THE INFLUENCE OF CROPPING SYSTEM, SOIL PROPERTIES AND MANAGEMENT ON BIOLOGICAL INDICATORS OF SOIL HEALTH IN WISCONSIN PASTURES

By

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Abstract

Opportunities to build soil health vary across cropping systems due to differences in crop rotation, carbon inputs and integration of livestock. The objective of this study was to evaluate how biological indicators of soil health compare across four common Wisconsin cropping systems: annual rotations with synthetic fertilizer inputs, annual rotations with manure applications, forage-based rotations that include a perennial legume or grass, and grazed pastures. Data (n=630) were compiled from three recent Wisconsin soil health assessments that analyzed soil organic matter (SOM), permanganate oxidizable carbon (POXC), autoclave citrate extractable protein (ACE), mineralizable carbon (MinC), and anaerobic potentially mineralizable nitrogen (PMN) in the top 15cm of the soil profile. In order of increasing values of soil health, systems typically ranked: annual rotations, annual rotations with manure, forage-based rotations, and pasture. SOM, ACE, MinC and PMN were greater in pastures compared to all other cropping systems and POXC was greater in pastures and forage-based rotations compared to annual systems. Additionally, POXC, MinC and PMN were greater in forage-based rotations compared to annual cropping systems, whereas SOM and ACE were not. POXC and MinC were the only measures that were greater in annual systems with manure, compared to those without. This study demonstrated that systems that incorporate perenniality and manure inputs were associated with greater biological indicators of soil health compared to annual cropping systems.

1. Introduction

Improving soil health in agricultural systems has been a central focus for farmers, agricultural and conservation professionals, and researchers. The definition of soil health as "the continued capacity of a soil to function as a vital living ecosystem that sustains plants, animals and humans" (Natural Resource Conservation Service; "NRCS"), becomes more complex when considering how best to apply it to the land. Five guiding principles have been identified to build healthier soil within agricultural systems: 1) minimize soil disturbance, 2) maximize biodiversity, 3) maximize soil cover, 4) maintain year-round living roots, and 5) integrate livestock (Franzluebbers et al., 2012; Karlen et al., 2019; Lehmann et al., 2020; Moebius-Clune, 2016; NRCS; Norris et al., 2020; Stott et al., 2021). Integrating livestock, which may include manure applications, growing forage crops, or grazing, is a recent addition to the soil health principles as a way to recouple plant and animal management for tighter nutrient cycling (Franzluebbers et al., 2012; Magdoff & van Es, 2021).

Opportunities to integrate these guiding principles vary by cropping systems, increasing from annual rotations to forage-based rotations, to pasture systems (Table 1.1). Grazed, perennial pastures inherently apply all five key soil health principles. Forage-based rotations that include a perennial crop have the benefit of living roots, reduced soil disturbance, and greater soil cover during that portion of the crop rotation, and typically receive manure inputs. Comparatively, annual rotations require intentional management decisions to integrate soil health principles into the cropping system. Building healthier soil in annual cropping systems can come from reduced tillage, residue management, cover cropping and manure use, which are most effective when applied together to the landscape (Culman et al., 2013; Morrow et al., 2016; Nunes et al., 2018; Nunes, Karlen, et al., 2020; Sprunger et al., 2020). To assess if integrating these principles amounts to measurable impacts to soil health and agronomic or environmental benefits, measuring soil health is a valuable tool to monitor changes over time.

Soil health indicators include simple measurements of soil biology, such as labile carbon (C) and nitrogen (N) pools, as well as C and N mineralization. Measurements of labile soil C and N reflect not only active C and N and potential nutrient availability, but also microbial activity, thus serving as biological indicators of soil health. To track changes in soil health, promoted measures of labile C and N include: permanganate oxidizable C (POXC), autoclave citrate extractable protein (ACE), mineralizable carbon (MinC) and anaerobic potentially mineralizable nitrogen (PMN) (Culman et al., 2012; Drinkwater et al., 1996; Franzluebbers et al., 2000; Hurisso et al., 2018). These measurements are evidence based, logistically feasible, cost effective, responsive to management, and agronomically valuable, underscoring their potential as soil health indicators (Amsili et al., 2021; Culman et al., 2013; Franzluebbers, 2018; Hurisso et al., 2016; Idowu et al., 2009; Morrow et al., 2016; Nunes et al., 2018; Nunes, Karlen, et al., 2020; van Es & Karlen, 2019; Wade et al., 2020).

In Wisconsin, cash grain and dairy operations are prevalent, and common cropping systems include annual rotations, forage-based rotations, and grazed pasture. Given that manure is a valuable and accessible nutrient source, annual systems with and without manure are well represented in Wisconsin, compared to other Midwestern states where annual rotations predominantly rely on synthetic fertilizer. Research at the Wisconsin Integrated Cropping Systems Trial (WICST) evaluated biological soil health across common Wisconsin cropping systems: three annual grain systems (continuous corn, corn-soybean, and organic grain), two forage-based systems with alfalfa (conventional and organic), and a well-managed, rotationally grazed pasture. Biological indicators of soil health did not differ among annual grain systems, or between forage systems, revealing that subtle shifts in management within cropping systems were insufficient to improve soil health (Diederich et al., 2019). Rather, large shifts in cropping system from annual grain to forage systems were necessary to achieve greater POXC, MinC and PMN (Diederich et al., 2019).

While long-term cropping system trials like WICST are invaluable to evaluate soil health while controlling for inherent environmental and soil properties, on-farm soil health evaluations are necessary to test these findings across a large network of operational farms and improve recommendations to farmers. Further, regional variability in soil health measurements attributed to differences in climate, soil, and cropping systems, underscores the need for a Wisconsin-specific soil health database (Crookston et al., 2021; Fine et al., 2017; Nunes, van Es et al., 2020). We compiled on-farm soil health data from three Wisconsin projects with the same research design to evaluate biological indicators of soil health across common cropping systems. To do this, fields were categorized into four cropping systems: annual rotations with synthetic fertilizer inputs, annual rotations that apply manure, forage-based rotations that include a perennial legume or grass, and grazed pastures. SOM and biological indicators of soil health, POXC, MinC, ACE and PMN, were measured to determine if there were measurable differences in soil health associated with cropping systems. We hypothesized that biological indicators of soil health would differ with cropping system, increasing from annual rotations to forage-based rotations, to pastures.

Table 1.1: Integration of key soil health principles by cropping system: annual rotations, forage-based rotations, and pasture

Cropping System			
	Annual	Forage-based	Pasture
	Inherently annual cropping systems do not meet soil health principles. Intentional management is required to increase soil health promoting practices and include:	Including a perennial in the rotation satisfies several soil health principles. During the annual portion of the rotation, opportunities to manage for soil health resemble those of annual cropping systems	Pastures inherently satisfy all soil health principles. Best management of pastures can improve how well these goals are met.
	Increasing integration of soil health principles →		
Minimize soil disturbance	Reduced or no-till	No-till in perennial crop	No-till
Biodiversity	Diverse crop rotation, cover crops	Includes annual and perennial crops	Typically consist of many grass, legume and forb plant species
Soil cover	Residue management, cover crops	Soil coverage and protection during the perennial crop	Perennials cover and protect the soil
Continual living roots	Cover crops	A portion of the rotation is perennial	Consistent continual living roots in perennial system
Livestock	Manure	Includes forage crop and (typically) manure	Grazing livestock

2. Materials and Methods

2.1 Study Design & Field Descriptions

This study used an on-farm, exploratory research design to evaluate soil health across Wisconsin agricultural systems (Figure 1.1). From 2015 through 2021, three projects employed this research design (see Richardson (2018), Malone (2022), and Augarten (2022) for complete details on each project). Richardson collected samples from 2015 through 2017 in fields planted to corn that year (n=218), Malone collected samples from 2019 through 2021 in fields planted to soybean that year (n=320), and Augarten collected samples in 2021 from pastures that had been grazed for at least one year (n=92). Data from all three studies were compiled, then divided into four cropping systems: annual rotations (n=223), annual rotations with manure (n=177), forage-based rotations (n=138), and pasture (n=92) (Table S1.1). Sites from Richardson (2018) and Malone (2022) included fields in the first three cropping systems. Cropping system differences were determined based on agronomic information reported by the farmers or crop advisors managing the fields. The majority of annual rotations were corn-soybean rotations, approximately half were no-till, and about half used cover crops in the past five years. Sites were predominantly silt loam soils (70%). Complete site descriptions are found in Richardson (2018) and Malone (2022).

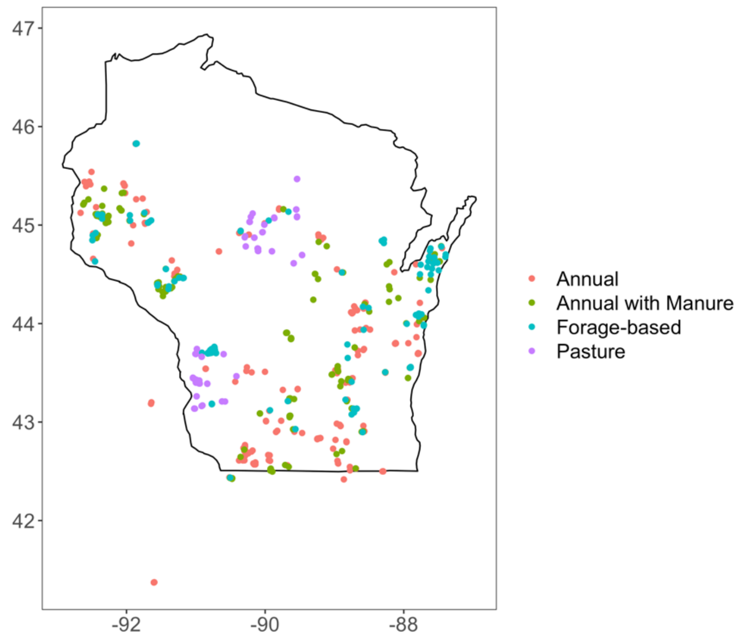


Figure 1.1: Map of sites included in this study (n = 630), categorized by cropping system. Annual, n= 214 (red); annual with manure, n=180 (green); forage-based, n=138 (blue); and pasture, n=92 (purple).

2.2 Soil sampling and handling

Between late April and mid-June (2015-2021) soils were sampled to a depth of 0-15 cm from a representative area using a probe with an internal diameter of 2.5 cm. Studies varied slightly in their soil sampling methodology (see Augarten, Malone, and Richardson for complete materials and methods). In brief, Richardson (2018), transferred samples to a freezer after sampling and within a month, samples were thawed and dried for one week at 32°C in a forced air drier, and ground through a 2-mm sieve. In 2019, Malone (2022) collected and immediately air-dried samples for one week before processing. However, in 2020 and 2021, samples were collected by farmers or crop advisors and mailed to University of Wisconsin-Madison prior to drying. Upon receipt, samples were air dried for ~1 week and ground through a 2-mm sieve. In Augarten, samples were kept on ice after sampling, transferred to a refrigerator within 12 h and, within 3 d, were dried in a forced air drier at 32°C and ground through a 1-mm sieve.

2.3 Soil analyses

Samples from all studies were analyzed for SOM via loss on ignition, POXC, MinC and PMN. Soils in Malone (2022) and Augarten (2022) were also analyzed for ACE protein (reduced site numbers for this analysis were: annual (n=174), annual with manure (n=101), forage-based (n=45), and pasture (n=92)).

Samples were sent to the UW Soil and Forage Analysis Lab in Marshfield, WI for routine pH, Bray-1 P, Bray-1 K, and SOM-LOI. PMN was analyzed in-house at University of Wisconsin-Madison, using the Drinkwater protocol for anaerobic PMN (Drinkwater et al., 1996). To calculate PMN, a 7-d anaerobic biological incubation was conducted and the amount of ammonium in the non-incubated soil was subtracted from that of the incubated sample.

Richardson analyzed POXC and MinC in-house, and Malone and Augarten sent samples to Ohio State University's soil test lab to measure POXC, ACE and MinC as part of the "Active Organic Matter" package. The same protocols were used by both labs. POXC was determined through oxidation with 0.2-*M* KMnO4, according to methods described by Culman et al. (2012) (modified analysis of Weil et al., 2003). MinC was measured through a 1-d incubation on soils at 50% water filled pore space (Franzluebbers, Haney, et al., 2000). ACE was measured through a chemical extraction using sodium citrate and autoclaving the sample (Hurisso, Moebius-Clune, et al., 2018).

2.4 Statistical analyses

Differences in soil measurements by cropping system were evaluated using analysis of variance (ANOVA). All data analysis was performed in RStudio version 2021.9.0.351 using R statistical software version 4.1.1 (R Core Team, 2020). ANOVA was performed using the aov() and summary() functions. When there was a statistical difference (p-value <0.05), Fisher's least significant difference (LSD) was used to determine how values differed among cropping systems.

3. Results & Discussion

Biological indicators of soil health increased from annual systems to forage-based rotations, to pastures (Figure 1.2, Table S1.2). All measurements, SOM, POXC, MinC, ACE and PMN, were greater in pastures compared to annual rotations, and all but POXC were greater in pastures compared to forage-based rotations, as well. While the response of SOM and ACE was muted and only differentiated between pastures and the other cropping systems, MinC, POXC and PMN differed among annual and forage-based rotations. POXC, MinC and PMN were greater in forage-based rotations than either annual cropping system. However, only the labile C measurements, MinC and POXC, differed between the annual systems and were greater in systems that included manure.

These results aligned with other regional studies in the United States. Findings from the Wisconsin Integrated Cropping Systems Trial demonstrated that POXC, MinC and PMN were consistently greater in forage and pasture systems compared to annual grain systems (Diederich et al., 2019). On-farm soil health assessments in New York reaffirmed the association between agricultural system and soil health and found that generally pastures and mixed vegetable systems had greater biological soil health than dairy cropping systems, followed by annual grain and processing vegetables (Amsili et al., 2021). Additionally, in a long-term cropping systems study in Ohio, Sprunger et al. (2020) observed increases in biological soil health in systems with greater crop diversity and perenniality.

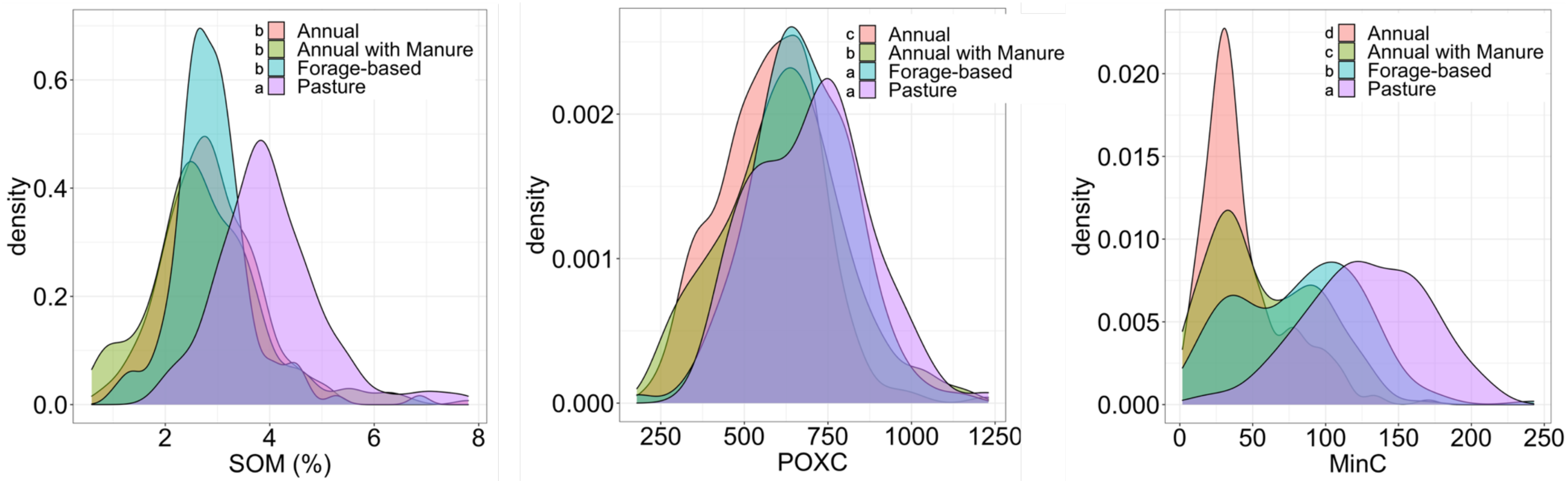
Benefits of perennial pastures to soil C and N pools over row cropping systems are well documented (Becker et al., 2022; Franzluebbers et al., 2012; Franzluebbers, Stuedemann, et al., 2000; Guillaume et al., 2021; Oates & Jackson, 2014; Rowntree et al., 2020; Sanford et al., 2012). Well-managed perennial pastures best integrate soil health building principles compared to annual and forage-based rotations; they minimize soil disturbance, maximize biodiversity, maximize soil cover, maintain year-round living roots, and integrate livestock. Perenniality, in pasture or even just as a rotation in a forage-based cropping system, protects the soil surface, reduces soil disturbance and provides extensive above- and below-ground C inputs, through continual living roots, longer growing seasons, fine root production and turnover, and root exudates (Cates et al., 2016; Franzluebbers & Stuedemann, 2015; Jackson et al., 1997; Sprunger et al., 2017; Teague & Kreuter, 2020). What sets pastures apart from all other cropping systems is their continuous perenniality, crop diversity (including multiple grass, legume, and forb species), and grazing, which stimulates greater above- and below-ground C inputs and provides manure deposition (Franzluebbers & Stuedemann, 2015; Teague & Kreuter, 2020). The adoption of well-managed pasture offers the greatest opportunity for improvements in SOM and biological soil health.

Our findings reflect that biological indicators of soil health have varying sensitivity to cropping system and soil health building practices. POXC, MinC, and PMN, were more sensitive to management compared to SOM, as observed in previous research (Culman et al., 2012, 2013; Hurisso et al., 2016; Stott et al., 2021; Wander & Drinkwater, 2000). MinC was the most sensitive indicator and differed across all four cropping systems, aligning with findings from other studies (Amsili et al., 2021; Diederich et al., 2019; Sprunger et al., 2020). However, our results also showed that soils from Malone (2022), which represent 81% of annual rotations, 57% of annual rotations with manure, and 33% of forage-based rotations, had lower MinC on average compared to Richardson (2018) (Table S1.3). We speculate that mailing in field moist samples without ice packs suppressed aerobic microbes (Malone, 2022; Moebius-Clune, 2016). Additionally, compared to other soil health indicators, MinC is associated with higher analytical, temporal and spatial variability and sensitivity (Crookston et al., 2021; Hurisso, Culman, et al., 2018; Morrow et al., 2016; Wade et al., 2018). Removing Malone MinC data from the statistical analysis revealed a similar trend in MinC, with annual rotations having lower values compared to forage-based rotations and pastures. However, there was no differentiation between annual systems with or without manure (Table S1.4). In the context of this simple system comparison study, we cannot deduce if the Malone data skewed the results to detect a difference in MinC between annual and annual with manure systems, or if it just provided more data that led to a difference being detected. This aspect of our dataset comparison highlights that MinC is sensitive to differences in soil sample handling, which needs to be considered for future soil health testing and practical applications for farmers.

Observed differences in indicator response to cropping system reaffirms that these measures of labile C and N are capturing nuanced information on soil health. MinC is more responsive to practices associated with SOM mineralization, such as manure use, integration of legumes, and perenniality, which likely contributed to greater differences in MinC among all cropping systems in this study (Amsili et al., 2021; Culman et al., 2013; Diederich et al., 2019; Hurisso et al., 2016; Sprunger et al., 2020). Comparatively, POXC is more associated with practices that promote SOM accumulation and stabilization, including reduced tillage and inputs of stable carbon (Hurisso et al., 2016; Nunes, Karlen, et al., 2020). In this study, forage-based rotations and pastures, which have reduced tillage and higher C inputs from crop biomass, were associated with greater POXC than annual rotations. Additionally, POXC was the only measurement that did not differ between forage-based rotations and pastures, which aligns with previous soil health work (Amsili et al., 2021; Diederich et al., 2019; Sprunger et al., 2020). Previous studies observed similar results for labile N measures: ACE did not differ between annual and forage-based rotations (Amsili et al., 2021), and PMN was more sensitive to cropping system or crop rotation, but less sensitive to nutrient source, like manure or compost (Culman et al., 2013; Diederich et al., 2019; Morrow et al., 2016).

Applying manure is a promoted soil health building practice, but in our study only POXC and MinC differed between annual systems with and without manure. Hurisso et al. (2016) demonstrated greater increases to MinC due to manure use, relative to POXC or SOM. In other studies, applying manure was associated with greater POXC (Min et al., 2003; Mirsky et al., 2008), or had no effect (Lewis et al., 2011; Wienhold, 2005). While manure is a valuable nutrient source in Wisconsin and often is promoted in annual systems to improve soil health, there are inconsistent effects of manure use on SOM, and C and N pools in previous research. Ten years of dairy manure applications had no effect to SOM in Wisconsin cash grain systems (Rui et al., 2020), while a meta-analysis of Midwest farms revealed that manure increased surface soil organic carbon by 39% (Nunes, van Es, et al., 2020). Manure type, including livestock species and manure consistency, can influence C accrual; while liquid manure has high N content and may promote SOM mineralization, solid manure has higher carbon-to-nitrogen ratio, which typically is more conducive to SOM gains (Eghball, 2002; Rui et al., 2020).

Manure applications are associated with greater soil health when used in conjunction with C inputs, like cover crops or crop residue (Aguilera et al., 2013; van Es & Karlen, 2019). In Wisconsin, Jokela et al. (2009) found that corn silage rotations with cover crops and dairy liquid manure had greater POXC than those with just manure. Stacking soil health practices like manure use, reduced soil disturbance and C inputs results in additive benefits to soil health (Aguilera et al., 2013; Franzluebbers & Stuedemann, 2015; Nunes, Karlen, et al., 2020).



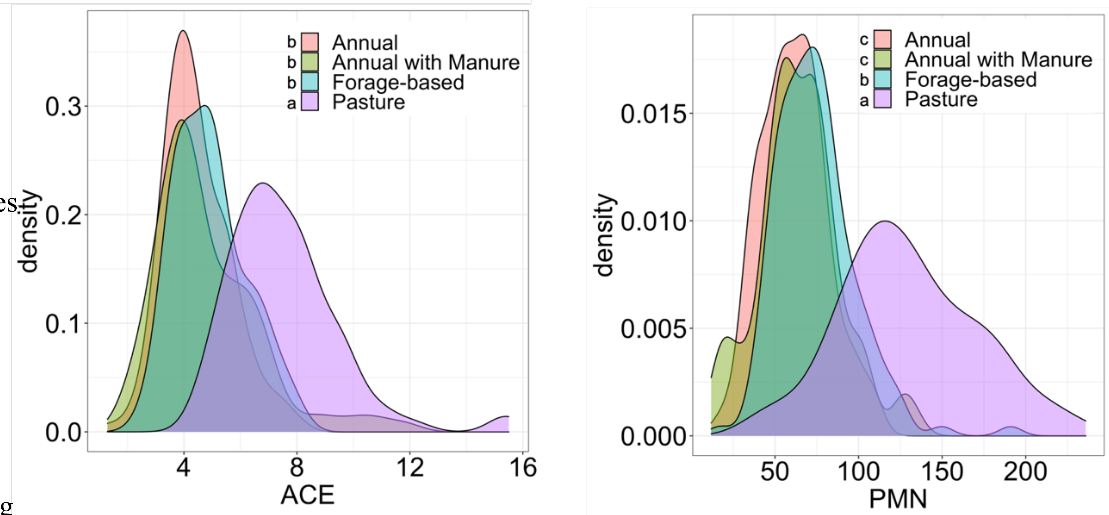


Figure 1.2 Density graphs of SOM, POXC, MinC, ACE and PMN by cropping system.
Density refers to the frequency of data for a given value. In the legend, letters next to the cropping system indicate significant differences (p-value < 0.05) as determined through ANOVA and Fisher's least significant difference. Cropping systems include: annual, n= 214 (red); annual with manure, n=180 (green); forage-based, n=138 (blue); and pasture, n=92 (purple).
SOM=soil organic matter (%); POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

4. Conclusion

This regional cropping system comparison of biological indicators of soil health clearly demonstrated that systems with perenniality are associated with greater soil health. Pastures, which include all soil health building principles, had the greatest value for SOM and biological indicators. When comparing a single soil health building practice (manure addition) in annual cropping systems, only POXC and MinC were greater in the manured sites. This supports the idea that single management changes may have limited gains in biological soil health. Therefore, within annual and forage-based cropping systems there is emphasis on stacking many soil health building practices: reduce soil disturbance, increase biodiversity, maintain continual living roots, protect the soil surface, and integrate livestock. As pasture soil health continues to serve as a goal for other cropping systems, continued research is needed to assess if gains in soil health indicators are feasible or realistic within annual cropping systems, or if larger shifts towards pasture-based systems are necessary.

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Supplementary Materials

Table S1.1: Site numbers for each cropping system and the proportion of sites from each project (Richardson, Malone, Augarten)

Cropping System	Annual	Annual with Manure	Forage-based	Pasture
Site numbers (n)	214	180	138	92
Project		%		
Richardson	19	43	67	0
Malone	81	57	33	0
Augarten	0	0	0	100

Table S1.2: Mean values (SD) and ANOVA results of biological soil health values by cropping system.

All results are significant at a p-value <0.001. Mean values followed by different letters were determined using Fisher's LSD. For SOM, POXC, MinC and PMN, the number of sites were: annual (n=214), annual with manure (n=180), forage-based (n=138), and pasture (n=92). ACE was only analyzed in two of the projects and reduced site numbers are: annual (n=173), annual with manure (n=102), forage-based (n=45), and pasture (n=92).

	SOM	POXC	MinC	ACE	PMN
Cropping System	%	--------- mg kg^{-1} --------		g kg^{-1}	mg kg^{-1}
Annual	2.9b (0.9)	576c (151)	43d (29)	4.5b (1.2)	61c (19)
Annual with manure	2.8b (1.1)	616b (183)	58c (37)	4.8b (1.9)	65c (25)
Forage-based	2.9b (0.8)	673a (152)	82b (43)	4.9b (1.3)	74b (24)
Pasture	4.0a (1.0)	701a (170)	128a (41)	7.6a (2.0)	130a (41)

SOM= soil organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon; ACE = autoclaved-citrate extractable protein; MinC = mineralizable carbon; PMN = potentially mineralizable nitrogen

Table S1.3: Mean values and ANOVA results of MinC for between Richardson and Malone studies.
All results are significant at a p-value <0.001. The difference in MinC between the two projects was analyzed within each cropping system. Mean values followed by different letters were determined using Fisher's LSD.

MinC (mg kg^{-1})			
	Annual	Annual with manure	Forage-based
Project			
Richardson	88a	92a	106a
Malone	33b	32b	33b

MinC = mineralizable carbon

Table S1.4: Mean values and ANOVA results of MinC by cropping system, excluding MinC data from the Malone study.
Results are significant at a p-value <0.001 and mean values followed by different letters were determined using Fisher's LSD. Adjusted site numbers are: annual (n=41), annual with manure (n=78), forage-based (n=93), and pasture (n=92).

Cropping System	n	MinC (mg kg^{-1})
Annual	41	88c
Annual with manure	78	92c
Forage-based	93	106b
Pasture	92	128a

MinC = mineralizable carbon

R Code

Univariate Statistics:

```
#Example for Annual cropping systems
#group soil C and N indicators
library(dplyr)
Indicators <- Annual %>%
  dplyr:: select(OM, POXC, ACE, MinC, PMN)

#analysis
library(pastecs)
library(psych)
summary(Indicators)
describe(Indicators)
stat.desc(Indicators)
```

ANOVA

```
library(agricolae)
model <- aov(OM ~ SystemGeneral, data= SystemsData)
summary(model)
lsd=LSD.test(model, c("SystemGeneral"), group=T)
lsd
```

Chapter 2: Soil health assessment of Wisconsin pastures reveal soil carbon and nitrogen metrics are sensitive to inherent soil properties, land use, and management

Abstract

Pastures, which include soil health building practices like perenniality, reduced soil disturbance and livestock integration, provide greater opportunities to improve soil health and benefit soil carbon (C) storage compared to row cropping systems. Given that most of the soil health research has been conducted in row cropping systems, it is vital to explore how grazing management can influence soil health and C and nitrogen (N) pools in order to provide graziers with best management recommendations. In Wisconsin, 92 pastures were evaluated for commonly used metrics, organic matter (OM), total carbon (TC), total nitrogen (TN), and carbon to nitrogen ratio (C:N), and four biological indicators of soil health, permanganate oxidizable C (POXC), autoclave citrate extractable protein (ACE), mineralizable carbon (MinC), and anaerobic potentially mineralizable nitrogen (PMN). The objectives of this study were to evaluate relationships and correlations among soil C and N measurements, and the relative importance of inherent soil properties, land use history, and management on these measurements. POXC, ACE and PMN had strong positive relationships with OM, TC, and TN (R^2=0.45-0.65), while MinC had weaker relationships with these measurements (R^2=0.2-0.35). Correlations between biological incubations (MinC and PMN, r= 0.82) and chemical extractions (POXC and ACE, r=0.68), were stronger compared to those between the two labile C (MinC and POXC, r=0.53) or N (PMN and ACE, r=0.58) measurements. These relationships suggest the soil health indicators reflect different components of biologically active soil fractions. With the exception of C:N, soil C and N measurements were positively related to pasture age (R^2=0.19-0.37) and were higher in continuously grazed pastures, which tended to be older, compared to rotationally grazed pastures. For rotationally grazed pastures (n=78), the relative importance of inherent soil properties, land use history, and management on soil C and N measurements was evaluated using random forest analysis. Pasture age explained more variation in OM, TC, TN, POXC, ACE, MinC, and PMN compared to all other factors. Random forest partial effects revealed rapid gains in predicted soil values after pasture establishment, with potential plateaus. Texture was important for MinC and PMN, with coarser soils corresponding with lower values, and C:N, which conversely, had a positive relationship with sand content. In rotationally grazed pastures, grazing management factors were important, especially for TC, TN, POXC and ACE, and predicted values corresponded with moderate to high grazing pressure over shorter grazing intervals with sufficient rest. Relationships among soil C and N metrics and soil and management factors demonstrated the importance of pasture age, texture, and well-managed rotational grazing on pasture soil health.

