Notably, the results may look different if this study examined soils from a short-term field experiment.

Soils sampled in June had significantly lower ACE protein concentrations and significantly higher LAP activity compared to soils sampled in May, July, and August averaged across all systems. Soils sampled in June also had significantly higher soil moisture than soils sampled in May, July, and August (Table S3). We speculate that the precipitation event that occurred prior to the June sampling timepoint disturbed ACE-occluded soil aggregates and stimulated LAP activity. However, soils sampled in June had significantly lower $NH_4^+$-N concentrations compared to May and August, which suggests that the amino acids produced via LAP activity were not directly mineralized through ammonification into $NH_4^+$-N. Instead, we speculate that the amino acids may have been assimilated by plants or microbes (Jones & Kielland, 2012; Jones et al., 2009; Wanek et al., 2010). Proteins and amino acids play an important role in SOM stabilization as proteins are the most abundant component of soil microbial biomass (Miltner et al., 2009), which contributes greatly to SOM formation (Miltner et al., 2012). Free amino acids can constitute up to half of the total N in soil (Friedel & Scheller, 2002) and in combination with small peptides constitute over half of the dissolved N in soil (Yu et al., 2002). In our study, the amino acids produced via LAP activity could have been immobilized by microbes into microbial biomass or immediately consumed by plant roots. While we did not measure N mineralization or microbial biomass, LAP activity is shown to positively correlate with both (Ali et al., 2021). Our study calls for further investigation of how ACE protein and SOM depolymerizing enzymes are related to complete N mineralization including the ammonification process.

The poplar and mown grassland systems showed the biggest discrepancies when $NH_4^+$-N and $NO_3^-$-N concentrations peaked across the growing season. The unfertilized poplar and mown grassland systems had the highest $NH_4^+$-N, ACE protein, and TN concentrations in May which may reflect that these low-input and low-management intensity systems promote the buildup of soil N in the forms of $NH_4^+$-N and ACE protein. On the other hand, the poplar system had the highest $NO_3^-$-N concentrations in June, and the mown grassland system had the highest $NO_3^-$-N concentrations in July. The increased $NO_3^-$-N concentrations in the poplar system following the precipitation event in June reflect the high N efficiency of poplar with respect to retaining $NO_3^-$-N in its root systems (Ball et al., 2005), as the system has not received fertilizer since plot establishment in 1989 (Syswerda et al., 2012). The poplar system retains $NO_3^-$-N and has high N use efficiency as evidenced by the miniuscule $NO_3^-$-N leached compared to the other systems (Syswerda et al., 2012). The differences in peaks of $NO_3^-$-N and $NH_4^+$-N with sampling timepoints across the growing season in the poplar and mown grassland systems system further emphasize the importance of considering $NO_3^-$-N and $NH_4^+$-N as functionally distinct inorganic N fractions.

## 4.5 | How does ACE protein correlate to other N-related soil health metrics?

We observed that ACE protein captured variability across the different systems that ranged in perenniality and diversity which shows its potential for being a strong indicator of soil health and regenerative agricultural practices. In our study, ACE protein was more consistently responsive to sustainable management practices including perenniality and crop diversity and fluctuated less across a single growing season than the other N-related soil health metrics. Previously, Sprunger et al. (2021) found that ACE protein was not impacted by tillage, perenniality, or manure applications across organic corn production systems in farmers' fields across the Eastern Corn belt using 4 years of crop rotation history and management data; however, we observed benefits of perenniality and crop diversity on ACE protein after 30 years of consistent long-term management. Concentrations of ACE protein were most correlated with TN and $NH_4^+$-N and to a lesser extent with $NO_3^-$-N and minimally correlated with LAP activity. The strong correlations between ACE protein and TN show that organically bound soil N content is strongly tied to total soil N status as organic N constitutes ~90% of TN (Kelley & Stevenson, 1995). Since ACE protein was so strongly correlated with $NH_4^+$-N and reflects the organically bound N in the soil (Hurisso & Culman, 2021; Rillig et al., 2007), ACE protein appears to act more as a sink of N rather than a pool of N that can be easily lost. However, further studies using stable isotopes would have to be conducted to prove this speculation. Ultimately, the trends in ACE protein may mean that it can reflect legacy soil organic N status across different management systems.