1. Introduction

There is increasing urgency to build soil organic matter (SOM) and soil health in our agricultural systems to provide essential ecosystem services such as water and nutrient cycling, agronomic performance, carbon sequestration, water quality, and erosion control (Gregorich et al., 1994; Idowu et al., 2009; Morrow et al., 2016; Wander & Drinkwater, 2000). The integration of pasture and grassland on the landscape allows for ample opportunities to provide these services, as well as the additional benefits of plant and microbial biodiversity, habitat, cultural and aesthetic opportunities, and economic resilience (Franzluebbers et al., 2012; Havstad et al., 2007; Teague & Kreuter, 2020).

In assessments of soil carbon and soil health, pastures typically outperform grain and forage cropping systems. Soil health research has identified five key soil health principles, all of which are included in well-managed pastures: 1) minimize soil disturbance, 2) promote biodiversity, 3) maximize soil cover, 4) maximize continuous living roots, and 5) integrate livestock (Franzluebbers et al., 2012; Karlen et al., 2019; Lehmann et al., 2020; Moebius-Clune, 2016; Natural Resource Conservation Service; Norris et al., 2020; Stott et al., 2021). Long-term trials (Diederich et al., 2019; Machmuller et al., 2015; Sanford et al., 2012; Sprunger et al., 2020), paired pasture-row crop studies (Becker et al., 2022; Franzluebbers, Stuedemann, et al., 2000; Nunes, Karlen, et al., 2020) and on-farm soil health assessments (Amsili et al., 2021; Augarten thesis chapter 1) demonstrate that pastures and/or perennial systems out-perform row cropping systems in SOC and biological soil health. However, given the diversity of pasture systems, there is potential for pasture- and grazing- specific management factors to influence soil C and soil health.

Grazing systems are dynamic and adaptive, ranging from continuous grazing to managed intensive rotational grazing (MIRG) and varying in grazing intensity (stocking density and residual pasture height) and grazing frequency (time in paddock and paddock rest period) (Lyon et al., 2011; Teague & Kreuter, 2020; Undersander et al., 2002). Understanding if, and how, grazing management practices influence soil health is imperative to provide farmers with research-based recommendations to improve economic and environmental outcomes. Previous research has elucidated that the effect of grazing management practices on surface soil organic carbon (SOC) is complex due to sensitivity to climate, soil type, pasture composition, grazing management, research design, and the interactions among these factors (Abdalla et al., 2018; Conant et al., 2017; Derner & Schuman, 2007; McSherry &

Ritchie, 2013; K. Zhang et al., 2015; Zhou et al., 2017). This underscores the need for regional assessments to improve our understanding of how these various factors can influence soil C and N pools.

Prior research in row cropping systems found that evaluating soil health revealed insight into the relationship between management and soil C and N, beyond that learned from solely measuring SOM or SOC. While it may take years to detect improvements in SOM due to shifts in management, labile pools of C and N, which cycle more readily, are sensitive to management and have proven effective as early indicators to changes in SOM (Culman et al., 2012, 2013; Hurisso et al., 2016; Wander & Drinkwater, 2000). Labile soil C and N measurements not only reflect active C and N and potential nutrient availability, but also microbial activity, thus serving as biological indicators of soil health. For soil health indicators to be valuable they must be logistically feasible, cost-effective, accurate and precise, sensitive to management, and of agronomic, economic or environmental value (Idowu et al., 2008; Moebius-Clune, 2016; Morrow et al., 2016).

Measures of labile C and N that meet these criteria and are often used in soil health assessments include two chemical extractions, permanganate oxidizable C (POXC) and autoclave citrate extractable protein (ACE), and two biological incubations, mineralizable carbon (MinC) and anaerobic potentially mineralizable nitrogen (PMN) (Culman et al., 2012; Drinkwater, et al., 1996; Franzluebbers et al., 2000; Hurisso et al., 2018). While all indicators represent soil C and N pools, they each relate differently to biological soil properties. POXC, often termed active carbon, reflects a more processed pool of C and is linked to soil C stabilization (Culman et al., 2012; Hurisso et al., 2016; Weil et al., 2003). MinC, or soil respiration, reflects the pool of C accessible to microbes and the potential microbial activity in the soil (Franzluebbers et al., 2000; Gregorich et al., 1997; Hurisso et al., 2016). ACE measures soil protein, which constitutes a large pool of soil organic N and has a strong association with SOM and soil aggregation (Hurisso, Moebius-Clune, et al., 2018). Lastly, PMN is a measurement of plant available N as a function of anaerobic microbial activity (Drinkwater et al., 1996; Gregorich et al., 1997).

Extensive research on these four indicators has been conducted in grain and forage cropping systems and demonstrated their responsiveness to management practices including tillage intensity, crop rotation diversity, organic amendments, residue management, and perenniality (Amsili et al., 2021; Hurisso et al., 2016; Idowu et al., 2009; Morrow et al., 2016; Nunes et al., 2018; Nunes, Karlen, et al., 2020). Additionally, they have been linked to corn and soybean yield and nutrient use efficiency (B. Crookston et al., 2022; Culman et al., 2013; Franzluebbers, 2018; Oldfield et al., 2022; van Es & Karlen, 2019; Wade et al., 2020). However, biological soil health research in pastures is lacking (Franzluebbers & Stuedemann, 2015), particularly in cool-season, temperate pastures (Arndt et al., 2022), which leaves untapped potential to better understand relationships among inherent properties, pasture and grazing management practices, and soil carbon and nitrogen pools in the Upper Midwest.

Measuring biological soil health in pasture systems through on-farm research serves as a complement to current research on pasture management and SOC. While long-term cropping system trials are invaluable to track changes to soil health over time, few studies extend beyond 20 years after pasture establishment (Byrnes et al., 2018). Controlled research studies on grazing management typically are for a few years, only providing short-term research findings (Teague et al., 2013). Additionally, compared to standardized management found in controlled research designs, on-farm assessments offer opportunities to evaluate a range of grazing management practices used by producers (Teague et al., 2013). In Wisconsin, grazing management varies, ranging from continuous grazing to 2-3 daily rotations, and includes variation in stocking density, rest period and residual pasture height (Lyon et al., 2011; L. Paine & Gildersleeve, 2011.; Undersander et al., 2002). A regional soil health assessment specific to cool-season, temperate pastures, and representative of regional climatic, soil and management factors can provide relevant information to Wisconsin graziers.

This study explored how soil C and N measures, OM, TC, TN, C:N, POXC, ACE, MinC, and PMN, varied across Wisconsin pastures of differing soil types, land use history and pasture and grazing management. The objectives were: i) to determine the relationships between bulk C and N measures, SOM, TC, TN, and C:N, and labile C and N measures, POXC, ACE, MinC, and PMN, and assess the value of biological indicators of soil health, and ii) to evaluate the relative importance of inherent soil properties, land use history and management practices on these metrics. Based on previous WI soil health assessments, for objective one we hypothesized that the biological indicators of soil health are related to the total C and N pools, but differ enough to suggest they reveal new information regarding C and N bioavailability. For objective two, we hypothesized that soil texture and pasture age are influential to soil C and N measures, but that certain management practices may be identified as beneficial to biological soil health, as well.

2. Materials & Methods

2.1 Study Design

The study used an on-farm, exploratory research design. Research was conducted in two regions in Wisconsin: "Kickapoo", named after the Kickapoo watershed, and "Marathon", comprising of sites mainly in Marathon County (Figure 2.1). Thirty-two farmers from these regions chose to participate in the study after discovering the project through local grazing networks, producer led groups, land and water conservation districts, and NRCS agents. A total of 92 pastures were sampled, all of which had been grazed for at least one year. Participants selected pastures based on their interests and to capture variation on their farm attributed to management, land history, soil type or productivity.

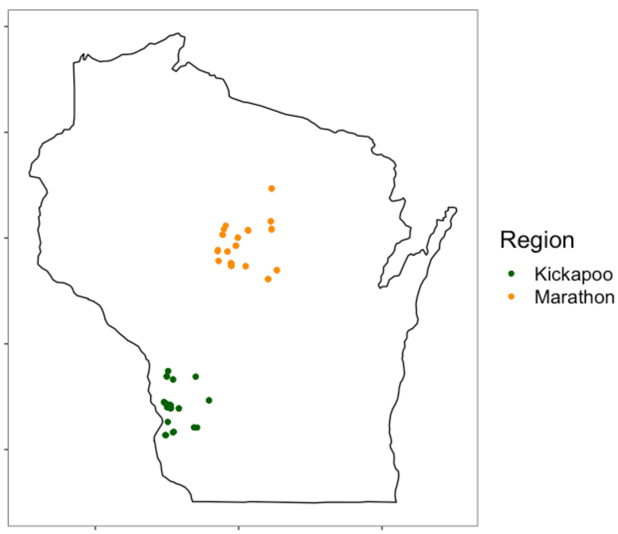

Figure 2.1: Map of sites included in this study (n = 92), grouped by two regions: Kickapoo (green), Marathon (orange).

2.2 Management Data Collection & Site Descriptions

Participants completed a comprehensive management questionnaire that included questions on land use history, pasture management and grazing practices specific to sampled pastures or paddocks. The questions were used to identify long-term, short-term, and immediate factors that may influence soil health indicators. With the understanding that grazing management and systems are dynamic and adaptive, producers reported typical values or averages across the season for their current operations. Table 2.1 includes a complete list of inherent properties and management practices included in the analysis.

Most sites were silt loam soils (73%) and well drained (57%). Farms included dairy (17%), dairy heifers (11%), beef (including cow/calf, stocker and finishing operations) (57%), and multispecies (15%) grazing. About half of the pasture sites were less than ten years old, with the remaining pastures ranging widely from 10 to 100 years old. 88% of the sites were rotationally grazed, while the remaining sites were continuously grazed. Sites varied in pasture and grazing management practices, including outwintering, nutrient inputs, haying, and stocking and rotation factors (Tables S2.1-S2.5).

Table 2.1: Soil, land use and management variables included in the data analysis. Sand, silt, clay, and pH were directly measured, and drainage class was identified using the Web Soil Survey. Land use history, grazing and pasture management factors were provided by the farmer. Stocking rate, stocking density and seasonal grazing pressure were calculated using animal units (total livestock weight in kg divided by 454 kg) and paddock size (ha).

Variable Category	Variable
Soil Properties	pH
	sand (%)
	silt (%)
	clay (%)
	Drainage Class
	Texture Group*
Land Use	Region
	Land ownership (owned or rented)*
	Farm Operation Type
	Pasture age (years)
	Previous land use
Grazing management	Grazing management category (rotational vs. continuous)**
	Days between grazing and soil sampling (days)
	Number of rotations
	Stocking Rate (animal units/total pasture hectares)
	Stocking Density (animal units/paddock hectares)
	Seasonal Grazing Pressure (stocking density x grazing days per season)
	Time in paddock per grazing event (days)
	Paddock rest period (days)
	Residual pasture height (cm)
Pasture Management	Percent legume cover
	Outwintered in the past five years
	Hay frequency in the past five years
	Synthetic fertilizer use in the past five years
	Synthetic fertilizer applied this year prior to sampling
	Synthetic fertilizer application frequency in the past five years
	Manure use in the past five years
	Manure applied this year prior to sampling*
	Manure application frequency in the past five years
	Lime applied in the past five years

*variables excluded in random forest analysis because greater than 90% of the data were the same value

**variables excluded in random forest, which only included rotationally grazed pastures

2.3 Soil sampling & in-field data collection

Soil samples were collected 2-11 June 2021. Within each pasture, a 50m x 50-m area was selected that was a uniform soil type and representative of the pasture. Coordinates from the center of the sampling area were recorded and used to retrieve drainage class from the NRCS Web Soil Survey.

At each site, 20 soil cores were taken to a depth 0-15 cm using a probe with an internal diameter of 2.5 cm, then combined into one composite sample. Samples were kept on ice for up to 12 hours and then transferred to a refrigerator for up to three days. Roots were removed from the soil sample and samples were then air dried and ground to pass through a 1-mm sieve.

At the time of soil sampling, visual in-field estimates of percent legume cover were taken using a 0.25-m^2 quadrat. Quadrats were laid out using three transects spaced 15 m apart and 5 replicates spaced 10 m apart within each transect. The 15 replicates were averaged, and this average was used for data analysis.

2.4 Soil Analysis

Soil samples were analyzed for pH, Bray-1 P, Bray-1 K, OM-loss on ignition (LOI), percent sand and clay, nitrate and ammonium, TC, TN, POXC, ACE, MinC and PMN. Samples were sent to the UW Soil and Forage Lab in Marshfield, WI for routine pH, Bray-1 P, Bray-1 K, OM-LOI, and texture analysis. Particle size analysis was conducted via the hydrometer method (Bouyoucos et al., 1962).

TC and TN were measured with a dry combustion method, using a Flash EA 1112CN Automatic Elemental Analyzer. Between 24.5 and 25.5 mg of finely ground soil was packed into 10 x 12 mm tin capsule prior to combustion. Total C was considered the same as organic carbon, after conducting an HCl fizz test, which demonstrated the lack of inorganic carbon in the samples.

Three of the soil health analyses, POXC, ACE and MinC were conducted at Ohio State University's soil test lab as part of the "Active Organic Matter" package. POXC was measured using protocols by Culman et al. (2012), adapted from Weil et al. (2003) protocol, where the carbon is oxidized with 0.2-*M* $KMnO_4$ and the color change is read on a spectrophotometer. MinC is determined through a one-day incubation. Soils were rewetted and incubated at 25°C for 24 hours, and then CO_2 evolution measured using an infrared gas analyzer (Franzluebbers, Haney, et al., 2000). Lastly, ACE was measured by adding sodium citrate, autoclaving the sample, extracting protein, and quantifying the concentration compared to a standard with a spectrophotometer (Hurisso, Moebius-Clune, et al., 2018).

PMN was analyzed in house at University of Wisconsin-Madison using the Drinkwater protocol for anaerobic PMN (1996). PMN was calculated by measuring the ammonium produced during a 7-d anaerobic incubation and subtracting from this the amount of ammonium already in the soil. For the incubated samples, 10mL of deionized water was added to 5mg of dried and ground soil, and then incubated at 40°C for 7 d (+/- 2 hours). After incubation, ammonium was immediately extracted by adding 40 mL of 2.5-*M* KCl to the sample, shaken for 1 h, centrifuged, and filtered. For the incubated samples, there were two technical replicates. The nonincubated samples were analyzed according to the same procedure, except that 10ml of deionized water and 40 mL of 2.5-*M* KCl were added to 5mg of soil just prior to shaking, without any incubation. The supernatant was stored frozen and sent to the UW Marshfield Soil and Forage Analysis lab within one month to quantify ammonium. The ammonium present in the nonincubated sample was subtracted from the average ammonium of two incubated samples, to calculate PMN. Nitrate and ammonium from the nonincubated sample were included as inorganic measurements of nitrogen.

2.5 Statistical Analysis

Data analysis was performed in RStudio version 2021.9.0.351 using R statistical software version 4.1.1 (R Core Team, 2020). Descriptive statistics were performed with R packages, Tidyverse (Wickham et al., 2019) and Psych (R Studio Team, 2022). Correlation among soil health values was analyzed with ggpairs()and corrplot() functions. Single factor analysis was run using one-way ANOVA and linear regression. For ANOVA, the aov() and summary() functions were used and when there was a statistical difference (p-value <0.05), Fisher's least significant difference (LSD) was conducted using the function LSD.test() from the agricolae package. For linear regression, lm() and summary() functions were used. Assumptions of linear models, normality, and equal and constant variance were assessed using QQ and residuals vs. fitted plots. In linear regression, explanatory variables were transformed when necessary to uphold assumptions. For select response variables, clay content, stocking density, seasonal grazing pressure, and number of rotations were transformed with a polynomial transformation. For some variables, no transformations could correct violations of normality and equal variance. Single factor analysis was run on transformed variables when applicable. A p-value less than or equal to 0.05 was considered statistically significant. All single factor analysis is included in supplementary materials.

Out of the 92 sites, 3 sites did not have complete management, and were removed when analyzing land use and management variables. Single factor analysis between response variables and inherent soil properties (n=92) and land use history (n=89) was run on the entire dataset. Afterwards, we explored rotationally grazed pastures separately. Given significant differences between continuously and rotationally grazed pastures and the desire to evaluate certain grazing management practices that apply only to rotationally grazed systems, random forest analysis was run on the rotationally grazed pasture subset (n=78).

Random forest analysis was used to evaluate the relative importance of inherent soil, land use, grazing management, and pasture management variables in rotationally grazed pastures. Random forest analysis, using randomForestSRC and ggRandomForests R packages, was selected because of its ability to handle exploratory research designs and its robustness to different data types (continuous and categorical), missing data, nonlinearity, and collinearity (Breiman, 2001; Ishwaran et al., 2008, 2021b; Ishwaran & Kogalur, 2022). Random forest creates many random regression trees with a bootstrapped dataset, then ranks the importance of the variables on whether and by how much the trees improved statistically with that factor present in the tree. The random forest analysis tries 1/3 of the variables at each split and selects the variable that adds the most predictive value to the model. 5000 trees were run to minimize error of the final model and the variability of error when re-running the model. A random seed was set to allow for reproducibility of the final model. Missing data were imputed; for categorical variables, the most common category was assigned, and for continuous variables the median value was assigned. However, in this dataset, there were very few missing data. Categorical variables were excluded if 90% of the observations had the same value (soil order, land ownership, and manure applied before sampling). Even if variables were correlated, they were included in the model to assess the relative importance among them.

The importance of each variable is measured using two methods: variable importance (VIMP) and average minimal depth. VIMP is based on whether and by how much the bootstrapped regression trees improved statistically with a given factor present in the tree. The most important variable has a relative importance of 1, and the succeeding variables of importance are relative to the most important variable. While VIMP calculates how important a variable was at improving the model, minimal depth evaluates how close to the root node the variable is in the regression tree. The average minimal depth reflects how predictive a variable is for the entire dataset, where a lower value indicates the variable typically appears closer to the root node in the regression tree. A higher VIMP rank compared to minimal depth rank indicates that the variable was important, but often at lower branches in the regression tree. Partial effect plots of important variables were created with the plot.variable() function, which varies the explanatory variable of interest and uses the random forest model to predict response values by calculating them over all remaining covariates, averaging and plotting them (Ishwaran et al., 2021).

This machine learning methodology was selected because it evaluates the relative importance of variables both as a significant effect, as well as how it interacts with the other variables. Additionally, by evaluating results from many trees, random forest analysis avoids the challenge of conditionality that is observed in regression tree analysis, that is whether or not a variable is important is dependent on the previous branches in the tree. Given the nature of the dataset and variables of interest, there is a high potential for interaction or correlation effects that may otherwise be overlooked in other statistical approaches.

3. Results

3.1 Soil Measurements

3.1.1 Summary Statistics

There was a wide range of values for all soil C and N measurements: OM% (2.0-7.7%), TC% (1.1-4.2%), TN% (0.09-0.37%), C:N (9.1-15.2), POXC (373-1230 mg kg^{-1}), ACE (4.5-15.5 g kg^{-1}), MinC (15.7-207.8 mg kg^{-1}), and PMN (36.9-235.9 mg kg^{-1}) (Table S2.6 & Table S2.7). OM, C:N and ACE were right skewed (0.96, 1.32, and 1.48 respectively) and had kurtosis greater than 1, indicating a peaked distribution. These deviations in normality were minor, so no transformations were performed and the non-transformed response variables were used in statistical analyses. The other metrics had normal distributions.

3.1.2 Relationships between biological indicators of soil health and OM, TC, TN and C:N

Pastures sites with complete soil data (n=92) were used for this analysis. Labile and bulk C and N measurements were all positively related, though the strength of relationships varied (Table 2.2, Figure S2.1). POXC had the strongest relationships with bulk pools, R^2 ranging 0.62-0.64, and MinC had the weakest relationships with bulk pools, R^2 ranging 0.25-0.37. Generally, bulk C and N explained greater variation in the chemical extractions (POXC and ACE), relative to the biological incubations (MinC and PMN). C:N had a negative relationship with biological incubations MinC and PMN (R^2 =0.16 and 0.14, respectively), but no relationship with POXC or ACE.

Table 2.2: Coefficient of determination (R^2) for labile C and N indicators vs. bulk C and N measurements.

	OM	TC	TN	C:N
	---------------------- R^2 ----------------------			
POXC	0.62	0.63	0.64	NS
ACE	0.52	0.60	0.53	NS
MinC	0.34	0.25	0.37	0.16
PMN	0.55	0.43	0.58	0.14

OM= soil organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon to nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1}); NS= not significant

3.1.3 Soil health indicator correlations

Positive correlations among biological indicators of soil health (POXC, ACE, MinC, and PMN) ranged from r=0.43 to r=0.82 (Figure 2.2). MinC and PMN had the strongest correlation (r=0.82), while the weakest correlations were between MinC and the chemical extractions (r=0.43 with ACE, r=0.53 with POXC).

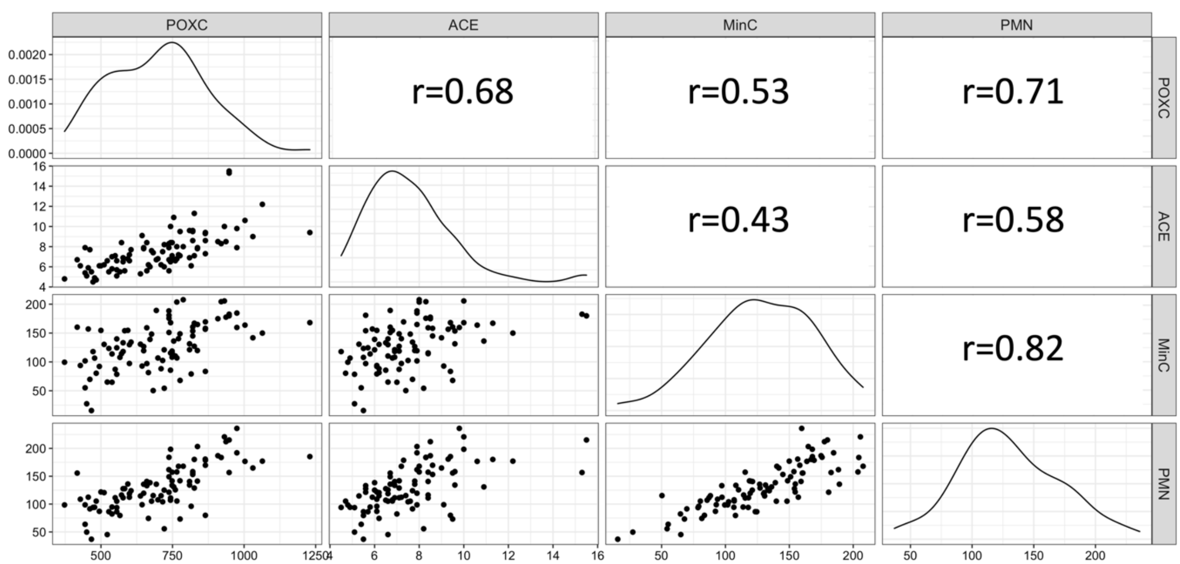

Figure 2.2: Scatterplots and correlation coefficients (r) among POXC, ACE, MinC and PMN

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

3.2 Soil Properties

Texture class and clay, silt and sand content had significant relationships with OM, TN, C:N, MinC, and PMN (Figure 2.3, S2.2, S2.3). Sand content was negatively related with soil C and N values, whereas silt had a positive relationship and clay content had a positive polynomial relationship, with values tapering at approximately 25% clay. Relationships between texture and biological incubations, MinC and PMN, were stronger compared to the other C and N measurements (R^2 values ranging from 0.3-0.4). POXC and ACE had no relationship with texture. POXC had a positive relationship with pH ($R^2 = 0.13$). Additionally, OM had a weak positive relationship with pH, while C:N had a negative relationship.

Poorly drained soils corresponded with higher OM, TC, MinC and PMN, but poor distribution of data among drainage class limits the ability to discern significant differences across all drainage classes (Figure S2.4).

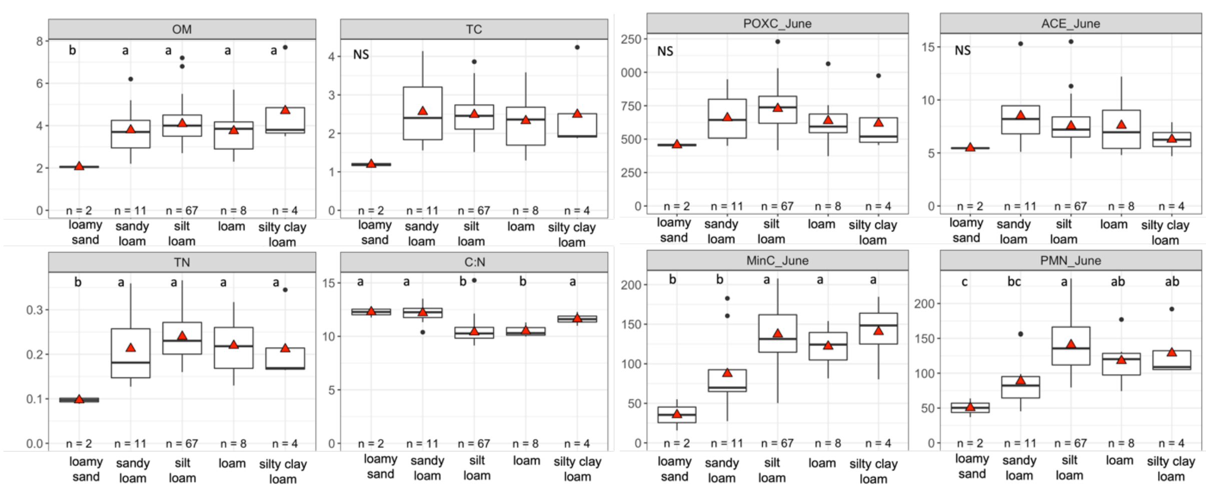

Figure 2.3: Boxplot of OM, TC, TN, C:N, POXC, ACE, MinC, and PMN by texture class.

Texture class was determined from measured sand and clay content. Texture classes include: loamy sand (n=2), sandy loam (n=11), silt loam (n=67), loam (n=8), silty clay loam (n=4). In the boxplot, the middle line indicates the median and boxes delimit first and third quartiles. Upper and lower whiskers represent 1.5 times the interquartile range or, if there were no observations beyond that range, the maximum and minimum values. Letters indicate significant differences (p-value < 0.05) among texture classes determined through ANOVA and Fisher's LSD. OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1}); NS=not significant

3.3 Pasture age & land use history

Pasture age had positive relationships will all factors except C:N, representing between 19 and 37% of the variation across response variables (Figure 2.4). However, the relationship between bulk pools produced slightly greater R^2 values (0.33-0.37) compared to labile pools (0.19 – 0.28). Sites previously fallowed had greater bulk and labile C and N compared to sites previously cropped. ACE was greater in sites previously in a row crop/hay rotation, compared to those that were exclusively hay, but no other soil metrics differed with prior agricultural system (Figure S2.5).

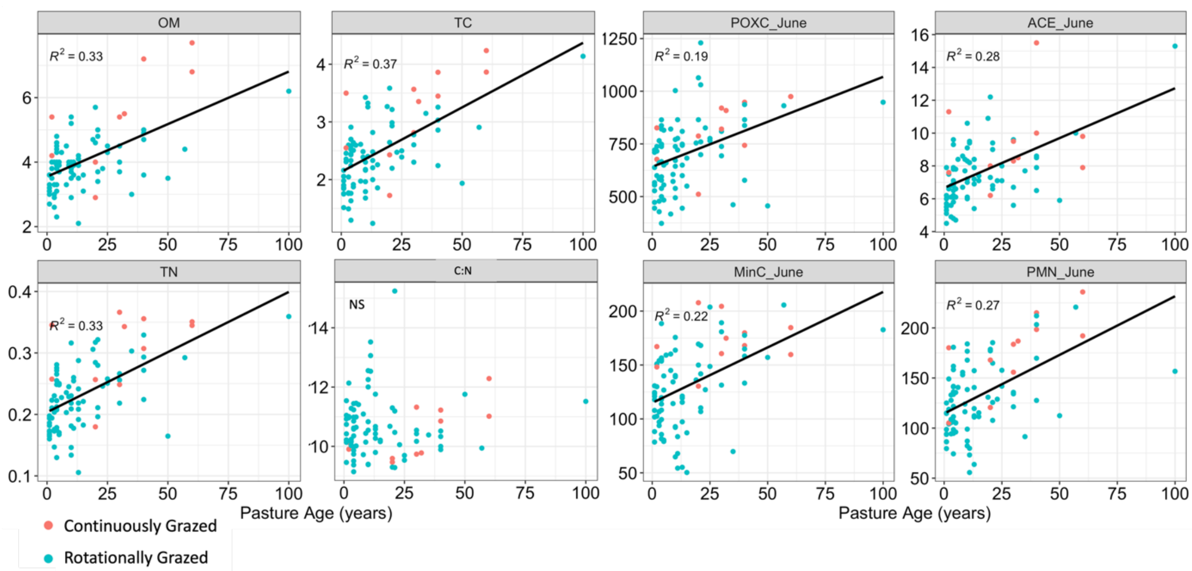

Figure 2.4: Scatterplots of bulk and labile C and N measurements and pasture age (years).
Points are colored blue for rotationally grazed pastures and red for continuously grazed. Regression lines and coefficient of determination (R^2) between pasture age and soil metrics are included for significant relations ($p<0.05$). All but the relationship between C:N and pasture age were significant.
OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1}); NS=not significant

3.4 Management

3.4.1 Continuous vs. Rotational Grazing

Pastures that were continuously grazed had higher values for total C and N pools (OM, TC and TN), as well as labile pools (POXC, ACE, MinC, PMN) (Figure 2.5). In this dataset, the continuously grazed pastures were often older pastures that had never been tilled for agricultural production. Continuously and rotationally grazed systems are inherently very different, and many grazing management practices do not apply to continuously grazed pastures. To effectively evaluate pasture and grazing management decisions that apply to rotationally grazed pastures, just those pastures (n=78) are evaluated in the random forest statistical analysis.

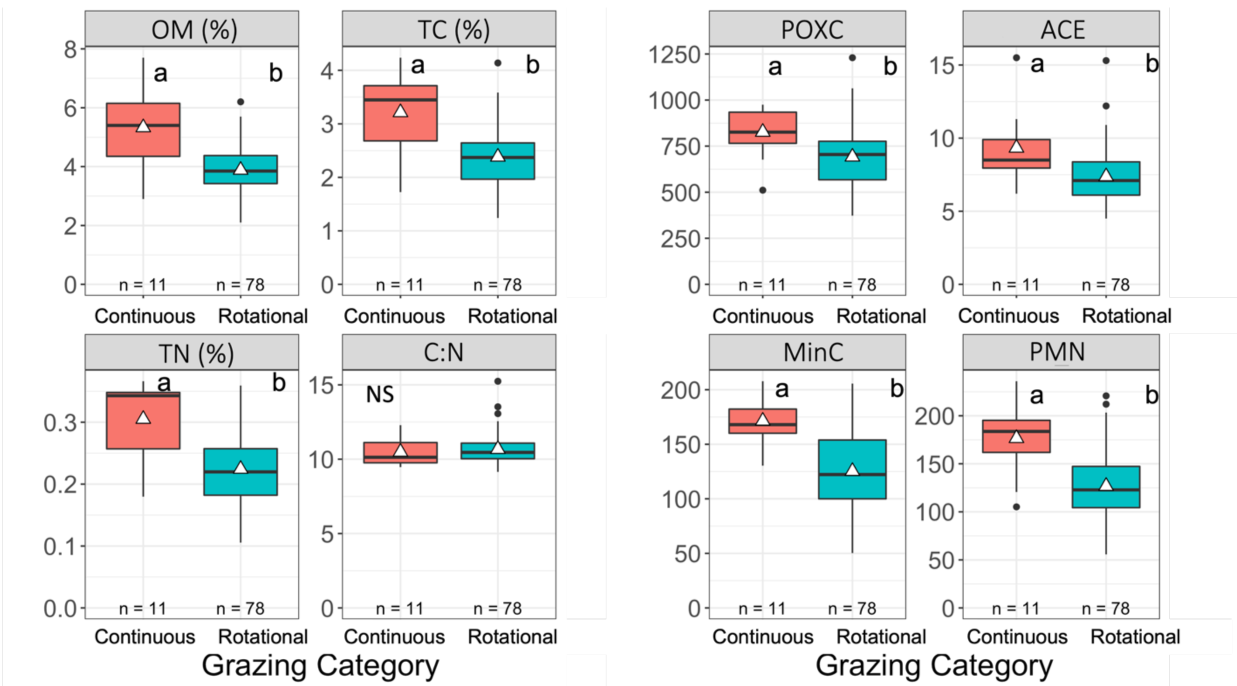

Figure 2.5: Boxplot of bulk and labile C and N measurements by grazing category
Sites were categorized as continuously or rotationally grazed pastures based on management data provided by the farmer. In the boxplot, the middle line indicates the median and boxes delimit first and third quartiles. Upper and lower whiskers represent 1.5 times the interquartile range or, if there were no observations beyond that range, the maximum and minimum values. Letters indicate significant differences (p-value < 0.05) among grazing category, determined through ANOVA and Fisher's LSD. OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1}); NS= not significant.

3.4.2 Management in rotationally grazed pastures

To evaluate grazing management practices specific to rotationally grazed pastures, the remaining analysis was conducted exclusively on rotationally grazed pastures (n=78). Due to a smaller sample size, rotationally grazed sites differed slightly in texture properties and pasture age compared to the full dataset; the same trends between soil C and N measures and texture and pasture age hold true, but p-values were weaker in the rotationally grazed data (Tables S2.8-S2.13). Sites that were outwintered, where livestock remained on the pasture over the winter and received inputs of bedding and feed, corresponded with higher OM, TC, TN, POXC and ACE. These soil measures, as well as MinC, were higher in sites that were never hayed. Synthetic fertilizer application frequency had a negative relationship with MinC and PMN (R^2= 0.07-0.11). Stocking density and seasonal grazing pressure had significant polynomial relationships with OM, TC, TN, POXC, ACE and PMN, where soil metrics increased with grazing pressure and then began to decrease at higher values (R^2= 0.06-0.20). Rest period had a weak negative relationship with TC, TN, POXC, MinC and PMN (R^2= 0.05-0.11) (Tables S2.8-S2.13).

Among rotationally grazed pastures, stocking rate, stocking density and seasonal grazing pressure were strongly correlated (r≥ 0.52) (Figure 2.6). Stocking density and time in paddock are negatively correlated (r=-0.42) since it is common to have higher stocking density for a shorter duration, or lower grazing pressure for a longer duration. Similarly, the number of rotations and rest period are negatively correlated (r=-0.54), since there are typically fewer grazing events if the paddock rests longer between grazing events. Higher stocking rate is associated with lower residual pasture height (r=-0.41), but this trend is not observed for stocking density. Pasture age has a moderate negative correlation with manure frequency (r=-0.27) and positive correlation with time in paddock (r=0.23). Accounting for inherent soil properties, there are also low to moderate correlations between sand content and legume composition (r=-0.21), stocking rate (r=-0.19), fertilizer frequency (r=0.19), and rest period (r=0.40).

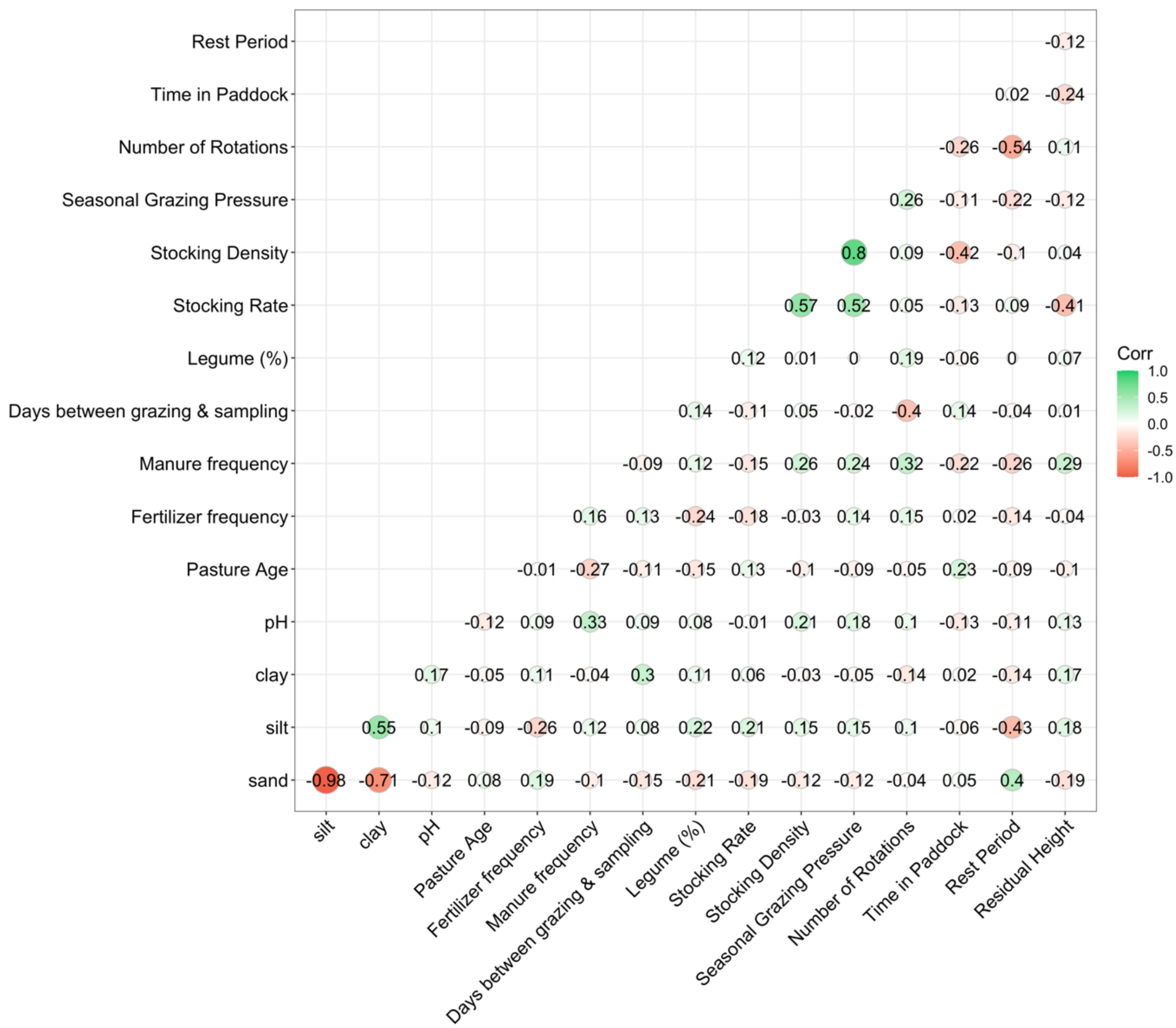

Figure 2.6: Correlation coefficients (r) between all soil and management exploratory continuous variables for rotationally grazed pastures (n=78).
Size and color of the circle behind each number represents the strength and direction of the correlation (green=positive, red=negative)

		OM	Bulk C & N TC	TN	C:N	POXC	Labile C & N ACE	MinC	PMN
Soil Properties	sand (%)		5		4			2	3
	silt (%)			5	3			4	2
	clay (%)				1		5	3	4
	Drainage Class								
	pH				2	3			
Land Use	Region								
	Farm Operation Type								
	Pasture age	1	1	1		1	1	1	1
	Previous land use					9	3	6	
Pasture Management	Legume (%)							5	
	Outwintered						4		
	Hay frequency								
	Fertilizer use								
	Fertilizer applied this year								
	Fertilizer application frequency								
	Manure use								
	Manure application frequency	2							
	Lime					4			
Grazing management	Days between grazing/sampling								
	Number of rotations								
	Stocking Rate								5
	Stocking Density		3	3		2			
	Seasonal Grazing Pressure			4		7			
	Time in paddock		4			5	2		7
	Rest period		2	2		6			6
	Residual pasture height					8			

VIMP Relative Importance
- 1
- 0.5-1
- 0.3-0.5
- 0.2-0.3
- 0.1-0.2
- <0.1

Figure 2.7: Summary table of important variables according to random forest analysis according to variable importance (VIMP) and average minimal depth (MD).
For each soil C and N measurement (column), the influence of each exploratory variable included in the analysis (rows) was determined by variable importance (VIMP) and average minimal depth (MD). VIMP represents how much the variable strengthened the model and corresponds to the cell color. The most important variable is assigned importance of 1 (colored green) and all other variables are given a relative importance based on how much it improved the model compared to the most important variable. Boxes are color coded based on VIMP for ease of interpretation (see key). The numbers in the cells correspond to ranking of importance according to average minimal depth (MD). MD reflects how close to the regression tree root node a variable appeared on average. Being closer to the root node signifies that, as a single split in the data, the variable explained larger variation in the dataset. Variables are ranked so that 1 corresponds with the variables closest to the root node. Only variables with minimal depth < 5 are numbered on the graph, as these are the most influential. See supplementary tables S14-S21 for exact values of relative importance and average minimal depth.

OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

3.5 Random Forest Analysis

3.5.1 Variables of importance

For all soil measurements the random forest analysis out of bag (OOB) R^2 ranged from 0.32-0.45, POXC and PMN having the highest R^2 (Tables S2.14-S2.21). Pasture age was the most important factor according to VIMP and minimal depth for all soil measurements except for C:N, which was most influenced by pH and texture (Figures 2.7, 2.8). Grazing and pasture management practices were more important for OM, TC, TN, POXC and ACE, whereas texture factors more were important for MinC and PMN. Compared to bulk measurements of C and N, biological indicators of soil health had more factors with VIMP relative importance greater than 0.2. Time in paddock was identified as the most important grazing variable, ranging in relative importance from 0.2-0.6 for MinC, TC, ACE, OM, and POXC, in increasing order. Time in paddock was typically ranked higher by VIMP compared to minimal depth, signifying its importance at lower branches of the regression trees due to its interaction with other factors, rather than being highly predictive for the entire dataset. After time in paddock, other important grazing and management practices include stocking density or rate, rest period, manure application frequency, and residual pasture height. Sand, silt, and clay content had relative importance between 0.35-0.80 for MinC and PMN, but less than or equal to 0.1 for OM, TC, TN, POXC and ACE. pH was an important variable for POXC and C:N.

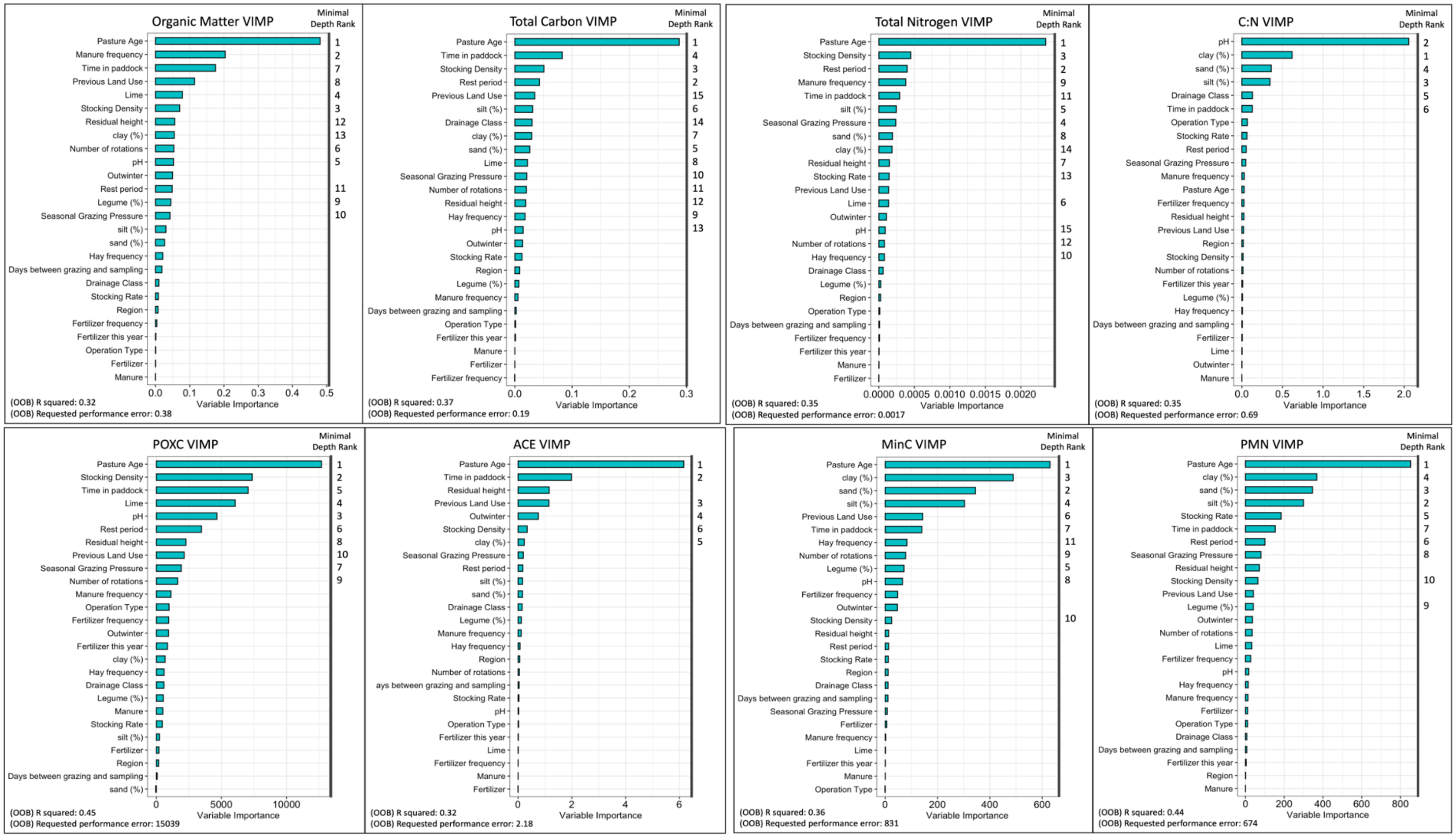

Figure 2.8: Random forest variable importance (VIMP) plots for soil C and N measurements in rotationally grazed pastures (n=78).

The list of factors are ordered by VIMP; higher factors were more important for explaining variation in the indicator. For variables identified as important according to the minimal depth method, rank of importance is along the righthand side of the graphs (1= most important). OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

3.5.2 Land use history: Pasture age & previous land use

The partial effect of pasture age shows rapid increases in predicted soil C and N values in the first 0-30 years after pasture establishment (Figure 2.9). However, over time the trend between pasture age and soil value differs by measurement. While predicted TN, POXC, MinC and PMN plateaued between 20-40 years, predicted OM, ACE and TC continued to increase with pasture age. Previous land use depicts similar trends as single factor analysis, with previously fallowed sites corresponding to greater soil health (Figure S2.6).

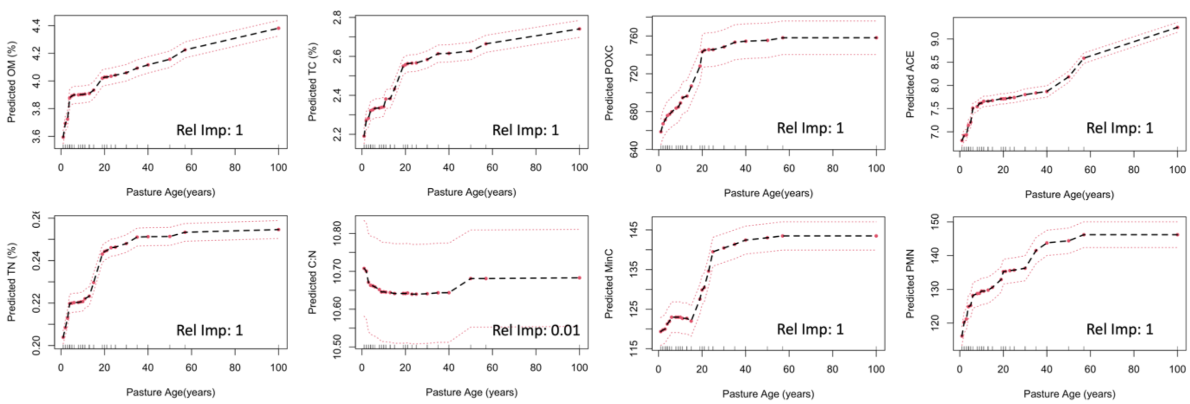

Figure 2.9: Partial effect of pasture age on OM, TC, TN, C:N, POXC, ACE, MinC and PMN
Predicted values are calculated as pasture age varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

3.5.3 Inherent soil properties

Texture content had similar trends in partial effect plots for all bulk and labile C and N measurements (Figures S2.7-S2.9), however, the importance of these texture properties is greater for MinC and PMN compared to the other soil measurements (Figure 2.10). Predicted values increased with clay content, plateauing between 10-20% clay content. Sand had a negative linear trendline and silt had a positive linear relationship with predicted MinC and PMN. Alternatively, C:N was greater at lower clay and silt content and higher at greater sand content (Figures S2.7-S2.9).

Predicted OM and POXC were lower in acidic soils with pH less than 6, which corresponded to sites that received applications of lime in the past five years. C:N shows a different trend, with a steep climb in predicted C:N when pH is greater than 7 (Figure 2.11).

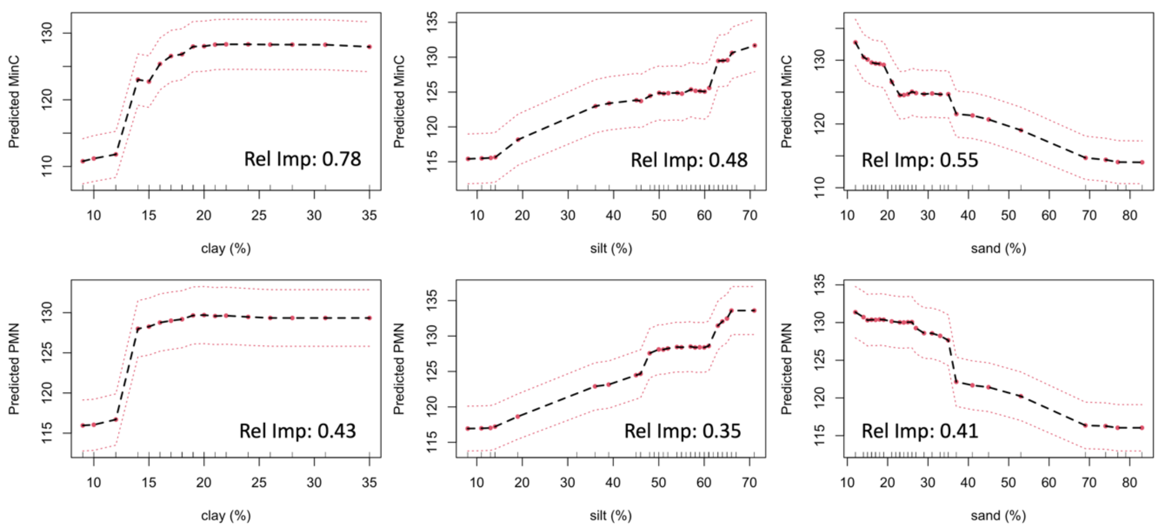

Figure 2.10: Partial effect of clay, silt, and sand on MinC and PMN
Predicted values of MinC and PMN are calculated as clay, silt and sand vary over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

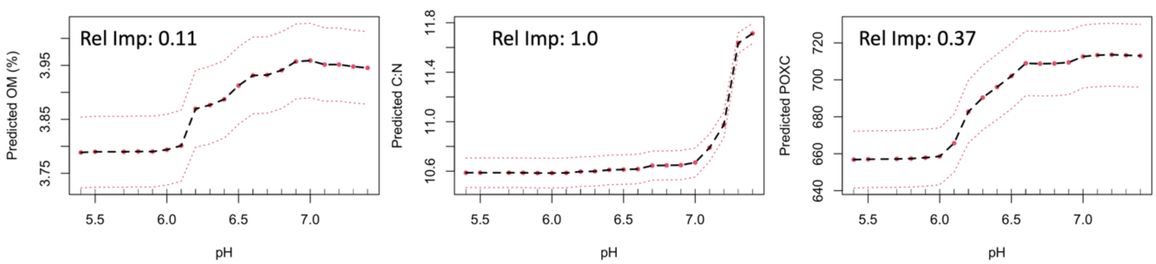

Figure 2.11: Partial effect of pH on OM, C:N, POXC
Predicted values of OM, C:N, and POXC are calculated as pH varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
OM= organic matter (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1})

3.5.4 Grazing management

Time in paddock less than one day was associated with greater predicted OM, TC, TN, POXC and ACE, but once grazing events were greater than 1d, there was no effect on predicted soil values (Figure 2.12). Comparatively, biological incubations, MinC and PMN, had weaker trends with time in paddock. Lower stocking density or stocking rate corresponded with lower predicted OM, TC, TN, POXC, and PMN (Figure 2.13). Predicted values for OM, TC, TN, and POXC increased until a stocking density of 50-100 AU/ha, at which point they plateaued. Predicted PMN and stocking rate followed the same trend, plateauing at 3 AU/ha. Predicted soil values increased with rest period, peaking around a rest period of 25-30 days, and then declining (Figure 2.14). At a residual pasture height greater than 20cm, predicted values of OM and ACE increased (Figure S2.10). Predicted OM and POXC was greater around 4-5 rotations per season, compared greater or fewer grazing events per season (Figure S2.11).

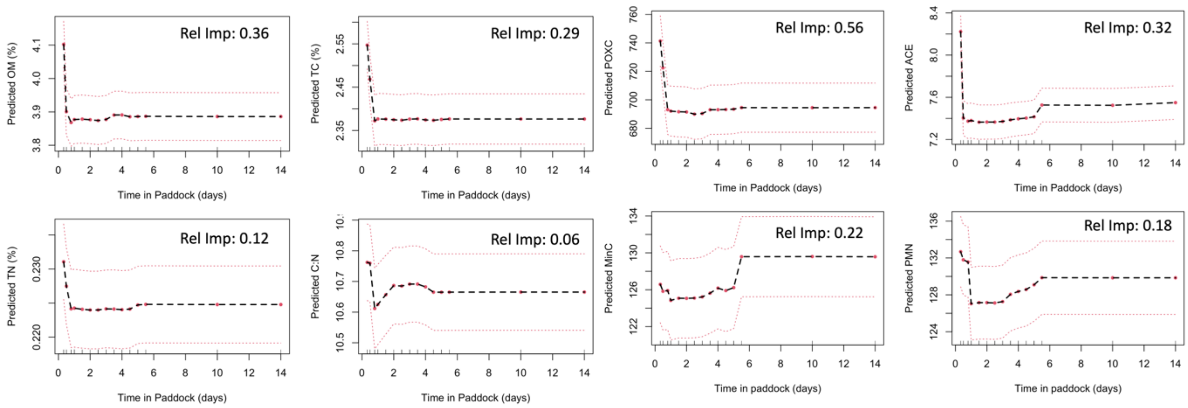

Figure 2.12: Partial effect of time in paddock (days) on OM, TC, TN, C:N, POXC, ACE, MinC and PMN

Predicted values are calculated as time in paddock per grazing event varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.

Rel Imp= relative importance according to VIMP

OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

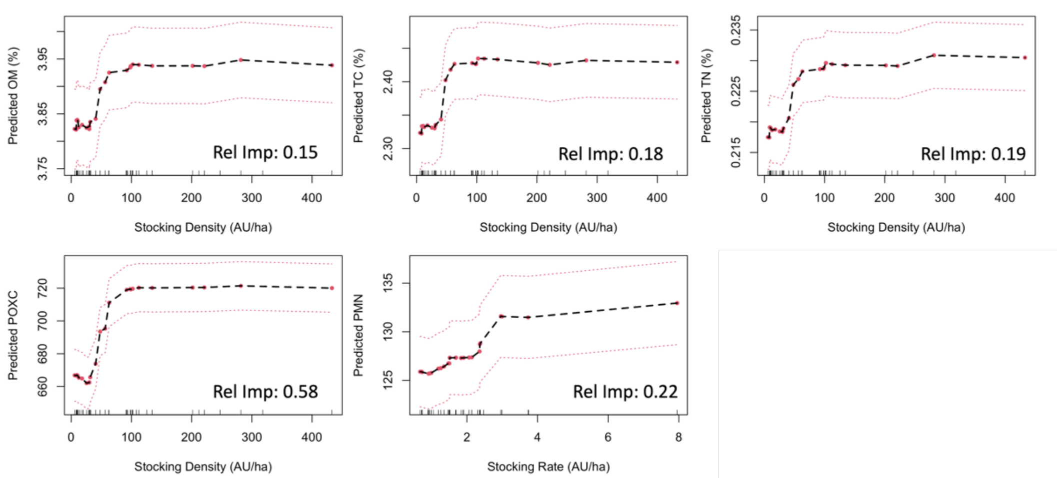

Figure 2.13: Partial effect of stocking density on OM, TC, TN, and POXC and stocking rate on PMN

Predicted values are calculated as stocking density or stocking rate varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.

Rel Imp= relative importance according to VIMP

OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); POXC = permanganate oxidizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

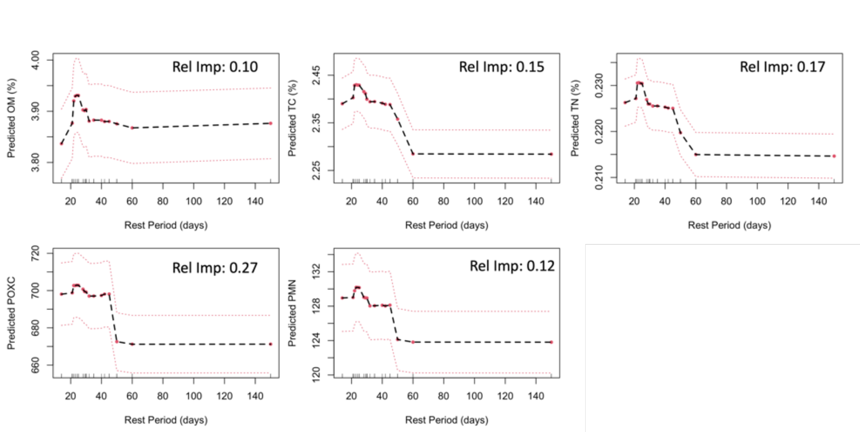

Figure 2.14: Partial effect of rest period on OM, TC, TN, POXC and PMN
Predicted values are calculated as rest period varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); POXC = permanganate oxidizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

Pasture management

Pasture management practices were not as important to soil C and N values as other factors. Predicted MinC increased as legume percentage increased to 15%, and then plateaued (Figure S2.12). Manure applied more than three times in the past five years corresponded with greater predicted OM, TN and POXC. The practice of outwintering livestock on the paddock in the past five years corresponded with greater predicted values for OM and ACE (Figure S2.13). Alternatively, haying, which removes biomass from the system, corresponded with lower predicted values of MinC (Figure S2.14).