### 4.6 | Which N-related soil health metrics are most closely associated with crop yield?

To assess how N-related soil health metrics correlate with crop productivity in a single growing season, we compared ACE protein, total soil N, inorganic N, and LAP activity with yield from soybean systems and aboveground biomass from the switchgrass system. In general, yields of the soybean-based systems were negatively correlated with N-related soil health indicators in the beginning of the season, and the correlation coefficients increased as time progressed. While we did not measure soil aggregate stability, we speculate that the increasing trends in correlations between yield and different soil N fractions across the growing season could reflect time-dependent relationships between plants and occluded soil N in microaggregates (Cates & Ruark, 2017).

Our study is the first to investigate how ACE protein relates to soybean yield and switchgrass biomass. However, the positive trends of ACE protein with soybean yield and switchgrass biomass at the end of the growing season in August in all systems coincide with previous research that has shown that ACE protein is positively linked to corn yield (Sprunger et al., 2019; Svedin et al., 2022; Wade et al., 2020) and crop yield in dryland cropping systems in coarse-textured soil (Sainju et al., 2022). We speculate that ACE protein contributed to increased plant productivity due to its role as the largest fraction of organically bound N that may be available to microbes through mineralization to provide plant-available inorganic N (Hurisso et al., 2018).

The differences in correlations of yield to the N-related soil health indicators show the importance of measuring soil N in the beginning of the growing season after planting has occurred. These differences also show how the N-related soil health indicators that are measured can impact the correlations observed with crop yield. It must be acknowledged though that this study is focused on soil health indicators that reflect N cycling, and it may be important to consider interactions between N fractions and select C fractions to interpret nutrient mineralization and relationships with crop yield. For example, soybean yields were better predicted by mineralizable soil C compared to total or inorganic soil N (Culman et al., 2013). Furthermore, permanganate oxidizable soil C was a better predictor of soybean yield in Malone et al. (2023) compared to mineralizable soil C, potentially mineralizable N, and ACE protein. Similarly, Svedin et al. (2022) reported that permanganate oxidizable C was a better predictor of corn yield compared to ACE protein or mineralizable soil C. However, the present study focuses on how ACE protein and other N-related soil health indicators correspond to differences in soybean yield and switchgrass biomass across a perenniality and management intensity gradient in a single growing season. Ultimately, the general positive trends of ACE protein with crop productivity show its potential for being an indicator of soil health and a reflection of N cycling and bioavailability in the plant–soil–microbe continuum. However, ACE protein must be investigated further for its relationship with plant productivity in other soil types and cropping systems, and we stress the importance of considering more than one soil health indicator to monitor soil health and maximize yield.

## 5 | CONCLUSIONS

The results of this study show the value of including ACE protein in the assessments of soil health and nutrient management practices that impact crop productivity. Measuring organic and inorganic fractions of soil N provided insight into how N cycling changes across a single growing season in different long-term management systems that differ in perenniality and crop diversity. Furthermore, we showed that ACE protein responded to increased perenniality and crop diversity across systems and was more stable across the growing season in individual systems compared to other N-related soil health metrics including TN, $NO_3^-$-N, and $NH_4^+$-N. Our study revealed that ACE protein is more strongly correlated with TN and $NH_4^+$-N compared to $NO_3^-$-N throughout a single growing season in a long-term management intensity experiment that ranges in synthetic N inputs, perenniality, and crop diversity. The increasing trend in correlation coefficients of N-related soil health indicators and yield/biomass in select systems over the course of a single growing season further shows the importance of considering how soil N fractions change over time and how these changes impact plant-available soil N. The results of this study are valuable to scientists and producers who manage different land uses that vary in management intensity, perenniality, and crop diversity. Moreover, this study can aid in a better understanding of the relationships between organically bound soil N and plant-available soil N, and how these distinct fractions relate to short-term outcomes, such as crop yield, and long-term outcomes, such as sustained soil health.

## AUTHOR CONTRIBUTIONS

**Katherine Naasko**: Formal analysis; investigation; visualization; writing—original draft. **Tvisha Martin**: Data curation; methodology; writing—review and editing. **Christian Mammana**: Data curation. **Jacob Murray**: Data curation. **Meredith Mann**: Data curation; methodology; writing—review and editing. **Christine Sprunger**: Conceptualization; funding acquisition; project administration; resources; supervision; writing—review and editing.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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