3.6 Sampling Time

Since soil sampling was confined to a two-week period to minimize temporal effects on biological indicators of soil health (Diederich et al., 2019; Hurisso, Culman, et al., 2018;

Hurisso et al., 2016), this study evaluated days between sampling time and the last grazing event as a variable. While grazing may have immediate effects on C and N dynamics and the resulting soil health measurements, single factor and random forest analysis did not indicate this factor was important.

4. Discussion

4.1 Biological indicators of soil health reveal additional information on C and N pools

OM, TC and TN only explained 25-64% of the variation in POXC, ACE, MinC, and PMN, which confirms prior research that bulk pools account for some, but not all, variation in the labile pools. Hurisso et al. (2018), found that dependent on site and sampling time, coefficients of determination between OM and POXC, MinC and ACE ranged 0.20-0.54, 0.19-0.36, and 0.24-0.61 respectively. In survey work of over 5,000 sites, Fine et al. (2017) reported correlations between OM and POXC, ACE and MinC (4-day incubation) of 0.72, 0.78, and 0.67 respectively. As in this study, previous research found chemical extractions had stronger relationships to bulk pools compared to biological incubations. In Morrow et al. (2016), SOC and TN were more correlated with POXC (r=0.93), compared to MinC (r=0.28, 0.31) or PMN (r=0.59, r=0.67). Soil health results from Sikora (2020), revealed strong relationships among POXC, SOC and SOM (R^2>0.54), but weak positive relationships between MinC and all other C-related indicators ($R^2 < 0.15$). Labile C and N measurements are positively related to bulk C and N, yet have remaining variability not attributed to those measurements, indicating that they are measuring different pools of C and N.

This study revealed positive correlations of varying strength among biological indicators of soil health, consistent with previous research (B. S. Crookston et al., 2021; Culman et al., 2013; Fine et al., 2017; Hurisso, Culman, et al., 2018; Hurisso et al., 2016; Morrow et al., 2016). A meta-analysis by Hurisso et al. (2016) demonstrated variability in the correlation between POXC and MinC that may be attributed to the site-specific factors and variation. Multisite surveys had weak relationships between POXC and MinC ($R^2 \leq 0.17$), as did single site studies on coarse textured soils (Hurisso 2016). Morrow et al. (2016) and Hurisso et al. (2018) also observed weaker correlations between POXC and MinC, r=0.43 and r=0.46 respectively, compared to correlations between among other labile pools and OM (r> 0.6). Relative to our study, Sikora observed weaker relationships among biological indicators of soil health (R^2=0.11-0.46), but trends are consistent that the strongest correlation was between the biological incubations PMN and MinC, and the weakest correlation was between POXC and MinC. This highlights that the chemical extractions and biological incubations reflect different biologically active C and N pools.

4.2 Pasture age is an important driver of soil C and N measures

Pasture age was the most important factor for soil C and N pools, which confirms findings from long-term trials that demonstrated similar gains in C in the soil surface with duration of well-managed pasture. The pasture system at the Wisconsin Integrated Cropping Systems Trial observed increases in soil total organic carbon (TOC) in the upper 15cm over a 20-year period, while common grain and forage systems lost TOC (Sanford et al., 2012). In an on-farm WI study, pasture age was positively related to SOC gains in pastures compared to a paired crop field (Becker et al., 2020). Similar relationships are observed for biological indicators of soil health. Rowntree et al. (2020) found that 20 years of well managed pasture in southeastern U.S. resulted in linear increases of soil health metrics with study duration: a 2-fold increase in microbial respiration, 5-fold increase in ACE protein, and 10-fold increase in active carbon, or POXC.

Corresponding with trend lines in the random forest partial effect plots, other studies found rapid increases in soil C and N measures after conversion of cropland to pasture, with decreasing gains over time (Franzluebbers, Stuedemann, et al., 2000; Guillaume et al., 2021). Using a chronosequence of grazed tall fescue, Franzluebbers, Stuedemann, et al., 2000 found that during the first ten years under pasture, soil organic C and total N accumulated at an average rate of 100 and 7.3 g m^2 yr^{-1}, after which accumulation of SOC and TN dropped to only 48 and 4.4 g m^2 yr^{-1} between 10-30 years after establishment, and 20 and 0.6 g m^2 yr^{-1} between 30-50 years after establishment. As SOC and soil health increases, it becomes more challenging to maintain comparable increases (Abdalla et al., 2018; Arndt et al., 2022; Guillaume et al., 2021; McSherry & Ritchie, 2013). While this study includes a range of pastures 1-100 years in age, there is a substantial skew towards younger pastures and greater research on pastures between 20-100 years old is necessary to understand the nature of the trend line between soil health and pasture age among older pastures.

4.3 Inherent properties were important for select soil measurements

4.3.1 Soil Texture

Soil texture was a strong driver for biological incubations, MinC and PMN, but contrary to other research, not for total C and N measures or chemical extractions. The partial effect trend lines for soil texture properties were consistent between the MinC and PMN: negative linear trends with sand content and positive trends with clay, until predicted values plateaued around a clay content of 15-25%. It is challenging to build SOM in coarse-textured soils, since sand particles do not form organo-mineral complexes as easily as clay (Arndt et al., 2022; Six et al., 2002; W. J. Wang et al., 2003). However, unlike this study, soil health assessments in other cropping systems show stronger relationships between texture and SOM and POXC, relative to MinC (Amsili et al., 2021; Fine et al., 2017; Hurisso et al., 2016). Other research suggests that the relationship between SOC and clay in pastures may be region and climate specific (McSherry & Ritchie, 2013). Lastly, most of the sites in our study were silt loams, so the lack of variability may have limited the ability to fully detect the relationship between texture soil health indicators.

4.3.2 pH

The strong positive relationship between POXC and pH has been observed in other Wisconsin soil health assessments (Malone, 2022; Richardson, 2018; Sikora, 2020). Though this relationship is not well studied (Gasch et al., 2020; Hurisso, Culman, et al., 2018), Richardson (2018) hypothesized that this relationship may be due to the permanganate oxidizing carbonates, inflating POXC values.

4.4 Grazing management variables were important factors for biological soil health indicators

4.4.1 Continuously vs. rotationally grazed pastures

Continuously grazed pastures, which constituted 12% of the sites, had higher bulk and labile C and N compared to rotationally grazed systems, despite substantial evidence on the benefits of rotational grazing over continuous grazing for pasture productivity and soil quality (Byrnes et al., 2018; Conant et al., 2017; Mosier et al., 2021; Oates et al., 2011; Oates & Jackson, 2014; Rowntree et al., 2020; Teague et al., 2013; T. Wang et al., 2018). In this study, continuously grazed pastures were typically older (average age of 30, compared to 14 years for rotationally grazed sites). Additionally, continuously grazed pastures in this study had low stocking rates which can help prevent overgrazing and soil degradation (Briske et al., 2008; Chen et al., 2015; Franzluebbers & Stuedemann, 2010; M. Zhang et al., 2018). Generally, greater C inputs and manure deposition, through outwintering, inputs of bedding and feed, and manure deposition can contribute to soil C and N (Yang et al., 2019). Another consideration is that this study evaluated C and N in terms of concentrations, not accounting for bulk density, which can be higher under continuous versus rotationally grazed pastures (Abdalla et al., 2018; Byrnes et al., 2018). Survey work is snapshot in time, and we are unable to discern if these continuously grazed pastures are gaining or losing soil health over time and/or if indicator values would be higher under rotational grazing practices when all other factors are held constant.

4.4.2 Grazing management

Random forest analysis elucidates the responsiveness of biological indicators to management properties compared to bulk pools, since they had more variables of higher relative importance. This response is a reflection that soil health indicators are more sensitive than OM or SOC to management (Culman et al., 2012, 2013; Hurisso et al., 2016; Wander & Drinkwater, 2000).

Partial effect plots demonstrated that greater soil health values corresponded with management intensive rotational grazing (MIRG) practices: medium to high grazing pressure, shorter grazing events, and adequate residual height, rest, and regrowth. In Wisconsin, studies have shown these practices to be beneficial to pasture health and productivity (Oates et al., 2011; Paine et al., 1999; Paine & Gildersleeve, 2011). The potential for plant health, C and N inputs, and soil quality to improve under these management practices is reflected in the partial effect trends between grazing management and biological soil health.

A shorter grazing event with higher stocking densities and sufficient rest, can facilitate greater forage production, equal manure distribution, and weed suppression (Franzluebbers et al., 2012; Oates et al., 2011; L. K. Paine et al., 1999; Teague & Kreuter, 2020; T. Wang et al., 2016). Teague et al. (2015) corroborated this study's findings that excessively long grazing events or recovery periods resulted in poorer plant recovery, which in turn may deter soil health. However, grazing management is context specific and dynamic to individual pastures and in-season variability (Abdalla et al., 2018; McSherry & Ritchie, 2013; Mosier et al., 2021; Oates et al., 2012). Therefore, other studies found benefits of lower stocking rates or longer rest periods than what was identified in this study to soil C (Abdalla et al., 2018; Mosier et al., 2021; T. Wang et al., 2018; T. Zhang et al., 2015). On the contrary, Becker et al. (2021) observed that rest period was negatively related to SOC, which they hypothesized was because pastures with higher SOC could support more frequent grazing events. Such confounding factors could also exist in this study, as it is possible that more productive pastures can support higher stocking densities and/or shorter rest periods.

4.5 Pasture management was not important to soil health values relative to other factors

Pasture management practices, including outwintering, haying, pasture composition, manure applications and synthetic fertilizer use had very small effects in this study. Though outwintering, associated with additional inputs of straw, hay and manure, and haying, or the removal of biomass, were significant in single factor analysis for OM, TC, TN, POXC and ACE, they were not identified as important variables in random forest analysis when all factors were considered. Other studies in grain systems identified the importance of aboveground biomass and retaining residue (Culman et al., 2012; Hurisso et al., 2016; Hurisso, Moebius-Clune, et al., 2018), and comparisons between rotationally grazed pastures and hay fields demonstrated lower C and N in exclusively hayed fields (Arndt et al., 2022; Franzluebbers, Stuedemann, et al., 2000; Oates & Jackson, 2014; Tilhou et al., 2021).

In this study, the additional manure use was only important to SOM and TN, with minimal importance to the other soil C and N measures compared to other factors. Manure often is promoted to build soil health in row cropping systems, but previous research shows inconsistent effects of manure on SOM and biological soil health (Bera et al., 2016; Hurisso et al., 2016; Jokela et al., 2009; Mikha et al., 2017; Nunes, van Es, et al., 2020; Rui et al., 2020; Yang et al., 2019). Given that grazed pastures already have high manure deposition there are likely minimal effects of manure applications in this study. Synthetic fertilizer frequency had a negative correlation with MinC and PMN in single factor analysis, though it was not important once all other factors were considered in the random forest.

Legume percentage was only weakly important to MinC, which benefited from a legume percentage of 15% or greater. Other studies found that crop diversity and legumes to have a significant effect on biological indicators of soil health, especially MinC, in both cropping and pasture systems (Arndt et al., 2022; Chen et al., 2001; Culman et al., 2013; Hurisso et al., 2016; Skinner et al., 2006). Pasture specific SOC and soil health work have identified pasture composition, including grass physiological type (C3 vs. C4), legume cover, species diversity within functional groups, and the percentage of improved grass and legume species as influential (McSherry & Ritchie, 2013; Skinner et al., 2006; Spiesman et al., 2018; Yang et al., 2019). Further exploration on the influence of pasture composition on biological soil health would be beneficial.

5. Conclusion

Pasture age, grazing management and soil texture were the most important factors in explaining variation in soil C and N measures. Results suggest that pasture age drives total and labile C and N pools and due to decreasing gains in predicted soil values over time, future research should explore whether there is a maximum threshold of these measurements in pastures. There is also evidence that within rotationally grazed systems, MIRG practices, such as higher stocking densities with shorter grazing intervals and sufficient recovery time, are associated with greater biological indicators of soil health. While only MinC and PMN were strongly influenced by soil texture, it is beneficial to continue to explore this relationship and benchmark soil health indicators accordingly.

Soil health testing is a potential tool for farmers to adjust management practices and track improvements to soil health over time. But since pastures inherently integrate all principles of soil health, the question of how soil health testing may fit into a grazier’s toolbox is uncertain. This study identified pasture age, soil texture, and grazing management as important variables to biological indicators soil health. However, out of these factors, only grazing management is within the grazier's control. MIRG, which has direct benefits to pasture health and productivity, aligned with soil health benefits for rotationally grazed pastures in this study, reaffirming the benefits of well-managed grazing to both pasture and soil health. Beyond grazing management, other pasture management factors, like manure and fertilizer inputs, legume coverage, outwintering, and haying, were not identified in the random forest, which may limit the tools available to graziers to improve pasture soil health. If practices that promote soil health have direct benefits to pasture productivity, regular soil health testing to evaluate the efficacy of soil health building practices may be less valuable in pastures compared to row cropping systems.

This study demonstrated that within pasture systems, biological indicators of soil health are variable, and sensitive to management as well as soil properties. But further value in soil health testing is dependent on the relationship between biological indicators of soil health and agronomic, economic, or environmental benefits. Therefore, it is important for soil health research to utilize a framework that links soil health indicators to desired soil functions and specific and pertinent outcomes, such as productivity, nutrient efficiency, soil C storage, greenhouse gas emissions, and water quality (Wade et al., 2022). Thus far, soil health research has been predominantly in row cropping systems, and it is imperative that pastures are represented in future research that evaluates relationships between soil health indicators, soil function and desired outcomes. Understanding the relationship between soil health indicators and essential ecosystem services is necessary to improve soil health in pasture soils and the broader agricultural landscape.

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Supplementary materials

Table S2.1: Categorical inherent properties, their levels and number of fields associated with each level.
Due to differences between continuous (n=11) and rotationally (n=78) grazed pastures, site descriptions are provided based on grazing category. One farm (3 sites) did not provide management and its inherent site descriptions provided under the category "unknown management". Texture class was determined based on measured sand, silt and clay content. Drainage class and soil order were retrieved from NRCS Web Soil Survey, based on GPS coordinates recorded at the center of the sampling area.

Inherent Site Properties		Site Category		
Factor	Levels	Rotationally Grazed Sites (n=78)	Continuously Grazed Sites (n=11)	Unknown Management (n=3)
		number of sites (n)		
Region	Kickapoo	38	8	0
	Marathon	40	3	3
Texture Class	loamy sand	1	0	1
	sandy loam	8	1	2
	loam	8	0	0
	silt loam	58	9	0
	silty clay loam	3	1	0
Drainage Class	poorly drained	0	2	0
	somewhat poorly drained	16	1	0
	moderately well drained	14	1	3
	well drained	45	7	0
	somewhat excessively drained	1	0	0
	excessively drained	2	0	0
Soil Order	Alfisols	68	10	0
	Entilsols	1	0	3
	Mollisols	1	1	0
	Spodosols	8	0	0

Table S2.2: Categorical management properties, their levels and number of fields associated with each level.
Due to differences between continuous (n=11) and rotationally (n=78) grazed pastures, site descriptions are provided based on grazing category. Management data was provided from the farmers.

Management Factors		Site Category	
Factor	Levels	Rotationally Grazed Sites (n=78)	Continuously Grazed Sites (n=11)
		number of sites (n)	
Operation Type	beef	44	7
	dairy	15	0
	heifers	7	3
	multispecies	12	1
Land Ownership	Owned	68	9
	Rented	10	2
Previous Land Use	fallow	5	3
	hay	14	0
	row crops	28	1
	row crops & hay	27	3
	unknown	4	4
Rotationally Grazed	Yes	78	0
	No	0	11
Outwintered in last 5 years	Yes	21	3
	No	57	8
Hay Frequency Category	Never	55	10
	Sometimes	13	1
	Often	10	0
Fertilizer applied in last 5 years	Yes	38	1
	No	40	10
Fertilizer applied this year	Yes	23	1
	No	55	10
Manure applied in last 5 years	Yes	28	0
	No	50	11
Manure applied this year	Yes	8	0
	No	67	11
	unknown	3	0
Lime applied in last 5 years	Yes	25	0
	No	53	11

Table S2.3: Univariate statistics for all independent continuous soil properties.
Data are separated into rotationally ("Rot") and continuously ("Cont") grazed pastures.

	pH		P		K		NO_3^-		NH_4^+		Sand		Silt		Clay	
			ppm								%					
	Rot	Cont	Rot	Cont	Rot	Cont	Rot	Cont	Rot	Cont	Rot	Cont	Rot	Cont	Rot	Cont
n	78	11	78	11	78	11	78	11	78	11	78	11	78	11	78	11
Minimum	5.4	6.0	5	10	30	47	1.1	1.4	3.4	4.1	12	14	8	30	9	16
1st Quartile	6.2	6.3	14	24	76	145	3.0	4.3	5.2	4.3	17	16	50	57	16	18
Median	6.4	6.6	23	42	114	188	4.9	5.8	6.2	5.6	23	18	58	61	18	20
Mean	6.5	6.5	37	93	138	240	6.1	6.6	7.3	6.7	29	21	53	58	18	21
3rd Quartile	6.8	6.7	37	142	168	278	7.6	6.7	9.1	9.0	31	20	61	64	20	23
Maximum	7.4	7.0	273	286	525	670	20.5	20.9	18.8	10.4	83	53	71	69	35	29
Std. Dev.	0.4	0.3	43	97	98	173	4.4	5.2	3.0	2.7	18	11	15	10	4	4
CV	0.07	0.05	1.16	1.03	0.71	0.72	0.73	0.79	0.41	0.40	0.62	0.52	0.29	0.18	0.23	0.18
Skewness	0.06	-0.18	3.16	0.88	2.07	1.23	1.28	1.7	1.45	0.27	1.84	2.03	-1.74	-1.63	0.8	0.73
Kurtosis	-0.25	-1.22	12.08	-0.88	5.11	0.74	1.16	2.22	2.03	-1.86	2.37	3.04	2.06	2.02	2.93	-0.2

Table S2.4: Univariate statistics for continuous management variables.
Data are separated into rotationally (“Rot”) and continuously (“Cont”) grazed pastures.

	Pasture Age		Fertilizer Frequency		Manure Frequency		Legume		Stocking Rate		Stocking Density		Seasonal Grazing Pressure	
	years		# applications in past 5 years				--- % ---		---------- AU ha-1 ----------				AU ha-1 * days	
	Rot	Cont	Rot	Cont	Rot	Cont	Rot	Cont	Rot	Cont	Rot	Cont	Rot	Cont
n	78	11	78	11	78	11	78	11	75	10	78	10	78	10
Minimum	1	2	0	0	0	0	0	0.2	0.7	1.1	6	1.1	38	82
1st Quartile	4	20	0	0	0	0	7.5	6.0	1.0	1.4	30	1.4	252	173
Median	9.5	30	0	0	0	0	14.5	10.9	1.7	1.6	91	1.6	454	229
Mean	13.6	30.6	1.5	0.5	0.8	0	19.9	10.1	2.1	1.8	102	1.8	585	298
3rd Quartile	19.8	40	3	0	1	0	31.0	14.8	2.4	1.8	122	1.8	628	355
Maximum	100	60	5	5	5	0	80.3	17.8	8.0	3.5	433	3.5	3031	835
Std. Dev.	15.9	19.4	2.0	1.5	1.4	0	16.3	5.6	1.5	0.7	102	0.7	568	224
CV	1.17	0.64	1.29	2.76	1.79	NA	0.82	0.55	0.72	0.38	1.00	0.38	0.97	0.75
Skewness	2.64	0.05	0.86	2.31	1.87	NA	1.07	-0.27	2.47	1.37	1.65	1.37	2.73	1.20
Kurtosis	9.69	-1.16	-0.94	3.94	2.64	NA	0.96	-1.38	7.16	0.87	2.36	0.87	8.30	0.53

Table S2.5: Univariate statistics for all continuous management factors, specific to rotationally grazed pastures.

	Days between grazing and sampling	Number of Rotations	Time in Paddock	Rest Period	Residual Pasture Height
			--------- days	---------	----- cm -----
n	73	78	78	78	78
Minimum	0	2	0.33	14	5.1
1st Quartile	13	3	1	30	10.2
Median	21	4	1	30	12.7
Mean	79	4.5	2.34	39	13.6
3rd Quartile	200	6	3.5	45	15.2
Maximum	260	10	14	150	27.9
Std. Dev.	97	1.9	2.33	25	4.1
CV	1.22	0.43	0.99	0.65	0.30
Skewness	0.9	0.93	2.56	3.22	0.54
Kurtosis	-1.05	0.4	8.34	11.54	0.77

Table S2.6: Univariate statistics for organic matter (OM)%, total carbon (TC)%, total nitrogen (TN)%, and carbon-to-nitrogen ratio (C:N); n=92.

	OM	TC	TN	C:N
	------------------- % -------------------			
n	92	92	92	92
Minimum	2	1.14	0.089	9.15
1st Quartile	3.4	1.96	0.182	10.01
Median	3.9	2.42	0.224	10.50
Mean	4	2.45	0.231	10.71
3rd Quartile	4.5	2.82	0.268	11.23
Maximum	7.7	4.24	0.366	15.24
Std. Dev.	1.02	0.65	0.062	1.06
CV	0.25	0.27	0.27	0.10
Skewness	0.96	0.51	0.24	1.32
Kurtosis	1.91	-0.05	-0.48	2.61

Table S2.7: Univariate statistics for permanganate oxidizable carbon (POXC), autoclaved-citrate extractable protein (ACE), mineralizable carbon (MinC) and potentially mineralizable nitrogen (PMN); n=92.

	POXC	ACE	MinC	PMN
	mg kg^{-1}	g kg^{-1}	---------- mg kg^{-1} ----------	
n	92	92	92	92
Minimum	372.7	4.5	15.68	36.94
1st Quartile	563.5	6.2	101.17	104.9
Median	718.1	7.2	128.47	125.36
Mean	700.9	7.6	127.92	130
3rd Quartile	816.5	8.4	158.44	157.41
Maximum	1229.8	15.5	207.76	235.91
Std. Dev.	170.1	2.0	41.08	41.14
CV	0.24	0.26	0.32	0.32
Skewness	0.31	1.48	-0.26	0.24
Kurtosis	-0.21	3.61	-0.33	-0.28

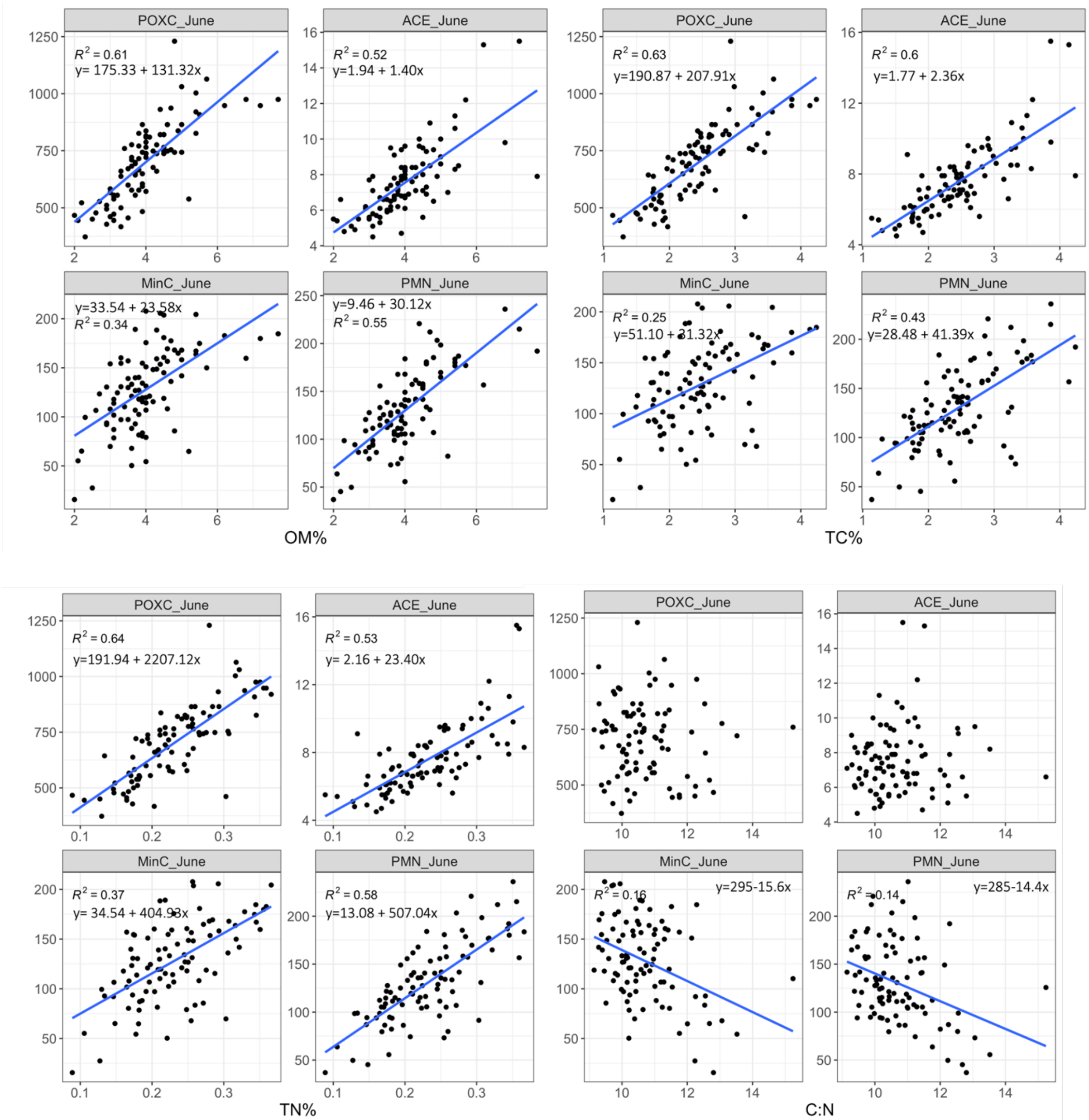

Figure S2.1: Scatterplots of biological soil health indicators (POXC, ACE, MinC and PMN) and i) organic matter (OM)%, ii) total carbon (TC)%, iii) total nitrogen (TN)%, and iv) carbon-to nitrogen ratio (C:N).

Linear regressions were conducted for each relationship and coefficients of determination (R^2) and regression equations are included in each figure. Estimates in the equations were statistically significant (alpha=0.05). POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

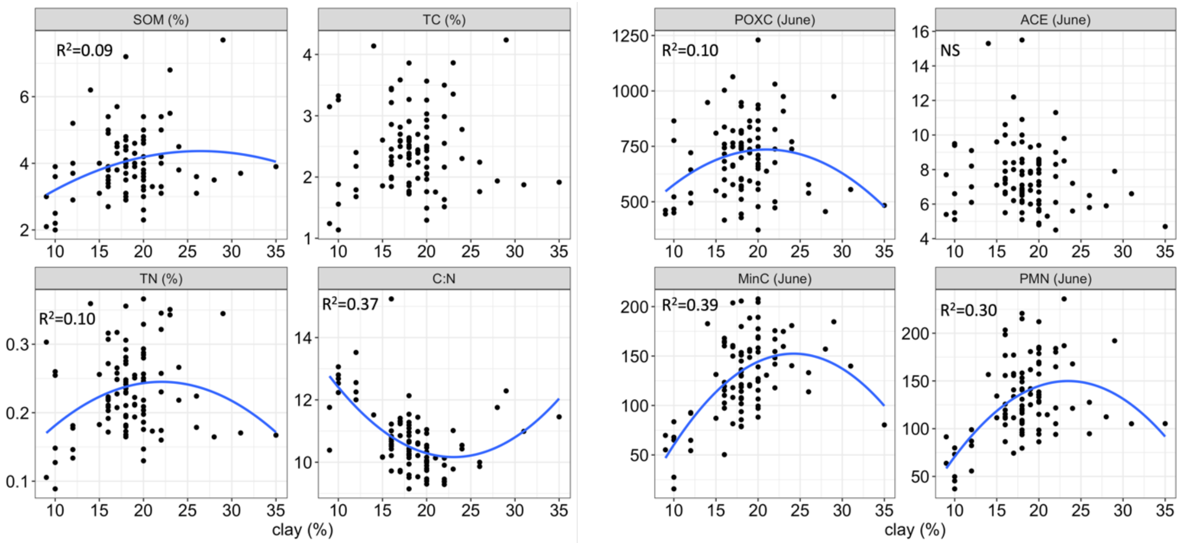

Figure S2.2: Scatterplots of clay content all soil C and N measurements OM, TC, TN, C:N, POXC, ACE, MinC and PMN.

Linear regressions were conducted for each relationship and if relationships were significant (alpha=0.05), coefficients of determination (R^2) and estimated equation were included. Clay content was transformed to include a polynomial term ($x+x^2$). The graph includes untransformed clay content on the x axis with the polynomial trendline. Estimates in the equations were statistically significant (alpha=0.05). OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1}); NS= not significant

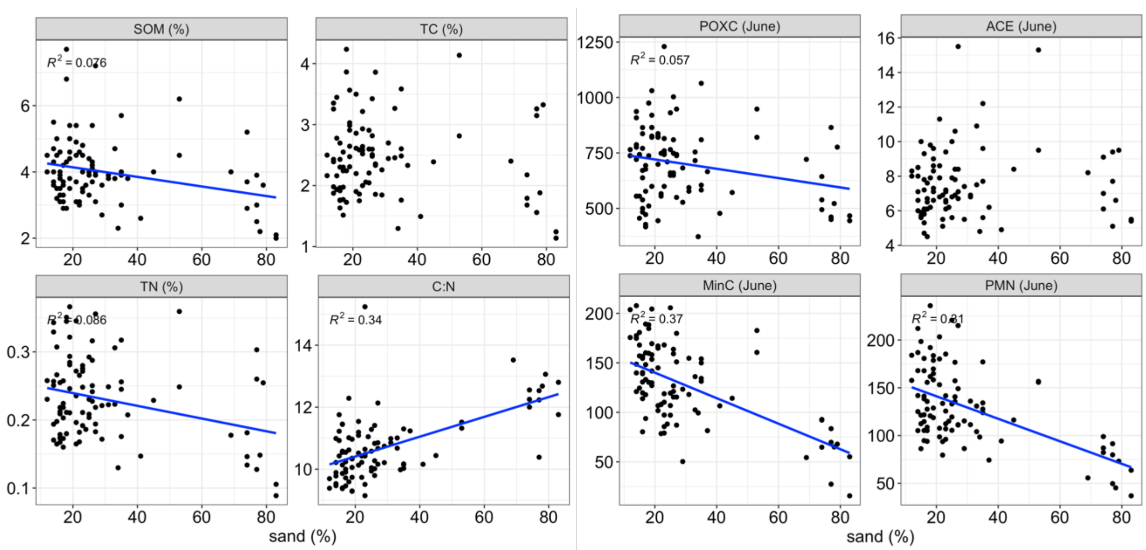

Figure S2.3: Scatterplots of sand content all soil C and N measurements OM, TC, TN, C:N, POXC, ACE, MinC and PMN.

Linear regressions were conducted for each relationship and if relationships were significant (alpha=0.05), coefficients of determination (R^2) and estimated equation were included. Estimates in the equations were statistically significant (alpha=0.05). OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

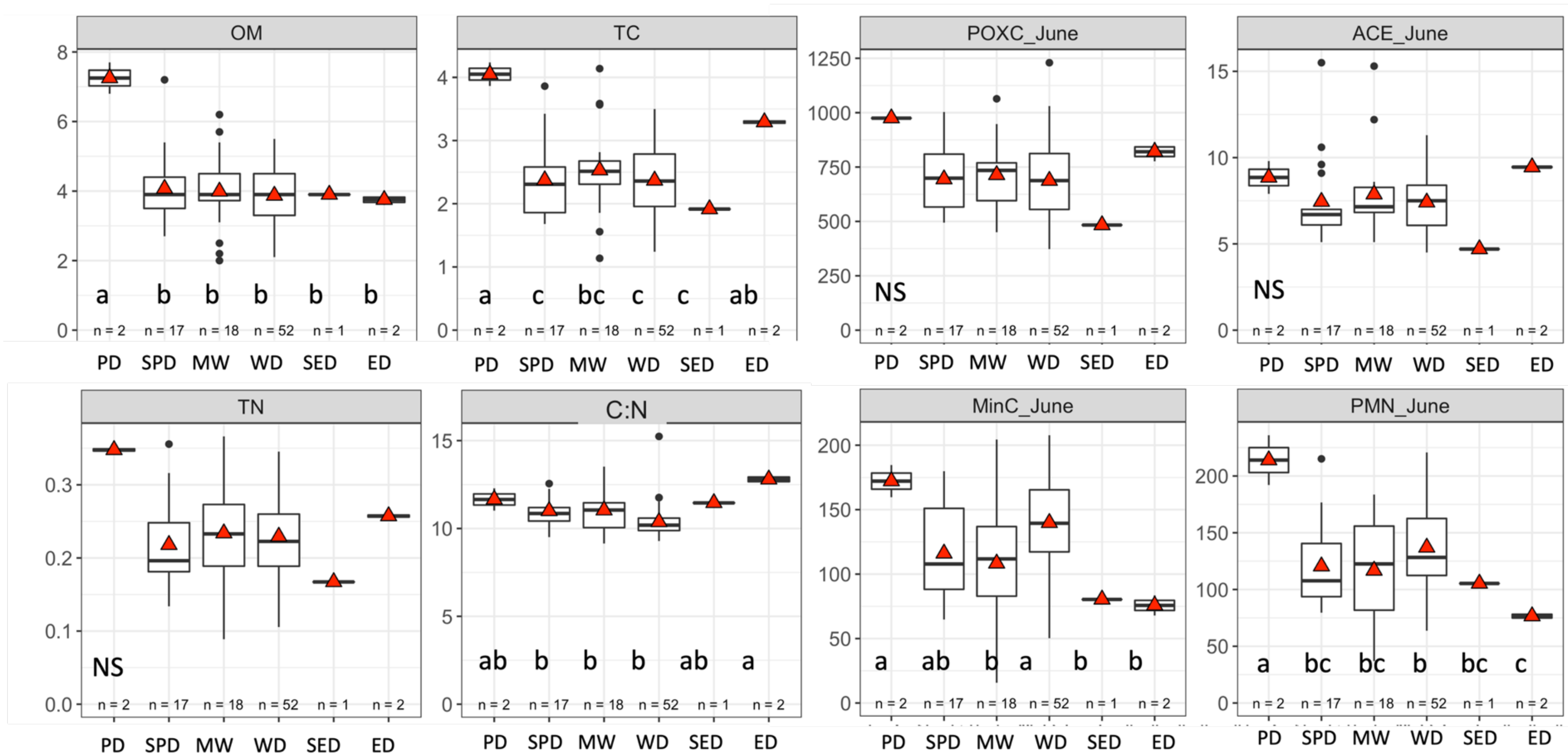

Figure S2.4: Boxplot of all OM, TC, TN, C:N, POXC, ACE, MinC, and PMN by drainage class. Drainage class was determined through the NRCS Web Soil Survey: PD= poorly drained (n=2), SPD=somewhat poorly drained (n=17), MW=moderately well drained (n=18), WD= well drained (n=52), SED=somewhat excessively drained (n=1), ED=excessively drained (n=2). In the boxplot, the middle line indicates the median and boxes delimit first and third quartiles. Upper and lower whiskers represent 1.5 times the interquartile range or, if there were no observations beyond that range, the maximum and minimum values. Letters indicate significant differences (p-value < 0.05) among previous land use, determined through ANOVA and Fisher's LSD. OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1}); NS= not significant

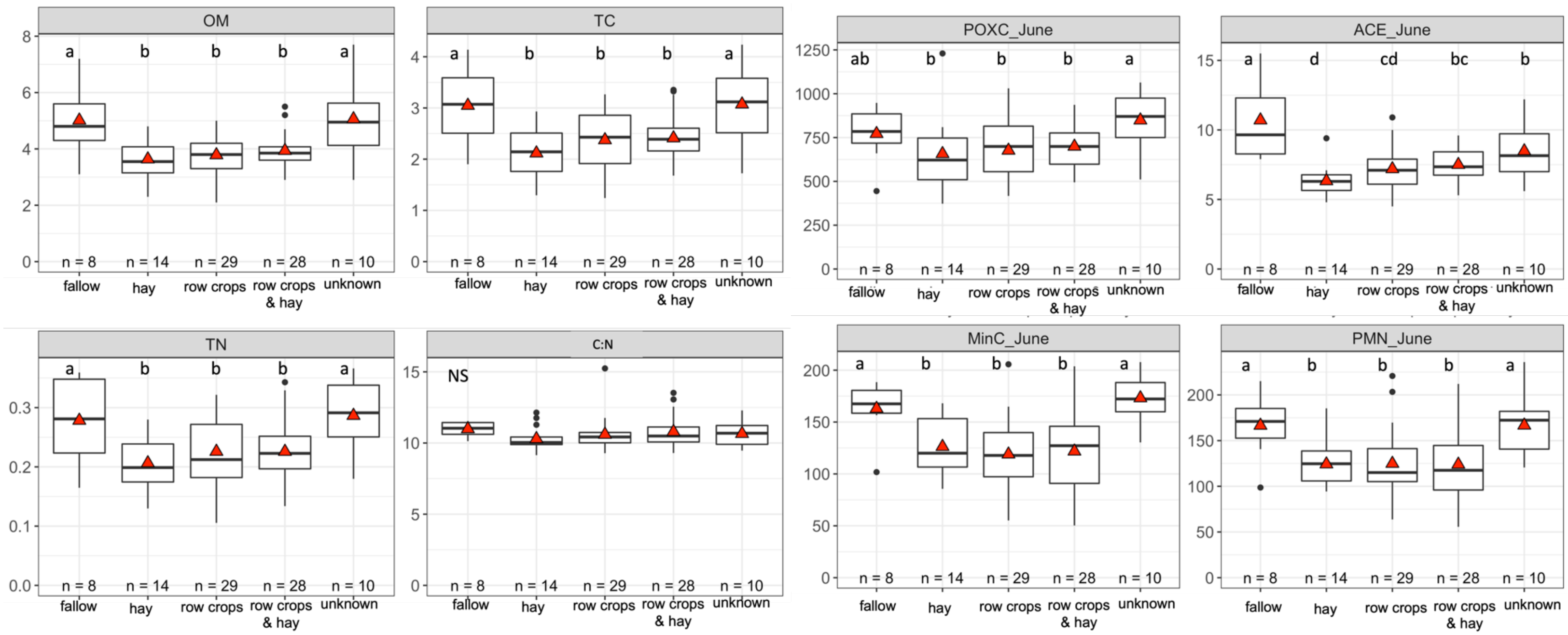

Figure S2.5: Boxplot of all OM, TC, TN, C:N, POXC, ACE, MinC, and PMN by previous land use. Previous land use was categorized as fallow hay, row crows, hay/row crop rotation, and unknown (when producers were unable to report land use prior to pasture). In the boxplot, the middle line indicates the median and boxes delimit first and third quartiles. Upper and lower whiskers represent 1.5 times the interquartile range or, if there were no observations beyond that range, the maximum and minimum values. Letters indicate significant differences (p-value < 0.05) among previous land use, determined through ANOVA and Fisher's LSD. OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1}); NS= not significant

Table S2.8: Analysis of Variance (ANOVA) results for texture class and drainage class for OM, TC, TN, C:N, POXC, ACE, MinC and PMN. Analysis was for rotationally grazed pastures (n=78).
Table includes p-values and, when p-value < 0.05, letters to indicate significant differences according to Fisher's LSD.

	Texture Class						Drainage Class					
		loamy sand	sandy loam	loam	silt loam	silty clay loam		somewhat poorly drained	moderately drained	well drained	somewhat excessively drained	excessively drained
n		1	8	8	58	3		16	14	45	1	2
	ANOVA p-value						ANOVA p-value					
OM (%)	0.149						0.326					
TC (%)	0.036	c	a	abc	ab	bc	0.016	b	a	b	b	a
TN (%)	0.044	c	ab	abc	a	bc	0.163					
C:N	< 0.001	ab	a	b	b	ab	0.005	b	b	b	ab	a
POXC (mg kg^{-1})	0.072						0.294					
ACE (g kg^{-1})	0.020	b	a	ab	b	b	0.035	ab	a	ab	b	a
MinC (mg kg^{-1})	0.005	b	b	ab	a	ab	0.014	b	ab	a	b	b
PMN (mg kg^{-1})	0.001	b	b	ab	a	ab	0.077					

OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

Table S2.9: Analysis of Variance (ANOVA) results for region and land ownership for OM, TC, TN, C:N, POXC, ACE, MinC and PMN. Analysis was for rotationally grazed pastures (n=78).
Table includes p-values and, when p-value < 0.05, letters to indicate significant differences according to Fisher's LSD.

	Region			Land ownership		Operation Type			
		Kickapoo	Marathon	Owned	Rented	Dairy	Heifers	Beef	Multispecies
n		38	40	68	10	15	7	44	12
	ANOVA p-value			ANOVA p-value		ANOVA p-value			
OM (%)	0.053			0.765		0.787			
TC (%)	0.026	b	a	0.217		0.423			
TN (%)	0.179			0.196		0.621			
C:N	0.040	b	a	0.337		0.751			
POXC (mg kg^{-1})	0.101			0.694		0.150			
ACE (g kg^{-1})	0.007	b	a	0.316		0.721			
MinC (mg kg^{-1})	0.001	a	b	0.497		0.817			
PMN (mg kg^{-1})	0.037	a	b	0.337		0.751			

OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

Table S2.10: Analysis of Variance (ANOVA) results for previous land use, outwintering, and hay frequency for OM, TC, TN, C:N, POXC, ACE, MinC and PMN. Analysis was for rotationally grazed pastures (n=78).
Table includes p-values and, when p-value < 0.05, letters to indicate significant differences according to Fisher's LSD.

	Previous Land Use						Outwinter			Hay frequency		
		Fallow	Hay	Row Crops	Row crops & hay	Unknown		No	Yes		Never	Sometimes	Often
n		5	14	28	27	4		57	21		55	13	10
	ANOVA p-value						ANOVA p-value			ANOVA p-value			
OM (%)	0.014	ab	c	c	bc	a	0.004	b	a	0.016	a	b	ab
TC (%)	0.028	ab	c	bc	bc	a	0.006	b	a	0.002	a	b	b
TN (%)	0.134						0.020	b	a	0.004	a	b	b
C:N	0.466						0.410			0.299			
POXC (mg kg^{-1})	0.195						0.020	b	a	0.012	a	b	b
ACE (g kg^{-1})	0.001	a	c	bc	b	ab	<0.001	b	a	0.047	a	b	ab
MinC (mg kg^{-1})	0.012	ab	bc	c	c	ab	0.537			0.010	a	ab	b
PMN (mg kg^{-1})	0.315						0.498			0.100			

OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

Table S2.11: Analysis of Variance (ANOVA) results for fertilizer use, fertilizer applied this year, manure use, manure applied this year, and lime use for OM, TC, TN, C:N, POXC, ACE, MinC and PMN. Analysis was for rotationally grazed pastures (n=78).
Table includes p-values and, when p-value < 0.05, letters to indicate significant differences according to Fisher's LSD.

	Fertilizer applied (5yrs)			Fertilizer applied this year			Manure applied (5yrs)			Manure applied this year			Lime applied (5yrs)		
		No	Yes		No	Yes		No	Yes		No	Yes		No	Yes
n		40	38		55	23		50	28		67	8		53	25
	ANOVA p-value			ANOVA p-value			ANOVA p-value			ANOVA p-value			ANOVA p-value		
OM (%)	0.133			0.207			0.654			0.005	b	a	0.001	a	b
TC (%)	0.305			0.128			0.379			0.112			0.001	a	b
TN (%)	0.092			0.333			0.756			0.025	b	a	0.001	a	b
C:N	0.051			0.316			0.099			0.130			0.773		
POXC (mg kg^{-1})	0.007	a	b	0.008	a	b	0.630			0.002	b	a	0.000	a	b
ACE (g kg^{-1})	0.543			0.143			0.231			0.293			0.194		
MinC (mg kg^{-1})	0.003	a	b	0.118			0.329			0.672			0.087		
PMN (mg kg^{-1})	0.003	a	b	0.400			0.980			0.104			0.003	a	b

OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

Table S2.12: Linear regression results for all continuous variables and organic matter (OM)%, total carbon (TC)%, total nitrogen (TN)%, and carbon-to-nitrogen ratio (C:N) of rotationally grazed pastures (n=78).
Table includes p-values, and if the relationship was significant (p-value <0.05), coefficient of determination (R2) and direction of relationship (+ or -). ▲ signifies that explanatory variables were transformed into a polynomial to fit assumptions of linear regression; for the trendline direction, the first sign corresponds to "x" and the second sign corresponds to "x^2".

	OM (%)			TC (%)			TN (%)			C:N		
	p-value	R^2	Slope	p-value	R^2	Slope	p-value	R^2	Slope	p-value	R^2	Slope
Soil Properties												
pH	0.007	0.09	+	0.067	0.04	+	0.398			0.006	0.10	+
Sand (%)	0.354			0.405			0.342			<0.001	0.28	+
Silt (%)	0.271			0.697			0.174			<0.001	0.27	-
Clay (%)	0.946			0.032	0.06	-	0.367			<0.001 ▲	0.31	-, +
Pasture management												
Pasture age	<0.001	0.21	+	<0.001	0.28	+	<0.001	0.27	+	0.848		
Fertilizer frequency (past 5 years)	0.293			0.883			0.806			0.438		
Manure frequency (past 5 years)	0.098			0.422			0.153			0.110		
Legume %	0.474			0.298			0.484			0.402		
Grazing management												
Days between grazing and sampling	0.726			0.392			0.575			0.509		
Stocking rate	0.623			0.892			0.447			0.038	0.06	-
Stocking density	0.007 ▲	0.12	+, -	0.004 ▲	0.14	+ , -	0.004 ▲	0.14	+, -	0.316		
Seasonal grazing pressure	0.088 ▲	0.06	+, -	0.035 ▲	0.09	+ , -	0.006 ▲	0.13	+, -	0.159		
Number of rotations	0.038 ▲	0.08	+ , -	0.020 ▲	0.10	+ , -	0.044 ▲	0.08	+, -	0.523		
Time in paddock	0.793			0.683			0.837			0.879		
Rest period	0.780			0.024	0.07	-	0.003	0.11	-	0.035	0.06	+
Residual pasture height	0.586			0.931			0.868			0.871		

Table S2.13: Linear regression results for all continuous variables and permanganate oxidizable carbon (POXC), autoclaved-citrate extractable protein (ACE), mineralizable carbon (MinC), and potentially mineralizable nitrogen (PMN) of rotationally grazed pastures (n=78).
Table includes p-values, and if the relationship was significant (p-value <0.05), coefficient of determination (R2) and direction of relationship (+ or -). ▲signifies that explanatory variables were transformed into a polynomial to fit assumptions of linear regression; for the trendline direction, the first sign corresponds to "x" and the second sign corresponds to "x^2".

	POXC			ACE			MinC			PMN		
	p-value	R^2	Slope	p-value	R^2	Slope	p-value	R^2	Slope	p-value	R^2	Slope
Soil Properties												
pH	0.001	0.15	+	0.984			0.984			0.560		
Sand (%)	0.266			0.051	0.05		<0.001	0.27	-	<0.001	0.23	-
Silt (%)	0.122			0.142			<0.001	0.27	+	<0.001	0.25	+
Clay (%)	0.433			0.002	0.12	-	<0.001	0.27	+, -	<0.001	0.21	+, -
Pasture management												
Pasture age	0.001	0.13	+	<0.001	0.29	+	<0.001	0.17	+	<0.001	0.16	+
Fertilizer frequency (past 5 years)	0.053	0.05	-	0.277			0.003	0.11	-	0.021	0.07	-
Manure frequency (past 5 years)	0.099			0.950			0.648			0.385		
Legume %	0.659			0.697			0.539			0.537		
Grazing management												
Days between grazing and sampling	0.621			0.502			0.257			0.531		
Stocking rate	0.423			0.884			0.069			0.007	0.09	+
Stocking density	<0.001▲	0.20	+, -	0.009▲	0.12	+, -	0.738			0.385		
Seasonal grazing pressure	0.017▲	0.10	+, -	0.036▲	0.09	+, -	0.460▲			0.030▲	0.09	+, -
Number of rotations	0.090▲	0.06	+, -	0.4536			0.050▲	0.08	+, -	0.104▲		
Time in paddock	0.530			0.954			0.306			0.741		
Rest period	0.042	0.05	-	0.774			0.047	0.05	-	0.035	0.06	-
Residual pasture height	0.651			0.400			0.953			0.628		

Table S2.14: Organic Matter random forest analysis results.
"VIMP rank", "Importance" and "relative importance" correspond with variables of importance (VIMP) analysis. Variables are ranked in order of variable importance (VIMP), which is calculated based on how important the variable was in explaining variation in the indicator value. "Minimal depth rank" and "minimal depth value" refers to the average minimal depth the factor appears in the regression trees. A minimal depth closer to 1 signifies that the factor was selected closer to the root node in the regression trees, and is more predictive of variation in the indicator as a single split.

Organic Matter Random Forest: Factor Importance					
	VIMP Analysis			Minimal Depth Analysis	
	Rank	Variable Importance	Relative Importance	Rank	Average Minimal Depth
Pasture age	1	0.48	1	1	2.59
Manure frequency	2	0.20	0.42	2	4.96
Time in paddock	3	0.18	0.36	7	5.35
Previous land use	4	0.11	0.24	8	5.39
Lime	5	0.08	0.16	4	5.08
Stocking density	6	0.07	0.15	3	5.08
Residual pasture height	7	0.06	0.12	12	5.81
Clay (%)	8	0.05	0.11	13	5.91
Number of rotations	9	0.05	0.11	6	5.24
pH	10	0.05	0.11	5	5.09
Outwinter	11	0.05	0.10		
Rest period	12	0.05	0.10	11	5.54
Legume (%)	13	0.04	0.09	9	5.41
Seasonal grazing pressure	14	0.04	0.09	10	5.49
Silt (%)	15	0.03	0.06		
Sand (%)	16	0.03	0.06		
Hay frequency	17	0.02	0.04	14	5.97
Days between grazing and sampling	18	0.02	0.04		
Drainage class	19	0.01	0.02		
Stocking rate	20	0.01	0.02		
Region	21	0.01	0.02		
Fertilizer frequency	22	0.00	0.01		
Fertilizer this year	23	0.00	0.00		
Operation type	24	0.00	0.00		
Fertilizer	25	0.00	0.00		
Manure	26	0.00	0.00		
(OOB) R squared: 0.32					
(OOB) Requested performance error: 0.38					

Table S2.15: Total carbon random forest analysis results.
"VIMP rank", "Importance" and "relative importance" correspond with variables of importance (VIMP) analysis. Variables are ranked in order of variable importance (VIMP), which is calculated based on how important the variable was in explaining variation in the indicator value. "Minimal depth rank" and "minimal depth value" refers to the average minimal depth the factor appears in the regression trees. A minimal depth closer to 1 signifies that the factor was selected closer to the root node in the regression trees, and is more predictive of variation in the indicator as a single split.

Total Carbon Random Forest: Factor Importance					
	VIMP Analysis			Minimal Depth Analysis	
	Rank	Variable Importance	Relative Importance	Rank	Average Minimal Depth
Pasture age	1	0.29	1	1	2.31
Time in paddock	2	0.08	0.29	4	4.71
Stocking density	3	0.05	0.18	3	4.53
Rest period	4	0.04	0.15	2	4.46
Previous land use	5	0.03	0.12	15	5.43
Silt (%)	6	0.03	0.11	6	5.02
Drainage class	7	0.03	0.11	14	5.39
Clay (%)	8	0.03	0.10	7	5.05
Sand (%)	9	0.03	0.09	5	4.91
Lime	10	0.02	0.08	8	5.14
Seasonal grazing pressure	11	0.02	0.07	10	5.18
Number of rotations	12	0.02	0.07	11	5.22
Residual pasture height	13	0.02	0.07	12	5.33
Hay frequency	14	0.02	0.06	9	5.16
pH	15	0.01	0.05	13	5.36
Outwinter	16	0.01	0.05		
Stocking rate	17	0.01	0.04		
Region	18	0.01	0.03		
Legume (%)	19	0.01	0.02		
Manure frequency	20	0.01	0.02		
Days between grazing and sampling	21	0.00	0.01		
Operation type	22	0.00	0.01		
Fertilizer this year	23	0.00	0.00		
Manure	24	0.00	0.00		
Fertilizer	25	0.00	0.00		
Fertilizer frequency	26	0.00	0.00		
(OOB) R squared: 0.37 (OOB) Requested performance error: 0.19					

Table S2.16: Total Nitrogen random forest analysis results.
"VIMP rank", "Importance" and "relative importance" correspond with variables of importance (VIMP) analysis. Variables are ranked in order of variable importance (VIMP), which is calculated based on how important the variable was in explaining variation in the indicator value. "Minimal depth rank" and "minimal depth value" refers to the average minimal depth the factor appears in the regression trees. A minimal depth closer to 1 signifies that the factor was selected closer to the root node in the regression trees, and is more predictive of variation in the indicator as a single split.

Total Nitrogen Random Forest: Factor Importance					
	VIMP Analysis			Minimal Depth Analysis	
	Rank	Variable Importance	Relative Importance	Rank	Average Minimal Depth
Pasture age	1	0.0023	1	1	2.01
Stocking density	2	0.0004	0.19	3	4.16
Rest period	3	0.0004	0.17	2	4.12
Manure frequency	4	0.0004	0.16	9	5.28
Time in paddock	5	0.0003	0.12	11	5.34
Silt (%)	6	0.0002	0.10	5	4.97
Seasonal grazing pressure	7	0.0002	0.10	4	4.52
Sand (%)	8	0.0002	0.08	8	5.16
Clay (%)	9	0.0002	0.08	14	5.41
Residual pasture height	10	0.0001	0.06	7	5.14
Stocking rate	11	0.0001	0.06	13	5.41
Previous land use	12	0.0001	0.06		
Lime	13	0.0001	0.06	6	5.12
Outwinter	14	0.0001	0.05		
pH	15	0.0001	0.04	15	5.50
Number of rotations	16	0.0001	0.03	12	5.39
Hay frequency	17	0.0001	0.03	10	5.32
Drainage class	18	0.0001	0.03		
Legume (%)	19	0	0.01		
Region	20	0	0.01		
Operation type	21	0	0.01		
Days between grazing and sampling	22	0	0.00		
Fertilizer frequency	23	0	0.00		
Fertilizer this year	24	0	0.00		
Manure	25	0	0.00		
Fertilizer	26	0	0.00		
(OOB) R squared: 0.35					
(OOB) Requested performance error: 0.0017					

Table S2.17: Carbon to nitrogen ratio (C:N) random forest analysis results. "VIMP rank", "Importance" and "relative importance" correspond with variables of importance (VIMP) analysis. Variables are ranked in order of variable importance (VIMP), which is calculated based on how important the variable was in explaining variation in the indicator value. "Minimal depth rank" and "minimal depth value" refers to the average minimal depth the factor appears in the regression trees. A minimal depth closer to 1 signifies that the factor was selected closer to the root node in the regression trees, and is more predictive of variation in the indicator as a single split.

C:N Random Forest: Factor Importance					
	VIMP Analysis			Minimal Depth Analysis	
	Rank	Variable Importance	Relative Importance	Rank	Average Minimal Depth
pH	1	2.05	1	2	3.63
Clay (%)	2	0.62	0.30	1	3.23
Sand (%)	3	0.36	0.18	4	4.23
Silt (%)	4	0.35	0.17	3	4.16
Drainage class	5	0.13	0.06	5	5.19
Time in paddock	6	0.13	0.06	6	5.21
Operation type	7	0.07	0.03		
Stocking rate	8	0.06	0.03		
Rest period	9	0.05	0.03		
Seasonal grazing pressure	10	0.05	0.02		
Manure frequency	11	0.03	0.01		
Pasture age	12	0.03	0.01		
Fertilizer frequency	13	0.03	0.01		
Residual pasture height	14	0.03	0.01		
Previous land use	15	0.02	0.01		
Region	16	0.02	0.01		
Stocking density	17	0.02	0.01		
Number or rotations	18	0.02	0.01		
Fertilizer this year	19	0.01	0.01		
Legume (%)	20	0.01	0.00		
Hay frequency	21	0.01	0.00		
Days between grazing and sampling	22	0.01	0.00		
Fertilizer	23	0.01	0.00		
Lime	24	0.00	0.00		
Outwinter	25	0.00	0.00		
Manure	26	0.00	0.00		
(OOB) R squared: 0.35					
(OOB) Requested performance error: 0.69					

Table S2.18: POXC random forest analysis results.
"VIMP rank", "Importance" and "relative importance" correspond with variables of importance (VIMP) analysis. Variables are ranked in order of variable importance (VIMP), which is calculated based on how important the variable was in explaining variation in the indicator value. "Minimal depth rank" and "minimal depth value" refers to the average minimal depth the factor appears in the regression trees. A minimal depth closer to 1 signifies that the factor was selected closer to the root node in the regression trees, and is more predictive of variation in the indicator as a single split.

POXC Random Forest: Factor Importance					
	VIMP Analysis			Minimal Depth Analysis	
	Rank	Variable Importance	Relative Importance	Rank	Average Minimal Depth
Pasture age	1	12684	1	1	2.93
Stocking density	2	7366	0.58	2	3.23
Time in paddock	3	7055	0.56	5	4.16
Lime	4	6062	0.48	4	3.60
pH	5	4651	0.37	3	3.45
Rest period	6	3466	0.27	6	4.31
Residual pasture height	7	2273	0.18	8	4.62
Previous land use	8	2142	0.17	10	4.98
Seasonal grazing pressure	9	1916	0.15	7	4.39
Number of rotations	10	1642	0.13	9	4.79
Manure frequency	11	1124	0.09		
Operation type	12	979	0.08		
Fertilizer frequency	13	950	0.07		
Outwinter	14	938	0.07		
Fertilizer this year	15	859	0.07		
Clay (%)	16	677	0.05		
Hay frequency	17	594	0.05		
Drainage class	18	585	0.05		
Legume (%)	19	534	0.04		
Manure	20	500	0.04		
Stocking rate	21	462	0.04		
Silt (%)	22	240	0.02		
Fertilizer	23	202	0.02		
Region	24	178	0.01		
Days between grazing and sampling	25	67	0.01		
Sand (%)	26	-52	0.00		
(OOB) R squared: 0.45					
(OOB) Requested performance error: 15039					

Table S2.19: ACE random forest analysis results.
"VIMP rank", "Importance" and "relative importance" correspond with variables of importance (VIMP) analysis. Variables are ranked in order of variable importance (VIMP), which is calculated based on how important the variable was in explaining variation in the indicator value. "Minimal depth rank" and "minimal depth value" refers to the average minimal depth the factor appears in the regression trees. A minimal depth closer to 1 signifies that the factor was selected closer to the root node in the regression trees, and is more predictive of variation in the indicator as a single split.

ACE Random Forest: Factor Importance					
	VIMP Analysis			Minimal Depth Analysis	
	Rank	Variable Importance	Relative Importance	Rank	Average Minimal Depth
Pasture age	1	6.17	1	1	2.30
Time in paddock	2	1.99	0.32	2	4.25
Residual pasture height	3	1.16	0.19		
Previous land use	4	1.15	0.19	3	4.29
Outwinter	5	0.75	0.12	4	4.69
Stocking density	6	0.34	0.06	6	5.03
Clay (%)	7	0.23	0.04	5	4.78
Seasonal grazing pressure	8	0.20	0.03		
Rest period	9	0.18	0.03		
Silt (%)	10	0.17	0.03		
Sand (%)	11	0.17	0.03		
Drainage class	12	0.15	0.02		
Legume (%)	13	0.12	0.02		
Manure frequency	14	0.12	0.02		
Hay frequency	15	0.07	0.01		
Region	16	0.07	0.01		
Number of rotations	17	0.05	0.01		
Days between grazing and sampling	18	0.04	0.01		
Stocking rate	19	0.03	0.01		
pH	20	0.02	0.00		
Operation type	21	0.02	0.00		
Fertilizer this year	22	0.01	0.00		
Lime	23	0.01	0.00		
Fertilizer frequency	24	0.00	0.00		
Manure	25	0.00	0.00		
Fertilizer	26	0.00	0.00		
(OOB) R squared: 0.32					
(OOB) Requested performance error: 2.18					

Table S2.20: MinC random forest analysis results.
"VIMP rank", "Importance" and "relative importance" correspond with variables of importance (VIMP) analysis. Variables are ranked in order of variable importance (VIMP), which is calculated based on how important the variable was in explaining variation in the indicator value. "Minimal depth rank" and "minimal depth value" refers to the average minimal depth the factor appears in the regression trees. A minimal depth closer to 1 signifies that the factor was selected closer to the root node in the regression trees, and is more predictive of variation in the indicator as a single split.

MinC Random Forest: Factor Importance					
	VIMP Analysis			Minimal Depth Analysis	
	Rank	Variable Importance	Relative Importance	Rank	Average Minimal Depth
Pasture age	1	630	1	1	2.67
Clay (%)	2	489	0.78	3	3.79
Sand (%)	3	345	0.55	2	3.65
Silt (%)	4	303	0.48	4	3.89
Previous land use	5	144	0.23	6	4.80
Time in paddock	6	140	0.22	7	5.06
Hay frequency	7	83	0.13	11	5.53
Number of rotations	8	78	0.12	9	5.49
Legume (%)	9	72	0.11	5	4.61
pH	10	66	0.11	8	5.09
Fertilizer frequency	11	47	0.08		
Outwinter	12	47	0.07		
Stocking density	13	25	0.04	10	5.52
Residual pasture height	14	13	0.02		
Rest period	15	13	0.02		
Stocking rate	16	12	0.02		
Region	17	11	0.02		
Drainage class	18	11	0.02		
Days between grazing and sampling	19	11	0.02		
Seasonal grazing pressure	20	8	0.01		
Fertilizer	21	6	0.01		
Manure frequency	22	2	0.00		
Lime	23	1	0.00		
Fertilizer this year	24	0	0.00		
Manure	25	0	0.00		
Operation type	26	-1	0.00		
(OOB) R squared: 0.36					
(OOB) Requested performance error: 831					

Table S2.21: PMN random forest analysis results.
"VIMP rank", "Importance" and "relative importance" correspond with variables of importance (VIMP) analysis. Variables are ranked in order of variable importance (VIMP), which is calculated based on how important the variable was in explaining variation in the indicator value. "Minimal depth rank" and "minimal depth value" refers to the average minimal depth the factor appears in the regression trees. A minimal depth closer to 1 signifies that the factor was selected closer to the root node in the regression trees, and is more predictive of variation in the indicator as a single split.

PMN Random Forest: Factor Importance					
	VIMP Analysis			Minimal Depth Analysis	
	Rank	Variable Importance	Relative Importance	Rank	Average Minimal Depth
Pasture age	1	852	1	1	2.48
Clay (%)	2	369	0.43	4	4.35
Sand (%)	3	346	0.41	3	4.04
Silt (%)	4	300	0.35	2	3.83
Stocking rate	5	184	0.22	5	4.82
Time in paddock	6	154	0.18	7	5.00
Rest period	7	101	0.12	6	4.94
Seasonal grazing pressure	8	80	0.09	8	5.17
Residual pasture height	9	72	0.08		
Stocking density	10	65	0.08	10	5.29
Previous land use	11	41	0.05		
Legume (%)	12	41	0.05	9	5.26
Outwinter	13	36	0.04		
Number of rotations	14	35	0.04		
Lime	15	33	0.04		
Fertilizer frequency	16	27	0.03		
pH	17	17	0.02		
Hay frequency	18	14	0.02		
Manure frequency	19	13	0.02		
Fertilizer	20	12	0.01		
Operation type	21	11	0.01		
Drainage class	22	7	0.01		
Days between grazing and sampling	23	7	0.01		
Fertilizer this year	24	4	0.00		
Region	25	1	0.00		
Manure	26	0	0.00		
(OOB) R squared: 0.44					
(OOB) Requested performance error: 674					

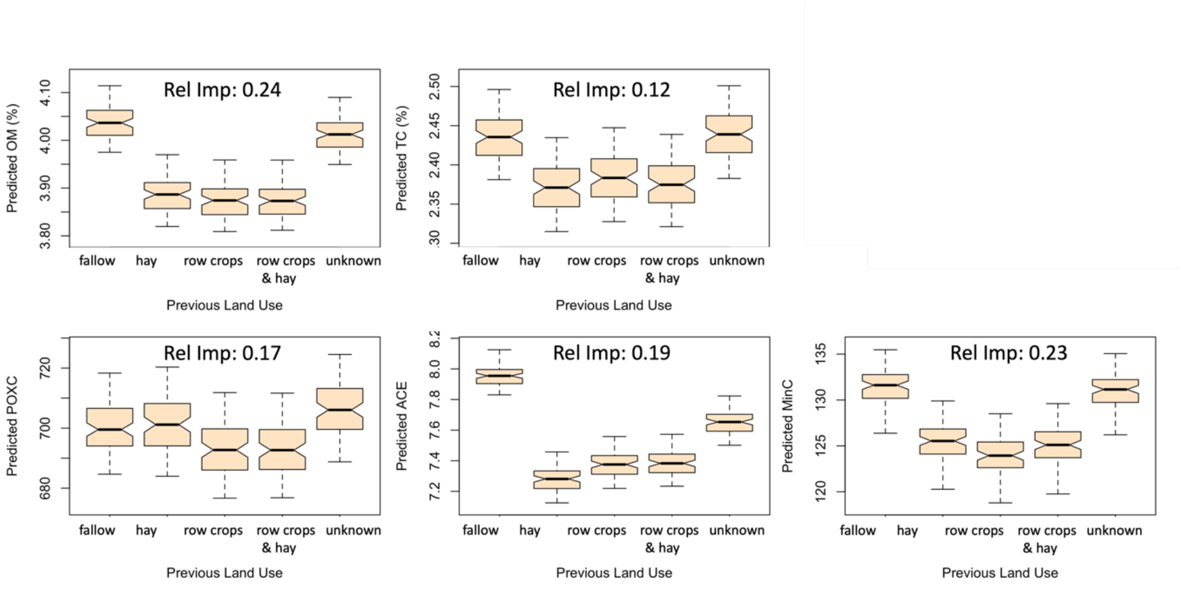

Figure S2.6: Partial effect of previous land use on OM, TC, POXC, ACE, and MinC
Predicted values are calculated for previous land use category over all remaining covariates, averaged, and plotted.

Rel Imp= relative importance according to VIMP
OM= organic matter (%); TC= total carbon (%); POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1})

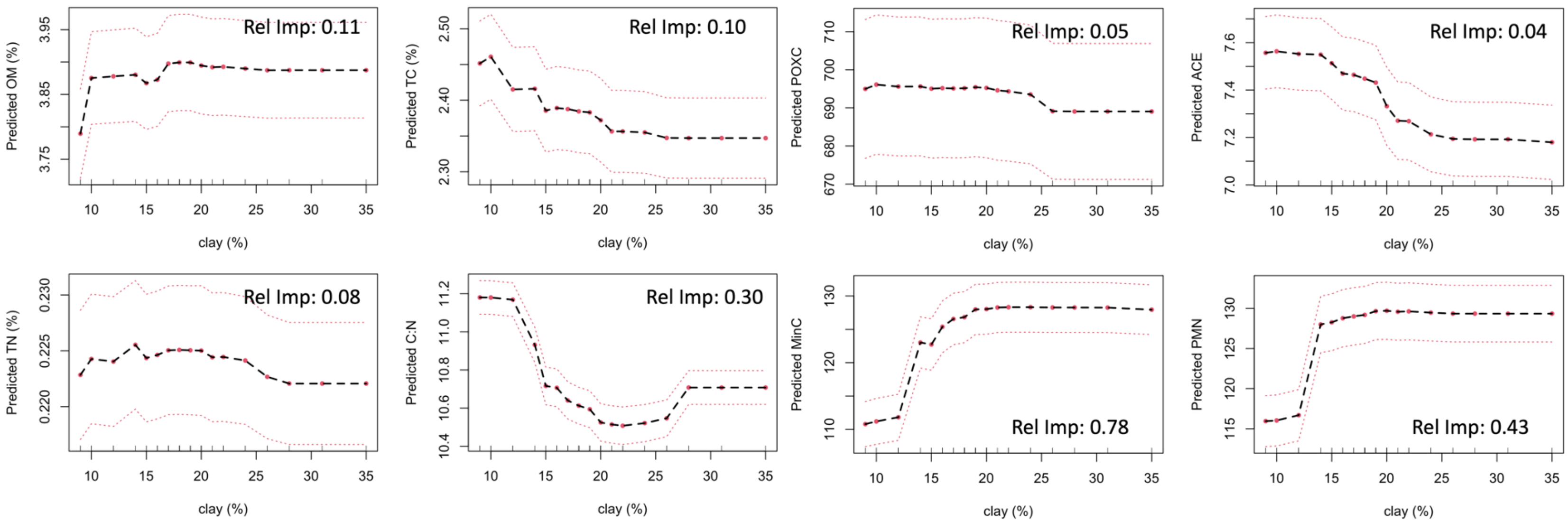

Figure S2.7: Partial effect of clay content on OM, TC, TN, C:N, POXC, ACE, MinC and PMN

Predicted values are calculated as clay (%) varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.

Rel Imp= relative importance according to VIMP

OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

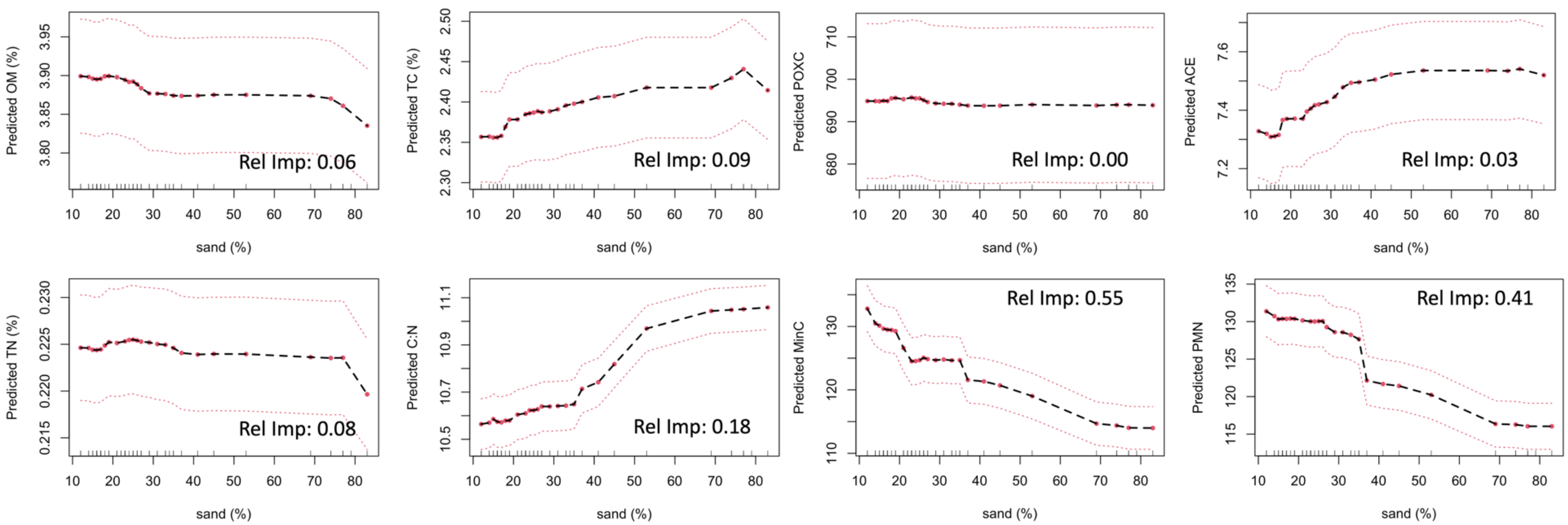

Figure S2.8: Partial effect of sand content on OM, TC, TN, C:N, POXC, ACE, MinC and PMN

Predicted values are calculated as sand (%) varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.

Rel Imp= relative importance according to VIMP

OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

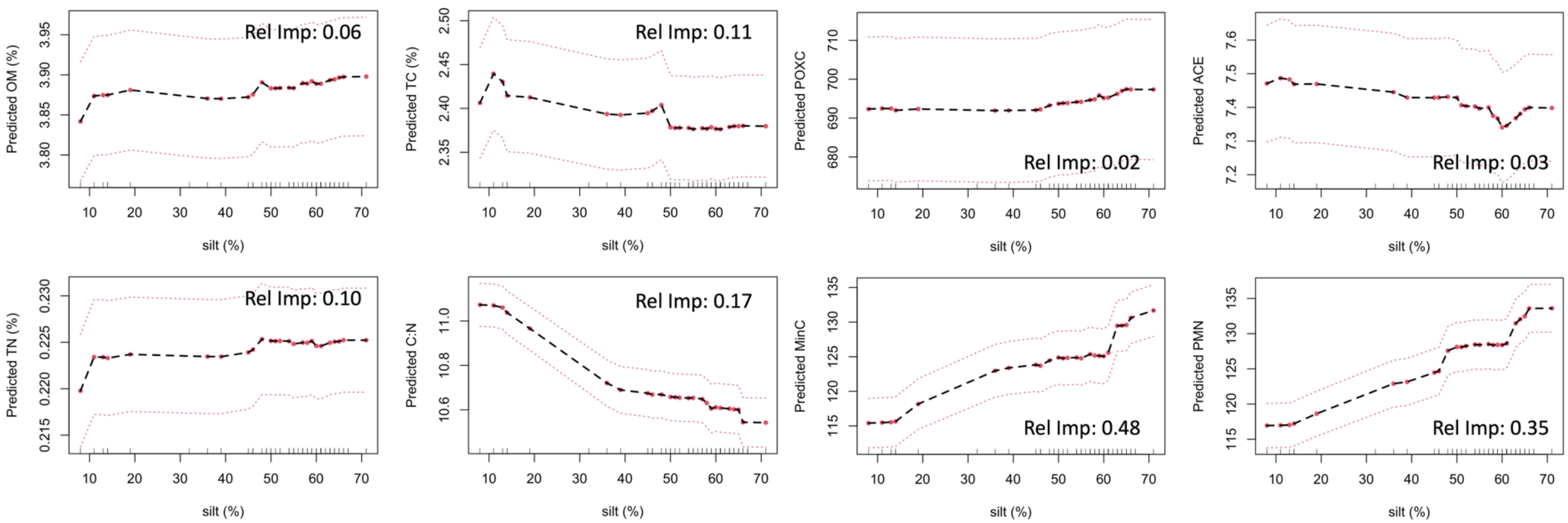

Figure S2.9: Partial effect of silt content on OM, TC, TN, C:N, POXC, ACE, MinC and PMN
Predicted values are calculated as silt (%) varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

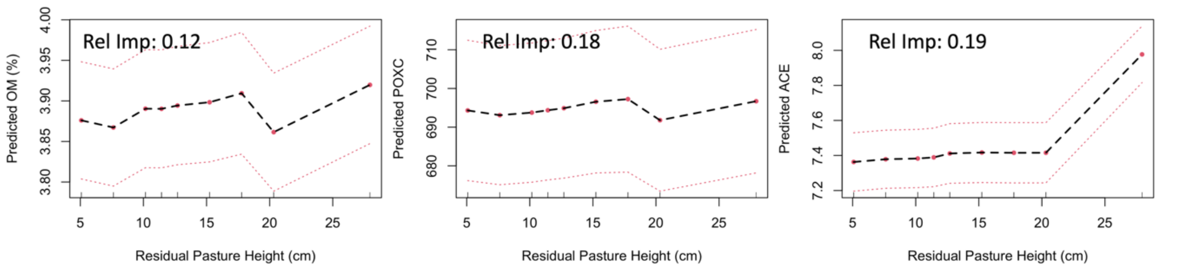

Figure S2.10: Partial effect of residual pasture height on OM, POXC, ACE
Predicted values are calculated as residual pasture height varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
OM= organic matter (%); POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1})

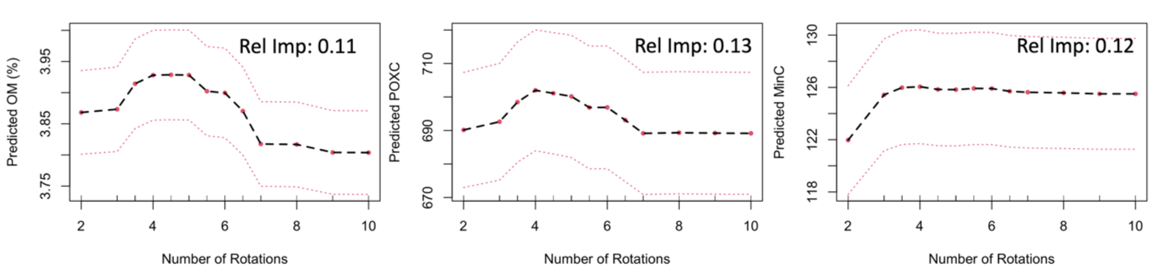

Figure S2.11: Partial effect of number of rotations on OM, POXC, MinC
Predicted values are calculated as number of rotations varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
OM= organic matter (%); POXC = permanganate oxidizable carbon (mg kg^{-1}); MinC = mineralizable carbon (mg kg^{-1})

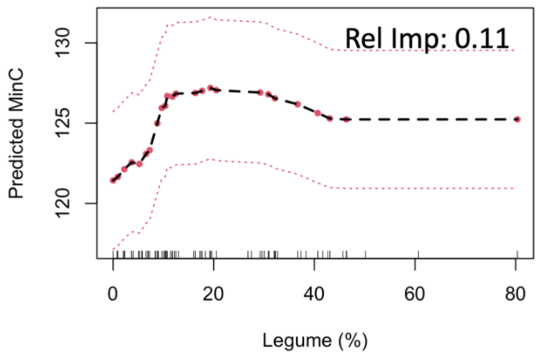

Figure S2.12: Partial effect of percent legumes on MinC
Predicted values are calculated as legume (%) varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
MinC = mineralizable carbon (mg kg^{-1})

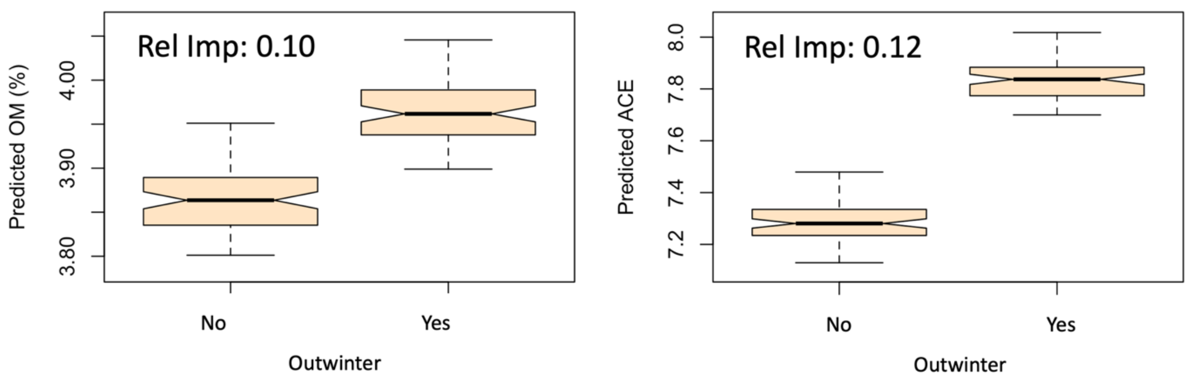

Figure S2.13: Partial effect of outwinter on OM and ACE
Predicted values are calculated for outwintering categories over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
OM= organic matter (%); ACE = autoclaved-citrate extractable protein (g kg^{-1})

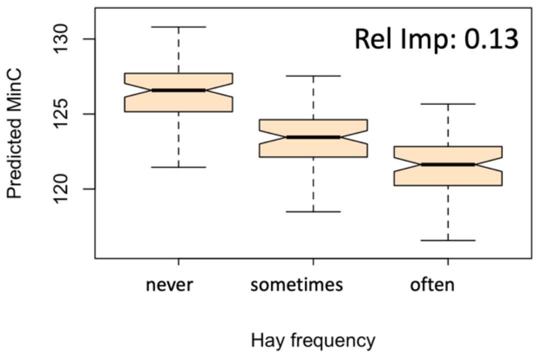

Figure S2.14: Partial effect of haying frequency on MinC
Predicted values are calculated for hay frequency categories over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
MinC = mineralizable carbon (mg kg^{-1})

R Code

Univariate Statistics:

```
#group soil C and N indicators
library(dplyr)
Indicators <- SH_da %>%
  dplyr:: select(OM, TC, TN, TCtoTN, POXC_June, ACE_June, MinC_June, PMN_June)

#analysis
library(pastecs)
library(psych)
summary(Indicators)
describe(Indicators)
stat.desc(Indicators)
```

Linear Regression:

```
m <- lm(POXC_June~OM, data=SH_da)
plot(m) #check assumptions of linear model
summary(m)
```

Correlation matrix:

```
library(GGally)
library(ggpubr)
library(ggplot2)
ggpairs(Indicators)
```

ANOVA

```
library(agricolae)
model <- aov(OM ~ Texture, data= SH_da)
summary(model)
lsd=LSD.test(model, c("Texture"), group=T)
lsd
```

Random Forest

```
#load packages
library(randomForestSRC)
library(ggRandomForests)

#set seed & make random forest
set.seed(220603)
OM_RF <- rfsrc(OM ~ Region+ pH + sand + clay + silt +
            DrainageClass+ OperationType_General + PastureYears + PreviousLandUse_cat +
            Legume_June+
            Fertilizer_thisYear + Fertilizer + Fertilizer_freq + Manure +
            Manure_freq + Lime + Overwinter  + HayFreq + DaysSinceGrazing_June +
                         StockingRate_ha + StockingDensity_ha +
                  SeasonStockingDensity_ha + NumberRotations +
            TimeInPaddock + RestPeriod + ResidualPastureHeight_cm,
           data = Rotation, ntree = 5000,
           na.action = "na.impute", nimpute = 1,
           importance = "anti",
           forest=TRUE)

#retrieve random forest results
OM_RF
```

```
#plot random forest results
plot(OM_RF)
plot(gg_vimp(OM_RF))

#minimal depth analysis
var.select(object = OM_RF, conservative = "low")

#plot partial effects
plot.variable(OM_RF, xvar.names = "PastureYears", partial = TRUE,
        xlab="Pasture Age(years)", ylab="Predicted OM (%)")
```

Chapter 3: Biological indicators of soil health are sensitive to sampling time in Wisconsin pasture

Abstract

Biological indicators of soil health are sensitive to sampling time and understanding temporal effects can shape how to use soil health testing in the future. While soil health testing is common in row cropping systems, uncertainty remains if, and when, soil health testing may be beneficial in pastures. Pastures in Wisconsin (n=92) were sampled for permanganate oxidizable carbon (POXC), autoclave citrate extractable protein (ACE), mineralizable carbon (MinC), and anaerobic potentially mineralizable nitrogen (PMN) in June and September to evaluate (i) seasonal effects on biological indicators of soil health, and (ii) differences in indicator responsiveness to soil, land use and management practices due to sampling time. All indicators were statistically different between sampling times. The effect of sampling time was largest for MinC, and the mean difference (September-June) was -45.78 mg kg^{-1}, compared to POXC (35.12 mg kg^{-1}), ACE (-0.29 g kg^{-1}), and PMN (-5.75 mg kg^{-1}). For ACE, MinC and PMN, relationships with bulk C and N were weaker in September compared to June, and R^2 values were less than those in June by 0.1-0.2. In September, pasture age had no relationship with MinC and weaker relationships with POXC, ACE and PMN (R^2<0.17), compared to June (R^2=0.19-0.28). While continuously grazed pastures had lower ACE and PMN in September compared to June, rotationally grazed pastures maintained these values between sampling times. The relative importance of inherent soil properties, land use, and management factors to biological indicators of soil health for both sampling times were evaluated using random forest analysis to explore seasonal effects. September results revealed findings not observed in June: a lower variable importance for pasture age for POXC, MinC,

and PMN, a negative relationship between MinC and synthetic fertilizer application, inconsistencies in trends between texture and MinC, and greater importance of rest period (for ACE and PMN) and residual pasture height (for MinC). Sampling time influenced how indicators relate to soil and management properties, and well-managed grazing may minimize in-season declines in soil health.

1. Intro

Soil health testing offers opportunities to evaluate and track the efficacy of management practices and improvements to soil health, but determining and standardizing methodology is necessary to make them reliable tools for farmers. Soil health indicators include simple measurements of soil biology, such as labile carbon (C) and nitrogen (N) pools as well as C and N mineralization. Biological indicators of soil health, including permanganate oxidizable carbon (POXC), autoclave citrate extractable protein (ACE), mineralizable carbon (MinC), and anaerobic potentially mineralizable nitrogen (PMN), reflect active C and N and microbial activity (Culman et al., 2012; Drinkwater, et al., 1996; Franzluebbers et al., 2000; Hurisso et al., 2018). These measurements have been identified and promoted based on criteria that they are logistically feasible, cost-effective, responsive to management, and of agronomic or environmental value (Culman et al., 2013; Franzluebbers et al., 2018; Hurisso et al., 2016; Idowu et al., 2008; Morrow et al., 2016; Nunes et al., 2018; van Es & Karlen, 2019; Wade et al., 2020; Wander & Drinkwater, 2000).

However, biological indicators of soil health also demonstrate temporal variability and seasonal sensitivity (Culman et al., 2013; Diederich et al., 2019; Hurisso et al., 2018; Nunes, Franzluebbers et al., 2016; van Es, et al., 2020). Previous research in row cropping systems found that POXC, MinC, ACE and PMN were greater in the middle of the growing season compared to spring or fall, though there is site-specific variation attributed to climate, soil properties, cropping system and management (Culman et al., 2013; Diederich et al., 2019; Hurisso, Culman, et al., 2018). Seasonal variability of biological soil health may be attributed to direct effects of climatic variables and indirect effects of plant growth and development (Stevenson et al., 2014). Though there is increasing effort to build soil health databases, sources of variability, particularly seasonal effects, are rarely included or considered (Crookston et al., 2021). As soil health testing is promoted to farmers, understanding the effect of sampling time is crucial to provide recommendations for best management practices.

The current recommendation for farmers interested in soil health testing is to consistently sample at the same time of year (Culman et al., 2013; Hurisso, Culman, et al., 2018; Morrow et al., 2016). In row crop systems, it is typical to soil sample earlier in the season (April-June), prior to fertilizer application and/or before it becomes too cumbersome to sample due to crop growth. However, depending on the sampling time, soil health test results may reveal different information about management effects on soil health or potential agronomic outcomes. Culman et al. (2013) found that as the season progressed from pre-plant to V10, biological indicators of soil health (POXC, MinC, and PMN) were more effective at predicting corn grain yield and total biomass. Other cropping systems, such as pastures, have greater flexibility with sampling time since it is easier to sample throughout the season. Therefore, soil health testing recommendations are not confined to a certain timeframe and would benefit from greater understanding into how sampling time influences soil health indicators and their relationships with management and agronomic outcomes.

Soil health research in temperate, cool-season pastures is limited, leaving uncertainty around the value and seasonal variability of soil health indicators in pastures. Seasonal patterns in plant growth and development differ in annual cropping systems and grazed, perennial pastures. In the upper Midwest, cool-season pastures attain most of their growth in the spring, declining in forage quantity and quality through the season (Brink et al., 2007; Oates et al., 2011; Paine et al., 1999; Riesterer et al., 2000). Late summer into early fall, often referred to as the “summer slump”, can be a particularly challenging time for graziers and requires well managed grazing to produce sufficient forage. Adapting grazing management to leave greater residual pasture height and provide adequate time for recovery and regrowth can help minimize summer declines in forage production (Oates et al., 2011; Paine et al., 1999). Seasonal variation in pasture growth and grazing management can impact biological soil health, and may influence how biological indicators of soil health are interpreted based on sampling time (Bardgett et al., 1997, 1999; Bardgett & Shine, 1999; Bardgett & Wardle, 2003; Jangid et al., 2008; Lin et al., 2020; Stevenson et al., 2014; Wardle et al., 2004).

This study explored how sampling time influenced biological indicators of soil health and their relationship with soil properties, land use and management practices. Using an on-farm soil health assessment approach, 92 Wisconsin pastures were sampled in early June and late September and analyzed for POXC, ACE, MinC, and PMN. These sampling times were selected for several reasons: to avoid periods of very low microbial activity common in early spring and late fall in Wisconsin (Diederich et al., 2019), to coincide with periods of high and low pasture productivity (Paine et al., 1999; Oates et al., 2011), and to evaluate effects of in-season grazing management. We hypothesized that biological indicators of soil health would differ based on sampling time and that sampling time may alter the relationships between indicators and soil properties, land use and management factors.

2. Materials and Methods

Field selection, soil sampling, lab analysis and statistical analysis were performed according to the methods outlined in *Chapter 2*, and can be found there in more detail. Briefly, 92 pastures were sampled in two regional clusters in Wisconsin: Kickapoo and Marathon. Soil samples were collected twice in 2021: between 2- 11 June and 17-26 September. From a 50m x 50m representative area, 20 soil cores were taken to a depth 0-15 cm and combined into one composite sample. Soil organic matter (OM), total carbon (TC), total nitrogen (TN), and the carbon-to-nitrogen ratio (C:N) were just analyzed in June and POXC, ACE, MinC, and PMN were analyzed in both June and September. OM, TC, TN, C:N were only analyzed once, since research shows there is less temporal variation for measurements of total N and C pools. POXC, ACE and MinC were analyzed at Ohio State University's soil test lab, according to protocols by Culman et al. (2012), Franzluebbers et al. (2000), and Hurisso et al. (2018), respectively. Anaerobic PMN was analyzed in-house according to the Drinkwater et al. (1996) protocol.

Temperature and precipitation data were collected from a central weather station in each region. Marathon weather data were retrieved from the Wausau weather station and

Kickapoo data were retrieved from the Viroqua weather station (National Weather Service). Precipitation data for August 2021 was missing at the Viroqua weather station.

Like in chapter two, descriptive statistics, linear regression, and analysis of variance (ANOVA) were performed with R statistical software version 4.1.1 (R Core Team, 2020). The relationships between the June and September biological soil health measurements were analyzed using a two-sided paired T-test, with the t.test() function. Correlation among soil health values was analyzed with the ggpairs() function. Linear regression was used to evaluate the relationship between the September biological indicators of soil health and OM, TC, TN, and C:N. For linear regression, lm() and summary() functions were used and assumptions of linear models, normality, and equal and constant variance were assessed using QQ and residuals vs. fitted plots. A p-value less than or equal to 0.05 was considered statistically significant.

Single factor and random forest analysis was used to evaluate how soil properties, land use and management factors explained variation in June and September measurements of labile C and N, in accordance with the statistical methods in chapter 2. The package randomForestSRC was used to evaluate the importance of each variable using two methods: variable importance (VIMP), which calculates how much a variable improved the random forest model when it was included, and the average minimal depth that the variable appears in the regression trees, which indicates how predictive that variable is for the overall dataset. Higher VIMP and/or lower minimal depth values correspond with factors that were important in explaining variation in the soil measures. The randomForestSRC and ggRandomForest packages were used to further analyze trends and generate partial effect plots for important variables.

3. Results

3.1 Weather

Monthly total precipitation and mean daily maximum and minimum temperatures were greatest in the summer months (Figure 3.1). From June through August 2021, the mean daily minimum was approximately 16°C and 15°C in Wausau (Marathon region) and Viroqua (Kickapoo region) respectively, and the mean daily maximum was approximately 28°C and 26°C respectively. Since August precipitation data were unavailable for the Viroqua weather station, the cumulative precipitation for June and July 2021 was 29.3cm for the Kickapoo region. For the Marathon region, the cumulative precipitation for June and July 2021 was 40.0 cm, and when including August (June through August 2021) it was 64.4cm. Seasonal trends in 2021 correspond with typical weather patterns at these weather stations (National Weather Service).

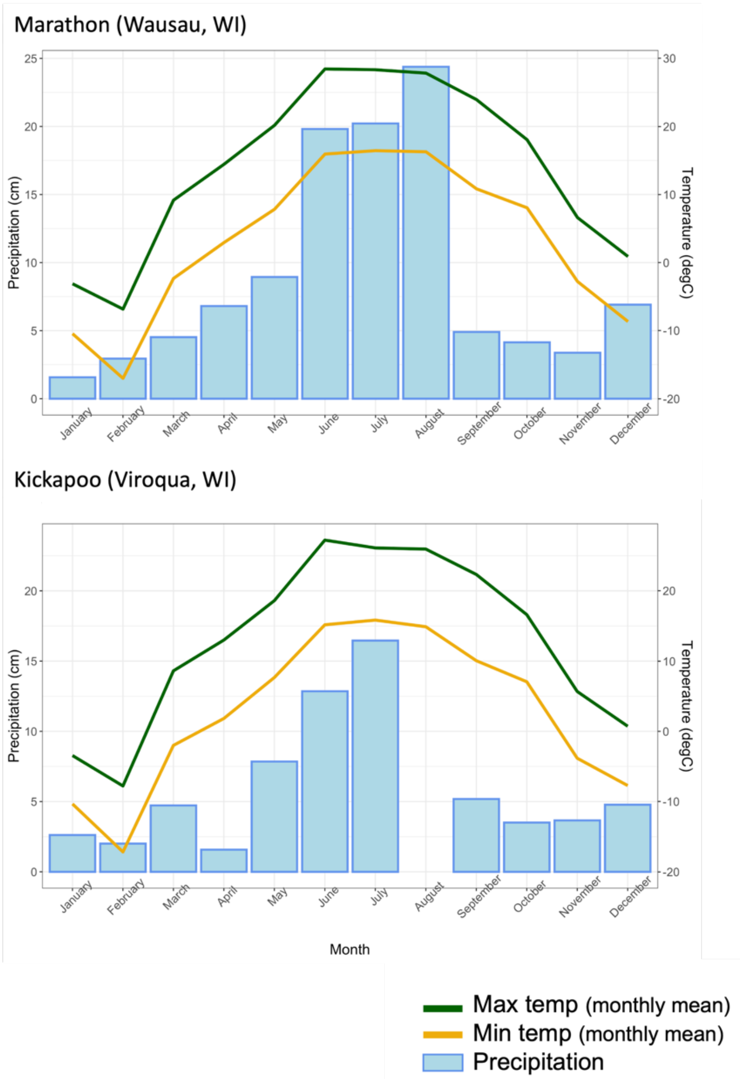

Figure 3.1 Monthly precipitation and temperature data (2021) in Wausau and Viroqua, Wisconsin.
Precipitation (cm) and temperature (°C) were measured at the Wausau and Viroqua weather stations to correspond to sites in the Marathon and Kickapoo regions respectively. Data were retrieved from the National Weather Service and the National Oceanic and Atmospheric Administration. No precipitation data was collected at the Viroqua weather station in August 2021, and thus is left blank in the figure.

3.2 Soil Measurements

3.2.1 Summary Statistics

There was a wide range of values for June and September measurements of labile C and N: POXC (373-1230 and 422-1183 mg kg^{-1}), ACE (4.5-15.5 and 4.0-13.7 g kg^{-1}), MinC (15.7-207.8 and 6.4-168.7 mg kg^{-1}), and PMN (36.9-235.9 and 37.6 mg kg^{-1}), June and September respectively (Tables S2.7 & S3.1). At both sampling times, skewness and kurtosis was just greater than 1 for ACE, indicating a slightly right-skewed, peaked distribution. The other metrics had normal distributions. Given that deviations in normality were minor, no transformations were performed, and the non-transformed response variables were used in statistical analyses.

3.2.2 Relationships between biological indicators of soil health and OM, TC, TN and C:N

In September, bulk C and N had the strongest relationships with POXC (R^2 =0.54-0.66), and the weakest relationships with MinC (R^2 ≤0.17), as was observed in June (Table 3.1). The relationships between ACE, MinC, and PMN and bulk C and N were weaker in September compared to June, and September R^2 values were less than those in June by 0.1-0.2 (Figure S3.1). In September, relationships between labile C and N and OM were stronger than those with TC and TN, but this was not observed in June. In September, only PMN had a weak, negative relationship with C:N (R^2=0.08). OM, TC, TN and C:N were measured on the soil samples collected in June, and while it is not expected for bulk C and N to vary through the season, it is possible that the weaker relationships with September measurements can be attributed to this.

Table 3.1: Coefficient of determination (R^2) for September labile C and N indicators vs. bulk C and N measurements

		OM	TC	TN	C:N
		----------------------------	R^2	----------------------------	
POXC					
	June	0.62	0.63	0.64	NS
	Sept	0.66	0.61	0.54	NS
ACE					
	June	0.52	0.60	0.53	NS
	Sept	0.43	0.39	0.30	NS
MinC					
	June	0.34	0.25	0.37	0.16
	Sept	0.17	0.15	0.14	NS
PMN					
	June	0.55	0.43	0.58	0.14
	Sept	0.58	0.39	0.48	0.08

OM= soil organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon to nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1}); NS= not significant

3.2.3 Soil health indicator correlations

All September biological indicators of soil health were positively correlated with one another. Correlations range from r=0.39-0.72 (Figure 3.2). The strongest correlations are between POXC and ACE (r=0.65) and PMN (r=0.72). The weakest correlations are between ACE and MinC (r=0.39) and PMN (r=0.41). Correlations are slightly lower for September values compared to June values, particularly for the correlations between PMN and MinC (r=0.57 vs. 0.82), and ACE (r= 0.41 vs. 0.58).

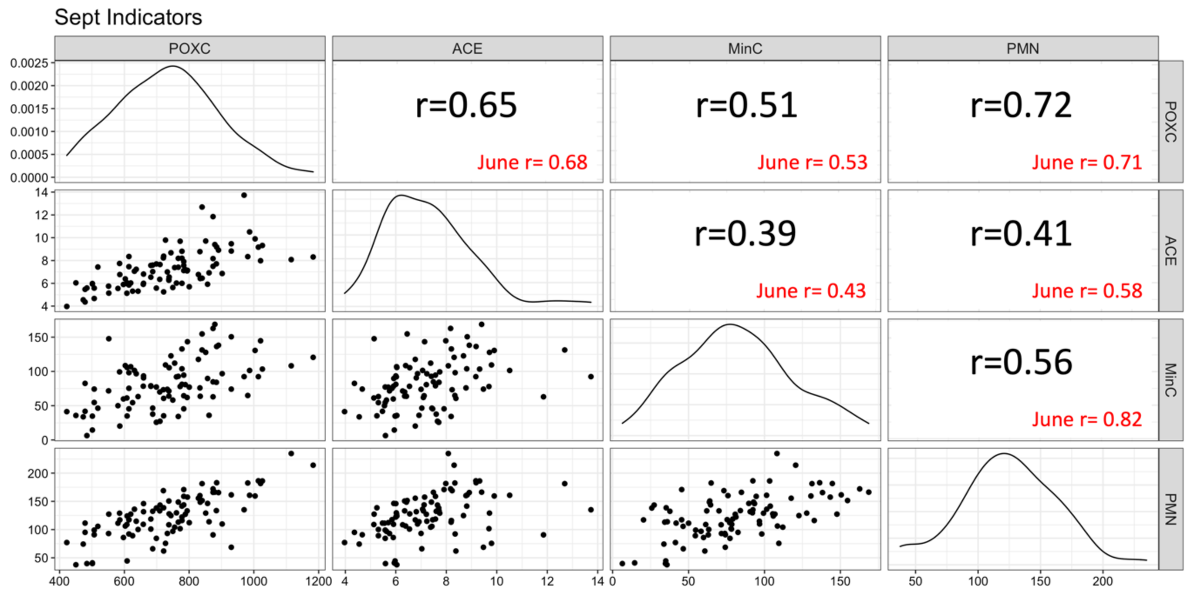

Figure 3.2: Scatterplots and correlation coefficients (r) among POXC, ACE, MinC and PMN.
Scatterplots, distribution curves and r values (in black) refer to the September measurements. June correlations are written in red for reference.
POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

3.3 Effect of sampling time

Correlations between sampling times were strong for POXC, ACE, and PMN (r > 0.84), but only r=0.41 for MinC (Table 3.2). The paired t-test revealed significant differences between June and September for all biological indicators of soil health (Table 3.3). While POXC was greater in September (with a mean difference of 35.12 mg kg^{-1}), the other indicators were lower in September, on average. Sampling time had the largest effect on MinC, with a mean difference of -45.78 mg kg^{-1}. When considering the difference between the sampling times relative to the June measurement, the average percent change in POXC, ACE, and PMN was small (3-7% with a standard deviation approximately 15%), compared to MinC, which had an average change of 32%, with a standard deviation of 31%.

Table 3.2: Correlation coefficients (r) between the June and September measures for POXC, ACE, MinC and PMN.

Indicator	Correlation (r)
POXC	0.84
ACE	0.84
MinC	0.41
PMN	0.87

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

Table 3.3: Two-sided paired T-test results for POXC, ACE, MinC and PMN evaluating the hypothesis that September-June values= 0.
Positive values indicate that September measurements were greater than June; Negative values indicate that September measurements were less than June. The test statistic, p-value, mean of difference and 95% confidence interval for the difference are included. To provide relative magnitude of the effect size, the table includes "% Difference", or the change in indicator values (%), calculated as: (September-June)/June. For the percent difference, the table includes mean, standard deviation (SD), and the range with minimum (min) and maximum (max) values). n=92

	Paired t-test			% Difference	
	p-value	Mean of difference	95% CI	Mean (SD)	[min, max]
POXC (mg kg^{-1})	<0.001	35.12	[15.71, 54.53]	7% (15%)	[-28%, 62%]
ACE (g kg^{-1})	0.011	-0.29	[-0.52, -0.07]	-3% (15%)	[-26%, 69%]
MinC (mg kg^{-1})	<0.001	-45.78	[-54.51, -37.04]	-32% (31%)	[-88%, 122%]
PMN (mg kg^{-1})	0.008	-5.75	[-9.97, -1.54]	-3% (16%)	[-59%, 46%]

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1}); SD= standard deviation; min=minimum; max=maximum

3.4 Soil properties

Sampling time had no effect on the relationship between texture and POXC, slight effects on the relationships between texture and ACE and PMN, and larger effects on the relationship between texture and MinC (Figure 3.3, Tables S3.2 & S3.3). ACE was greater in sandy loams compared to all other texture classes in September, while in June, texture class was not significant. ACE had a weak negative relationship with clay content (R^2=0.07) in September, which was not observed in June.

In September, MinC did not differ in sandy loams, loams, and silty clay loams, whereas in June, MinC was lower in loamy sands and sandy loams compared all other texture classes. Relationships between MinC and sand, silt and clay content were weaker in September (R^2 = 0.05-0.10) compared to June (0.35-0.39).

PMN had stronger relationships with texture in September compared to June. In September, PMN was lower in loamy sands and sandy loams than all other texture classes, but June showed no difference in PMN in sandy loams, loams, and silty clay loams. Relationships between PMN and sand, silt and clay were slightly stronger in September (R^2=0.38), compared to June (0.3-0.31).

pH was only significant for POXC at both sampling times and POXC had a stronger positive relationship with pH in September (R^2=0.21) compared to June (R^2=0.13).

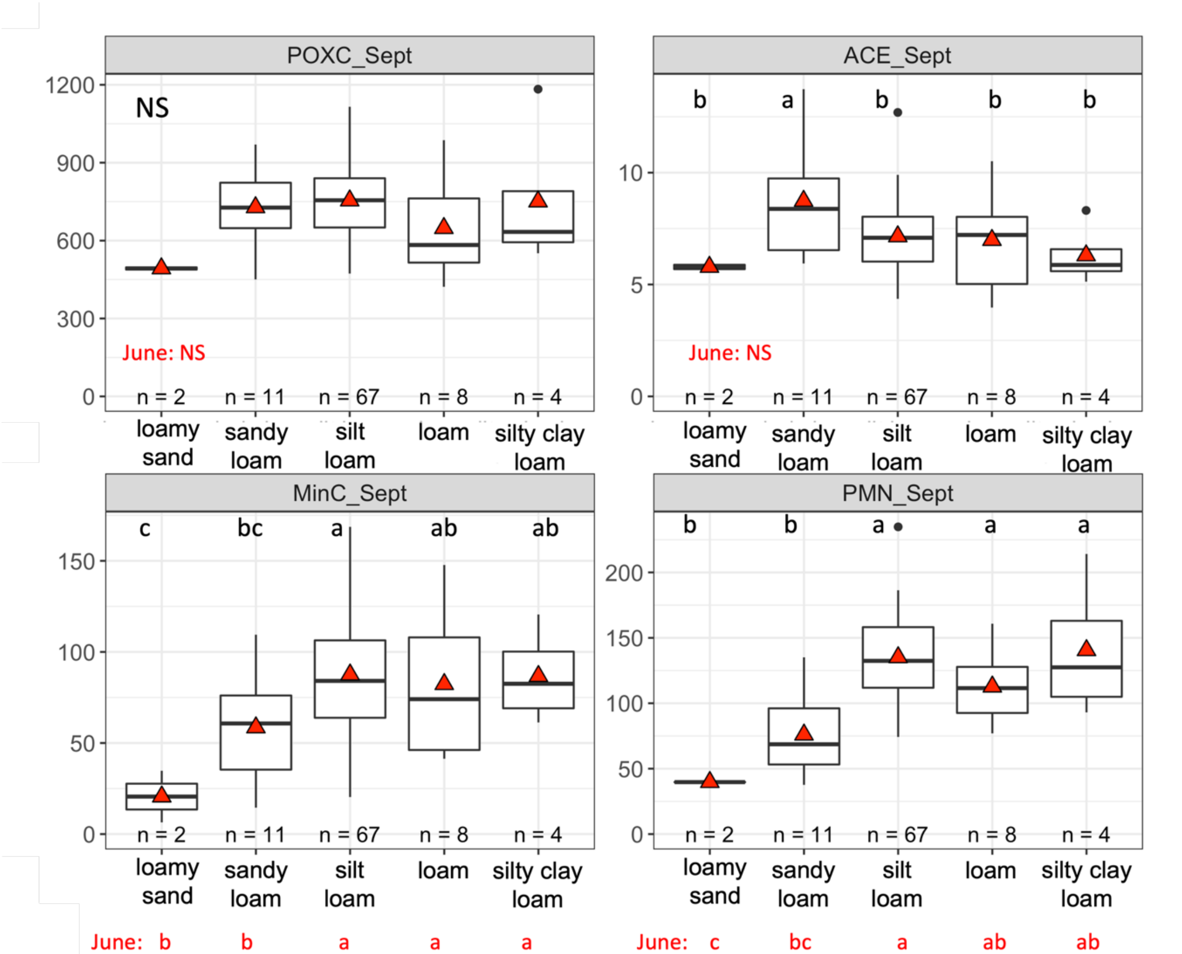

Figure 3.3: **Boxplot of September measurements of POXC, ACE, MinC, and PMN by texture class.** Texture class was determined from measured sand and clay content. Texture classes include: loamy sand (n=2), sandy loam (n=11), silt loam (n=67), loam (n=8), silty clay loam (n=4). In the boxplot, the middle line indicates the median and boxes delimit first and third quartiles. Upper and lower whiskers represent 1.5 times the interquartile range or, if there were no observations beyond that range, the maximum and minimum values. Letters indicate significant differences (p-value < 0.05) among texture classes determined through ANOVA and Fisher's LSD. Letters along the top of the graph in black correspond with the boxplot and September measurements; letters in red along the bottom correspond with June ANOVA results, for reference. POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1}); NS=not significant

3.5 Land use: pasture age and previous land use

For POXC, ACE and PMN, only 16-17% of the variation in September measurements was explained by pasture age, compared to 19-28% in June. MinC, which had R^2 = 0.22 in

June, did not have a significant relationship with pasture age in September (Figure 3.4). At both sampling times, POXC, ACE and PMN were greater in sites previously fallowed compared to those cropped prior to pasture establishment; however for MinC, only June values were greater in previously fallowed sites (Table S3.3).

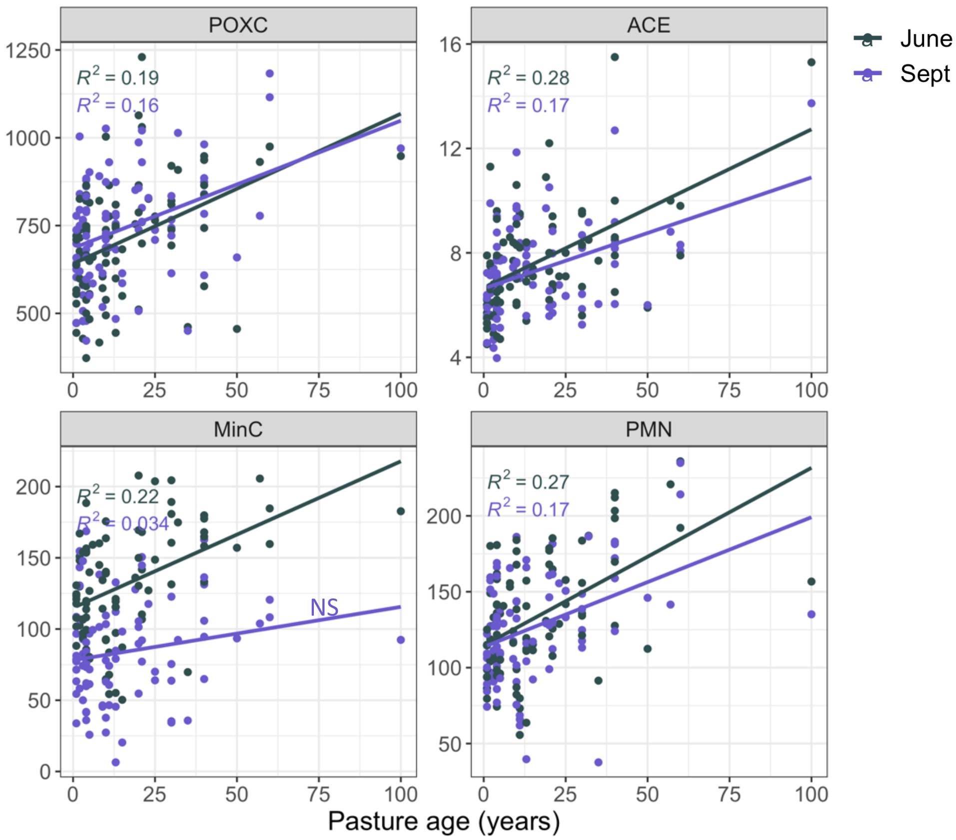

Figure 3.4: Scatterplots of June and September POXC, ACE, MinC and PMN and pasture age (years).
Points are colored blue for rotationally grazed pastures and red for continuously grazed. Regression lines and coefficient of determination (R^2) between pasture age and soil metrics are included for significant relations ($p<0.05$). All but the relationship between MinC and pasture age were significant. June R^2 values for the relationship between pasture age and June soil measurements are included in red for reference.
OM= organic matter (%); TC= total carbon (%); TN= total nitrogen (%); C:N= carbon-to-nitrogen ratio; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})
NS=not significant

3.6 Management

3.6.1 Grazing System

In September, continuously grazed pastures were associated with greater POXC and PMN, whereas in June, all labile C and N measurements were greater in continuously grazed pastures compared to those rotationally grazed (Figure 3.5). A paired t-test was conducted on continuously and rotationally grazed pastures separately to evaluate the effect of sampling time on biological indicators of soil health (Table 3.4). In continuously grazed pastures, there was no change in POXC and decreases in ACE (average= -1.26 g kg^{-1}), MinC (-74.84 mg kg^{-1}), and PMN (-18.90 mg kg^{-1}) from June to September. Comparatively, in rotationally grazed pastures, POXC increased (32.37 mg kg^{-1}), ACE and PMN were unchanged, and MinC decreased (-43.14 mg kg^{-1}), but to a lower magnitude than in continuously grazed sites, from June to September.

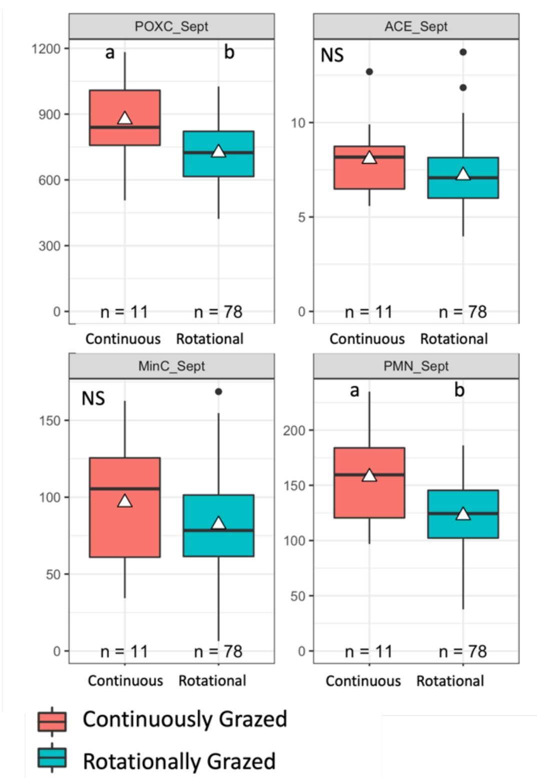

Figure 3.5: Boxplot of POXC, ACE, MinC and PMN by grazing category
Sites were categorized as continuously or rotationally grazed pastures based on management data provided by the farmer. In the boxplot, the middle line indicates the median and boxes delimit first and third quartiles. Upper and lower whiskers represent 1.5 times the interquartile range or, if there were no observations beyond that range, the maximum and minimum values. Letters indicate significant differences (p-value < 0.05) among grazing category, determined through ANOVA and Fisher's LSD. June soil values (not included here) were significantly higher in continuously grazed pastures compared to rotationally grazed pastures for all POXC, ACE, MinC and PMN
POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1}); NS= not significant.

Table 3.4: Two-sided, paired T-test results (based on grazing category) for POXC, ACE, MinC and PMN evaluating the hypothesis that September-June values= 0.
Paired t-test analysis was conducted on continuously grazed (n=11) and rotationally grazed (n=78) pastures to evaluate if results differed based on grazing system. Positive values indicate that September measurements were greater than June; Negative values indicate that September measurements were less than June. The test statistic, p-value, mean of difference and 95% confidence interval for the difference are included. A p-value < 0.05 was significant; when p-value > 0.05, we did not reject the null hypothesis that the June and September values were equal and did not report a mean difference or 95% CI.

	Continuously Grazed (n=11)			Rotationally Grazed (n=78)		
	p-value	Mean of difference	95% CI	p-value	Mean of difference	95% CI
POXC (mg kg^{-1})	0.183			0.003	32.37	[11.37, 53.37]
ACE (g kg^{-1})	0.002	-1.26	[-1.96, -0.57]	0.133		
MinC (mg kg^{-1})	<0.001	-74.84	[-101.45, -48.23]	<0.001	-43.14	[-52.43, -33.84]
PMN (mg kg-1)	0.011	-18.90	[-32.33, -5.46]	0.082		

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1}); CI=confidence interval

3.6.2 Pasture and grazing management in rotationally grazed systems

To evaluate grazing management practices specific to rotationally grazed pastures, the remaining analysis was conducted exclusively on rotationally grazed pastures (n=78). Due to a smaller sample size, rotationally grazed sites differed slightly in texture properties and pasture age compared to the full dataset. The same trends for texture and pasture age hold true, but p-values were weaker for the relationships between pasture age and POXC and PMN, and stronger between texture properties and ACE (Tables S3.4-S3.8).

Single factor analysis identified certain management practices as significant to September biological indicators of soil health (Tables S3.4-S3.8). Relationships between labile C and N measures and outwintering, haying, and stocking density were similar for June and September sampling times. Significant relationships observed in September but not in June included MinC and fertilizer application frequency (R^2=0.12, negatively related) ACE and rest period (R^2= 0.10, positively related), and MinC and residual pasture height (R^2=0.08, positively related).

		POXC		ACE		MinC		PMN	
		J	S	J	S	J	S	J	S
Soil Properties	sand (%)				6	2	1	3	4
	silt (%)				7	4	7	2	5
	clay (%)			5	2	3	5	4	3
	Drainage Class								
	pH	3	1				3		
Land Use	Region								
	Farm Operation Type								
	Pasture age	1	3	1	1	1		1	1
	Previous land use	9	7	3		6			
Pasture Management	Legume (%)		8			5			
	Outwintered			4	5				
	Hay frequency				4				
	Fertilizer use								
	Fertilizer applied this year								
	Fertilizer application frequency						2		
	Manure use								
	Manure application frequency								
	Lime	4							
Grazing management	Days between grazing/sampling								
	Number of rotations						4		
	Stocking Rate							5	
	Stocking Density	2	2						
	Seasonal Grazing Pressure	7	6						
	Time in paddock	5	4	2				7	
	Rest period	6			3			6	2
	Residual pasture height	8	5				6		

VIMP Relative Importance
1
0.5-1
0.3-0.5
0.2-0.3
0.1-0.2
<0.1

Figure 3.6: Summary table of important variables to September soil health indicators from random forest analysis, according to variable importance (VIMP) and average minimal depth (MD).

For each soil C and N measurement (column), the influence of each exploratory variable included in the analysis (rows) was determined by variable importance (VIMP) and average minimal depth (MD). VIMP represents how much the variable strengthened the model and corresponds to the cell color. The most important variable is assigned importance of 1 (colored green) and all other variables are given a relative importance based on how much it improved the model compared to the most important variable. Boxes are color coded based on VIMP for ease of interpretation (see key). The numbers in the cells correspond to ranking of importance according to average minimal depth (MD). MD reflects how close to the regression tree root node a variable appeared on average. Being closer to the root node signifies that, as a single split in the data, the variable explained larger variation in the dataset. Variables are ranked so that 1 corresponds with the variables closest to the root node. Only variables with minimal depth < 5 are numbered on the graph, as these are the most influential. See supplementary tables for exact values of relative importance and average minimal depth.; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

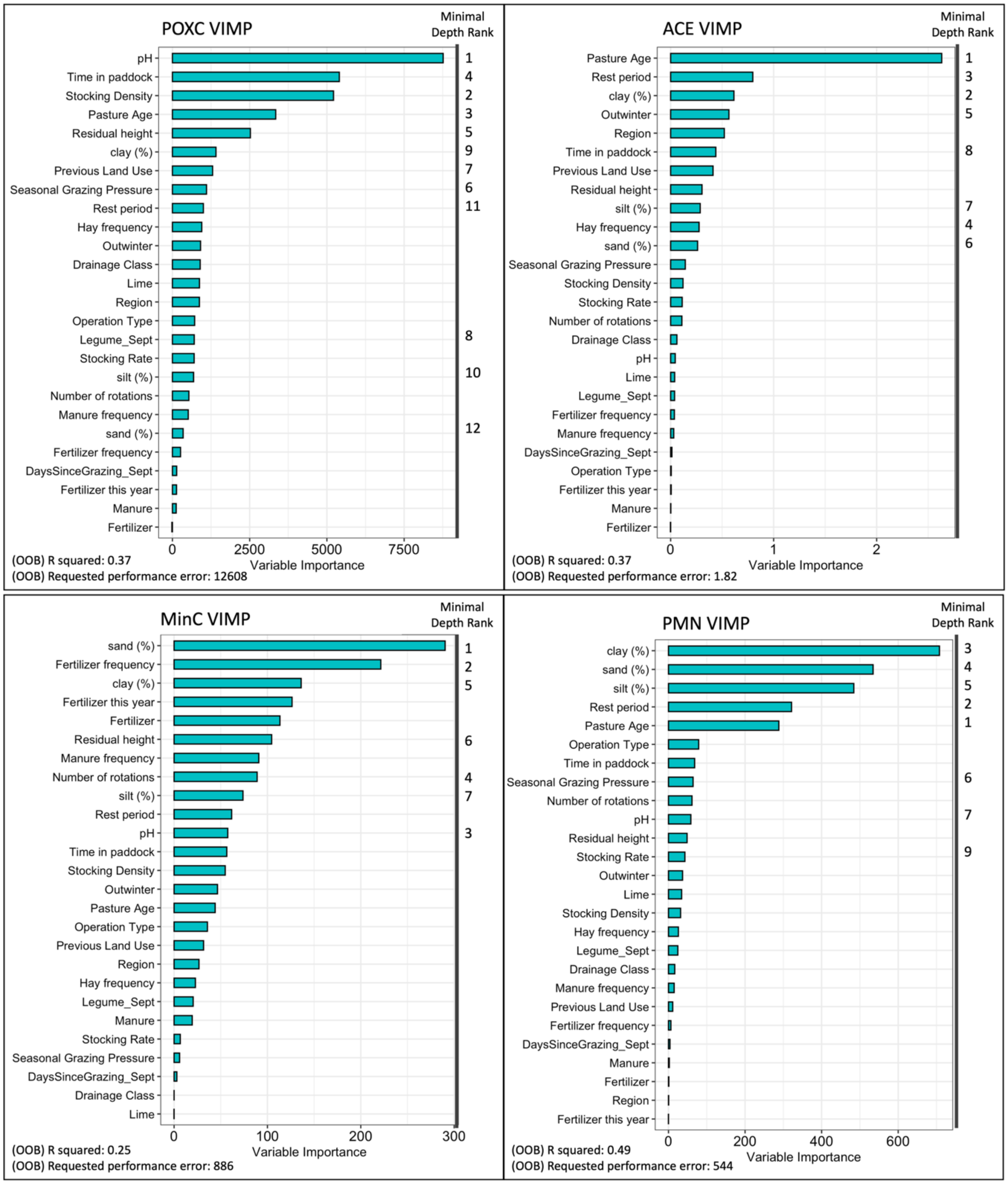

Figure 3.7: Random forest variable importance (VIMP) plots for September POXC, ACE, MinC and PMN in rotationally grazed pastures (n=78).

The list of factors are ordered by VIMP; higher factors were more important for explaining variation in the indicator. For variables identified as important according to the minimal depth method, rank of importance is along the righthand side of the graphs (1= most important). Exact values of relative importance and average minimal depth are provided in supplementary materials; POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

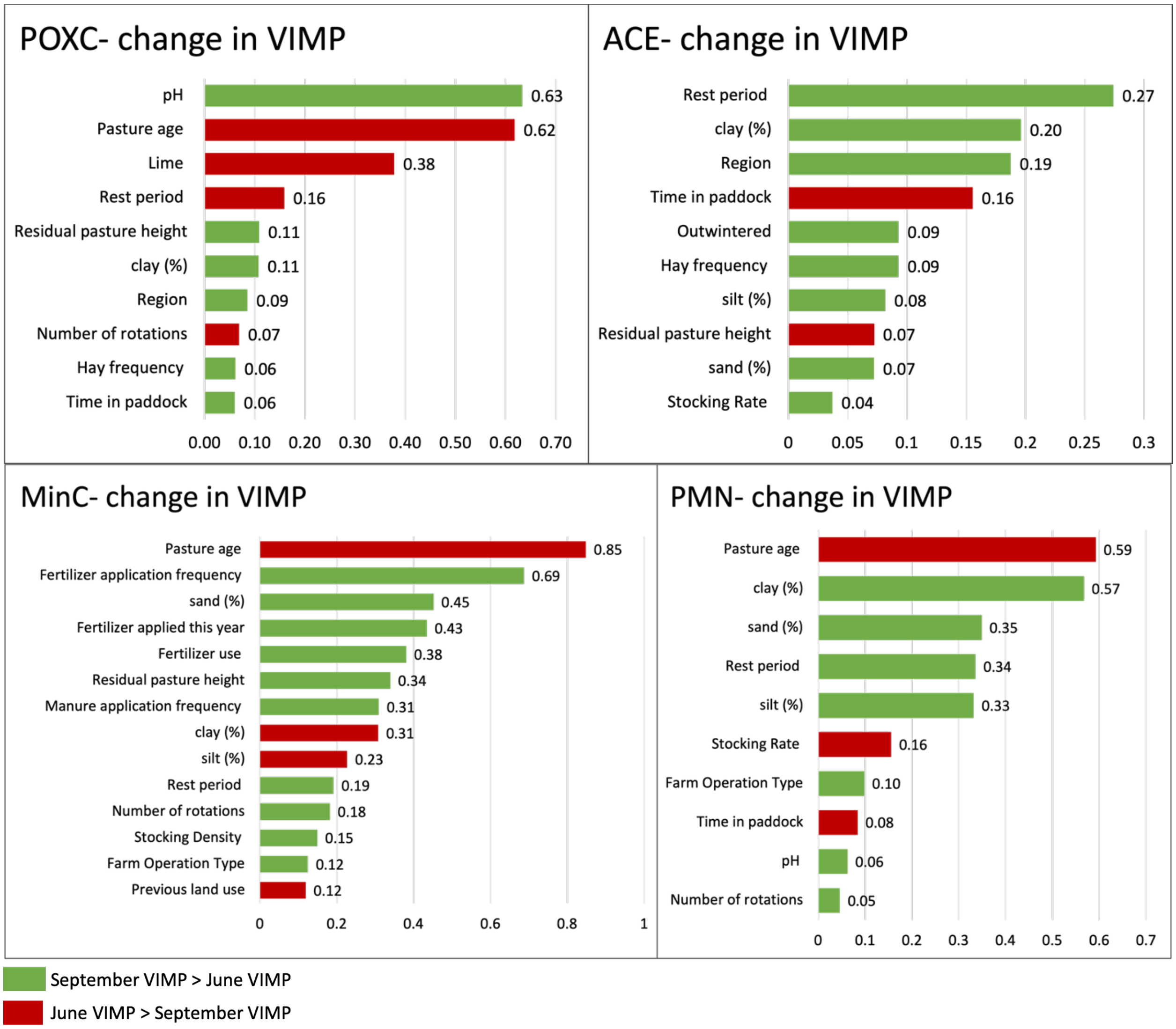

Figure 3.8: Change in relative importance from June to September for select factors for POXC, ACE, MinC and PMN.

The difference in VIMP relative importance was calculated as: September relative importance – June relative importance. If September relative importance was greater, bars are colored green; if June relative importance was greater, bars are colored red. Factors graphed are those with the largest difference in relative importance.

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

3.7 Random Forest

Random forest analysis out of bag (OOB) R^2 were comparable between the two sampling times (R^2=0.25-0.49 in September compared to R^2 =0.32-0.45 in June) (Tables S3.9-S3.12). An exception was the MinC random forest, which had a R^2=0.25 in September compared to R^2 =0.35 in June. For MinC and PMN, average minimal depth values for the most predictive variable were lower in September (3.38 and 3.58, respectively) compared to June (2.67 and 2.48, respectively). This signifies that variation in September MinC and PMN was not well explained by a single explanatory variable. Rather, many factors explained variation in data at lower branches of the regression trees.

3.7.1 Variables of importance

For each soil measurement in September, the most important variables by VIMP and minimal depth respectively, were: POXC (pH for both), ACE (pasture age for both), MinC (sand for both), PMN (clay, pasture age) (Figures 3.6 & 3.7). Factors that were important to biological indicators of soil health in both June and September include: texture for MinC and PMN; pH for POXC; pasture age for ACE, PMN and POXC (but to a lower magnitude in September); and stocking density and time in paddock for POXC. For other variables, sampling time had a large effect on changes in relative importance (Figure 3.8). Factors with notable differences in relative importance include pasture age, fertilizer frequency, rest period, and residual pasture height.

POXC had the most consistent random forest results between the two sampling times. Aside for pH being more important and pasture age being less important in September compared to June, the relative importance of texture and management factors and the trends between POXC and important variables were similar between the two sampling times.

Like June, ACE was most influenced by pasture age and had fewer variables of high relative importance. However, from June to September, the VIMP relative importance of rest period and clay content increased by 0.27 and 0.20 respectively, and were more important according to average minimal depth.

Sampling time had the largest effect on MinC random forest results. Pasture age decreased from a relative importance of 1.0 in June to 0.15 in September. Sand content increased, and silt and clay decreased, in importance according to VIMP and minimal depth. Even though texture was consistently important, trends between MinC and texture differ by sampling time. Fertilizer application frequency increased in relative importance for MinC from 0.08 in June to 0.76 in September; and residual pasture height increased in relative importance from 0.02 to 0.36.

Aside for stocking rate which had a lower relative importance for PMN in September compared to June, the variables of importance were consistent between sampling times. At both sampling times, pasture age was the most important variable according to minimal depth, signifying it best described variation in PMN compared to the other factors. Texture properties were consistently important between sampling times, but with higher VIMP relative importance in September. Rest period had higher importance in September (VIMP relative importance=0.45; minimal depth ranking=2), relative to June (VIMP relative importance=0.12; minimal depth ranking=6).

3.7.2 Pasture age

Trends between pasture age and predicted values of POXC, ACE and PMN were consistent between June and September; partial effect plots demonstrated rapid increases in predicted soil values after pasture establishment, with predicted POXC and PMN plateauing at around 30-40 years and ACE continuing to increase (Figure S3.2). Due to lower relative importance of pasture age in September compared to June, predicted MinC did not have a trend with pasture age, and POXC and PMN partial effect plots had higher standard deviations and lower effect sizes depicted on the y-axis.

3.7.3 Inherent soil properties

MinC, which was highly influenced by sand content at both sampling times, had differing trendlines based on sampling time. While June showed strong relationships between MinC and sand content (negative), silt (positive), and clay (positive with a plateau), September partial effect plots show predicted MinC increased until a sand content of 20% and then plateaued (Figure 3.9). In September, predicted MinC trends with silt and clay were weak with high standard deviation. While texture was important throughout the regression trees in September, there was no strong trend that represented directionality between texture and MinC, as there was in June.

Only observed in September, predicted ACE declined with clay content, until it leveled at 20% clay content (Figure S3.3). Alternatively, trends between PMN and texture, and pH and POXC were consistent between sampling times (Figures S3.4, S3.5).

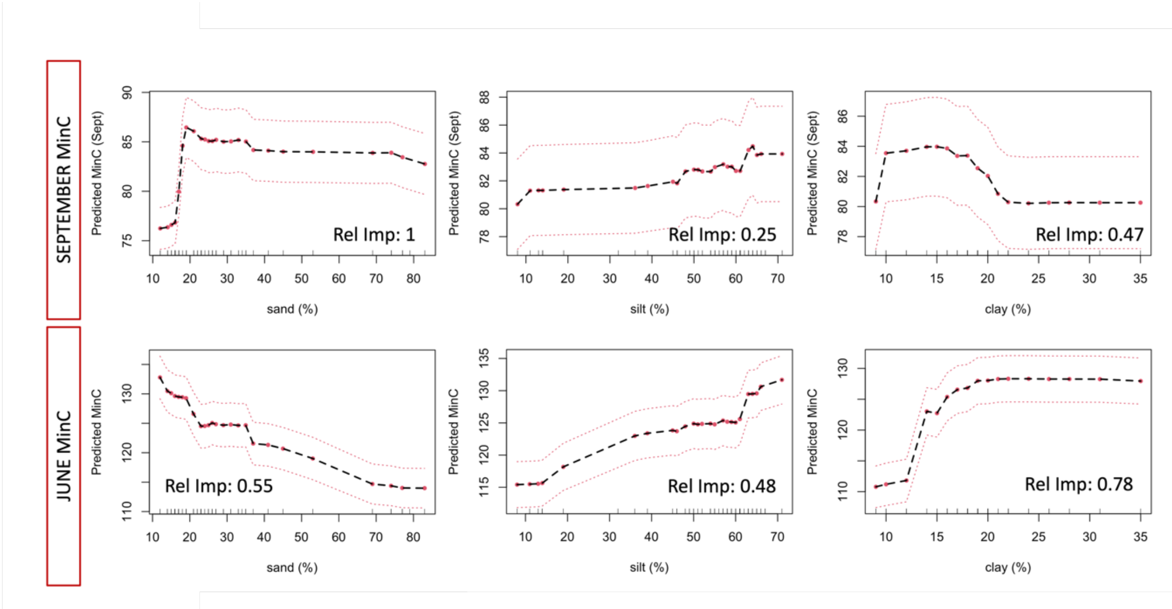

Figure 3.9: Partial effect of sand, silt and clay on MinC for June and September.
(Top) Predicted values of September MinC calculated as sand, silt and clay vary over observed values.
(Bottom) Predicted values of June MinC calculated as sand, silt and clay vary over observed values.
Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations. Rel Imp= relative importance according to VIMP
MinC = mineralizable carbon (mg kg^{-1})

3.7.4 Grazing management

In September, partial effect plots reaffirm the benefits of well-managed rotational grazing. POXC maintains the same trends with time in paddock and stocking density as June: greater predicted POXC corresponded with grazing events less than one day and stocking density greater than 100 AU/ha (Figure S3.6).

Rest period had a greater relative importance for September measures of ACE and PMN compared to those in June, but partial effect trendlines differ between the two labile N measures (Figure 3.10). In September, predicted ACE increased with rest periods greater than 60 days and PMN trendlines mirror those in June, where predicted soil health peaked around

25-30 days and decreased when rest periods were greater than 40 days. These trends are highly influenced by one farm (3 sites) that had a rest period of 140 days, meaning there was no grazing event between June and September. These sites had increases in POXC and ACE, but no change to MinC and PMN between the two sampling times (Figure S3.7).

Predicted MinC in September increased with residual pasture height until 15cm, at which point it plateaued (Figure 3.10). Conversely, residual height was not important to June measures of MinC. Trends between residual height and predicted ACE and POXC were the same in June and September: predicted ACE increased at residual height greater than 20cm and POXC shows no consistent trend.

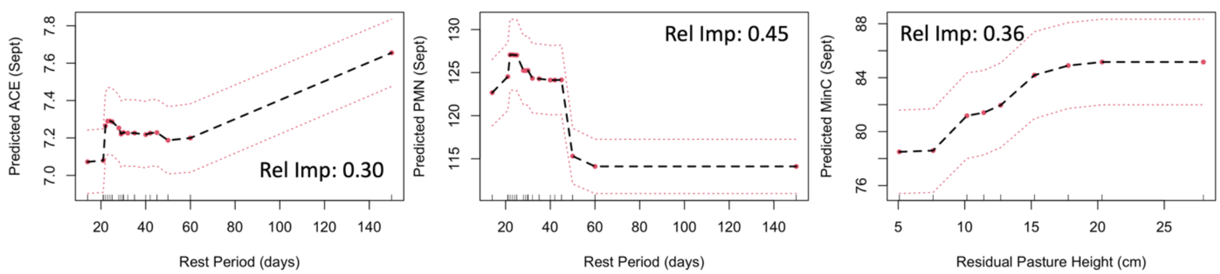

Figure 3.10: Partial effect of grazing management practices on September ACE, PMN, MinC. (left) Predicted values of ACE as rest period varies; (center) Predicted values of PMN as rest period varies; (right) Predicted values of MinC as residual pasture height varies.

Predicted values are calculated as rest period or residual pasture height varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.

Rel Imp= relative importance according to VIMP

ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

3.6.5 Pasture management

Sampling time greatly influenced the relationships between MinC and synthetic fertilizer and manure application frequency (Figure 3.11). Predicted MinC declined with increasing fertilizer use in the past five years, whereas three or more manure applications in the past five years corresponded with higher predicted MinC.

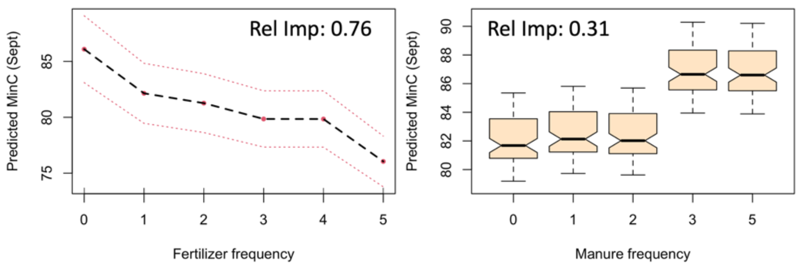

Figure 3.11: Partial effect of (left) fertilizer application frequency and (right) manure application frequency on predicted September MinC
Predicted values are calculated as fertilizer or manure frequency varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
MinC = mineralizable carbon (mg kg^{-1})

4. Discussion

4.1 Biological indicators of soil health were sensitive to seasonal variability

All indicators, particularly MinC, differed from June to September and the magnitude of the differences corresponded with previous work. Diederich et al. (2019) reported seasonal differences in POXC, MinC and PMN of approximately 100 mg kg^{-1}, 40 mg kg^{-1}, and 30 mg kg^{-1}, respectively, in well-managed pasture. Culman et al. (2013) found maximum differences of approximately 150 mg kg^{-1} for POXC, 10-20 mg kg^{-1} for MinC, and 5-25 mg kg^{-1} for PMN in annual grain systems under different management treatments. Hurisso et al. (2018) reported similar trends for POXC and MinC, 70-100 mg kg^{-1} and 6-20 mg kg^{-1}, respectively, and 0.5-1.4 g kg^{-1} for ACE.

The heightened temporal variability of MinC is consistent with other research findings across various agricultural systems (Crookston et al., 2021; Hurisso, Culman, et al., 2018; Menefee et al., 2022; Morrow et al., 2016; Wade et al., 2018). Hurisso et al. (2018) measured soil health indicators in corn fields throughout the growing season and observed temporal variation was the greatest in MinC (CV=22-37%), compared to OM-LOI (16–25%), POXC (9–21%), and ACE protein (7–13%). Crookstone et al. (2021) found soil respiration (measured with the Solvita test and 4-day respiration) had greater seasonal variability, particularly in coarse soils, compared to other total and labile C and N measurements. Alternatively, Diederich et al. (2019) found that PMN was more sensitive to sampling date and the interaction between sampling date and cropping system than POXC or MinC.

4.2 Seasonal changes in biological soil health attributed to direct effects of the soil environment and indirect effects of plant growth

ACE, MinC and PMN were greater in June compared to September, which confirmed previous studies in this region that demonstrated peaks in biological indicators of soil health in July and August. Heightened biological soil health is attributed to direct effects of conducive temperature and precipitation on microbial activity and indirect effects of plant growth stages (Culman et al., 2013; Diederich et al., 2019; Stevenson et al., 2014; Wade et al., 2018). Abiotic conditions, such as soil moisture and temperature, influence the composition and activity of the microbial community (Fierer, 2017). MinC and PMN, which are biological incubations and proxies for biological activity, are more sensitive to seasonal variation due changes in weather and additions of easily decomposable substrates (Franzluebbers et al., 1994). Peak values of labile C and N and microbial activity, typically observed during the summer in Midwestern agricultural systems, have been attributed to greater temperature and precipitation that foster a conducive soil environment for microbial activity, as well as greater C inputs due to plant growth (Campbell et al., 1999; Culman et al., 2013; Franzluebbers et al., 1994; R. I. Griffiths et al., 2003; Kennedy et al., 2005). In contrast, Campbell et al. (1999) observed that MinC and PMN were negatively correlated with soil moisture and temperature in a semiarid grassland in Canada. They found that conducive environmental conditions stimulated decomposition of soil C, and without sufficient inputs of fresh C, there was less substrate to be detected in the laboratory biological incubation. This underscores the importance of C inputs from plant growth to promote microbial activity, as well as increase labile C and N.

Typical trends in forage productivity in Wisconsin's cool season pastures align with observed fluctuations in biological indicators of soil health. The seasonal growth pattern of cool-season pastures, which peak around June and decline through the season (Brink et al., 2007; Oates et al., 2011; Paine et al., 1999; Riesterer et al., 2000) has indirect effects on the microbial community (Bardgett et al., 1998; Franzluebbers et al., 1994; Stevenson et al., 2014). Plant growth influences the microbial community through many mechanisms and feedbacks: plant nutrient uptake, litter decomposition, quality of C and N inputs, root turnover, and root exudates (Bardgett & Shine, 1999; Bardgett & Wardle, 2003; B. S. Griffiths et al., 2004; Hamilton et al., 2008; Kennedy et al., 2005; Wang et al., 2016; Wardle et al., 2004). Extensive root systems in pastures and seasonal patterns of root growth and turnover are particularly influential to biologically active fractions of C and N (Bardgett et al., 1998; Chen et al., 2015; Corre et al., 2002; Hamilton et al., 2008; Jackson et al., 1997; Lovell et al., 1995). In grazed pastures, feedbacks and interactions among weather, plant, livestock, and soil are dynamic through the season and can contribute to in-season variability of biological indicators of soil health.

4.3 June measurements were more related to total pools of C and N and pasture age

In June compared to September, biological indicators of soil health, particularly the biological incubations, had stronger relationships with total C and N and pasture age. When sampled in June, soil measurements were approaching typical peak values for the region, which we hypothesized strengthened the relationships among soil C and N metrics (Diederich et al., 2019). Weaker relationships among September measurements may indicate that after peak soil health values, other factors account for greater variation among indicators. The relationships between MinC and other soil C and N measures were particularly weak in our study, confirming observations in Crookston et al. (2021) that indicators with low temporal variation (OM, C:N, POXC, ACE) and those with high temporal variation (Solvita 24-hr respiration test and 4-day mineralizable C incubation) were weakly correlated. Though weaker relationships between September soil health values and total C and N may be attributed to seasonal fluctuations in our study, only analyzing for bulk C and N in June could also impact results due to sampling bias or seasonal changes in total pools (Crookston et al., 2021; Culman et al., 2013; Diederich et al., 2019).

When sampling closer to peak activity in June, pasture age explained more variation in the biological indicators of soil health compared to September. In our study, pasture age had a stronger relationship with OM, TC and TN, explaining 33-37% of the variation in bulk C and

N, compared to any labile C and N measure (chapter 2). In general, labile C and N measurements have greater variation unexplained by pasture age compared to total pools. Given weaker relationships between September labile C and N and pasture age, there may be in-season factors contributing to greater variation in the values.

4.4 Relationships between inherent soil properties and biological indicators soil health vary based on sampling time

The effect of sampling time had inconsistent effects on relationships between inherent soil properties and biological indicators of soil health. For POXC, ACE and PMN, these relationships were stronger in September compared to June (pH with POXC, and texture with ACE and PMN). The negative relationship between ACE and clay content was also observed by Amsili et al. (2021). They reported that ACE was lower in loam and fine texture groups compared to coarse and silt loam soils, which they attributed to lower extraction efficiency in soils with higher clay content (Giagnoni et al., 2013). However, this relationship typically was not found in other studies (Crookstone et al., 2021; Hurisso et al., 2016).

Results for MinC depict uncertain trends with texture properties. While sand was consistently an important variable in June and September random forest analysis, the trend lines for the two sampling times differ and demonstrate the absence of a clear relationship between MinC and texture that holds through the season. Additionally, Crookstone et al. (2021) showed indicators exhibited dissimilar temporal variation according to soil texture. Temporal CV for indicators, particularly Solvita and 4-day respiration, was greater in coarse, compared to medium, and then fine textured soils. In this study, soils with a higher sand content exhibited different trends in MinC between June and September, compared to medium and fine textured soils.

4.5 Temporal changes in soil health may be attributed to in-season grazing management

While the perceived benefits to soil health of rotational grazing over continuous grazing were not reflected by June measurements of labile C and N, evaluating the change in measures across the sampling times underscored the benefits of well-managed grazing. On average, rotationally grazed pastures had an increase in POXC throughout the season and maintained ACE and PMN between sampling times. Well-managed rotational grazing stimulates nutrient cycling, increases forage quantity and quality, improves plant community composition, and evenly distributes nutrient deposition from livestock, thus supporting C storage in the soil surface (Byrnes et al., 2018; Conant et al., 2017; Mosier et al., 2021; Oates et al., 2011; Paine et al., 1999; Teague & Kreuter, 2020; Wang et al., 2016). Grazing management can impact soil biology through indirect effects to litter quality and quantity, carbon allocation, litter decomposition due to manure deposition, and root growth (Bardgett & Shine, 1999; Bardgett & Wardle, 2003; Hamilton et al., 2008; Kennedy et al., 2005; Patra et al., 2005; Wang et al., 2016; Wardle et al., 2004).

Random forest results demonstrated that among rotationally grazed pastures, sufficient rest and residual pasture height were important factors for September measures of biological indicators of soil health and may be valuable management practices to maintain soil health throughout the grazing season. To maintain productivity in the summer slump, cool-season pastures require longer rest periods as the season progresses (Brink et al., 2007; Paine et al., 1999). Providing sufficient rest and regrowth, particularly during periods of lower production, can promote plant growth, root exudation and belowground C inputs, which is likely to promote biological soil health (Oates et al., 2011, 2012; Piñeiro et al., 2010; Teague et al., 2015; Wang et al., 2016). Due to plant-grazing-soil interactions and feedbacks, in-season management may influence changes to labile C and N throughout the season (Wang et al., 2016).

4.6 Sampling time revealed a negative relationship of fertilizer use and MinC

Results showed that biological incubations, especially September MinC, were negatively related to synthetic fertilizer use. Sufficient nitrogen is beneficial for plant growth, greater C inputs and soil C stabilization (Snyder et al., 2009; Sprunger et al., 2020). While some studies show that synthetic inputs can benefit SOC and TN in grasslands or have no effect (Conant et al., 2017; Hassink, 1994; Menefee et al., 2022), others show negative impacts. Previous studies demonstrate that synthetic fertilizer can deter root growth rates, impair microbial communities or influence temporal variation in microbial activity (Bardgett et al., 1998, 1999; Diederich et al., 2019; Franzluebbers et al., 1994; Jangid et al., 2008; Lovell et al., 1995). When compared to pastures that received manure inputs, those with synthetic fertilizer had lower PMN, microbial biomass, bacterial diversity and abundance, and greater seasonal variation in microbial communities (Dahal et al., 2021; Franzluebbers et al., 1994; Jangid et al., 2008). Dahal et al. (2021) observed that the relationship among soil health indicators in pasture sites varied based on the use of synthetic nitrogen fertilizer versus broiler litter, demonstrating potential variation due to fertilizer and manure use. Diedreich et al. (2019) observed that the use of synthetic fertilizer disrupted temporal trends in MinC and PMN, and that synthetic fertilizer may influence seasonal trends in C mineralization more than other management practices like tillage or crop rotation. Research suggests that fertilizer use may relate to lower MinC, as well as increase temporal variation of the indicator.

5. Conclusion

This research demonstrated the seasonal variability of biological indicators of soil health in pasture systems and the need to standardize sampling time for soil health testing. Biological indicators of soil health were greater in June, when weather conditions were most beneficial to pasture growth and microbial communities, compared to September. Dependent on sampling time, interpretation of soil health test results would differ greatly for MinC, but only moderately for POXC, ACE and PMN. Important factors for MinC differed greatly by sampling time, and sampling later in the season revealed that MinC had no relationship with pasture age, an inconsistent relationship with texture, and a negative relationship with fertilizer use. Comparatively, POXC, ACE and PMN's variables of importance were consistent, but their relative ranking varied due to weaker relationships with pasture age in June compared to September. If only examining June or September soil health measurements, values in continuously grazed pastures were greater than or equal to those in rotationally grazed sites. However, evaluating the change in soil health through the grazing season highlighted the benefits of rotational grazing over continuously grazing to maintain labile C and N values through the season. Additionally, within rotationally grazed systems, sufficient rest period and residual height had a larger effect on September soil measurements, when plant growth requires greater rest and recovery, compared to June.

To determine if soil health testing in pastures is valuable, and if so, what sampling time poses the most benefit, biological indicators of soil health must be linked to agronomic or environmental value. While links between soil health tests and agronomic productivity have been established for corn and soybean, this has not been explored in pastures. Seasonal declines in pasture productivity can deter economic resilience and livestock health, which underscores the importance of grazing management practices that minimize the summer slump in forage production. Results highlight how seasonal variation in soil health correspond with typical trends in pasture productivity and relate to well-managed grazing. Evaluating relationships between biological indicators of soil health and pasture productivity throughout the season can elucidate if relationships between biological soil health and pasture productivity align or vary during times of rapid plant growth or declining production. Understanding if and what relationships may exist between seasonality, pasture productivity, grazing management and soil health will determine if measuring soil labile C and N is valuable in pastures, and how to select soil health indicators and sampling time to best evaluate and/or manage for pasture productivity and economic return.

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Supplementary Materials

Table S3.1: Univariate statistics for September measurements of permanganate oxidizable carbon (POXC), autoclaved-citrate extractable protein (ACE), mineralizable carbon (MinC) and potentially mineralizable nitrogen (PMN); n=92.

	POXC	ACE	MinC	PMN
	mg kg^{-1}	g kg^{-1}	-------- mg kg^{-1}--------	
n	92	92	92	92
Minimum	422.1	4.0	6.41	37.63
1st Quartile	614.1	6.0	59.47	100.72
Median	737.5	7.1	78.38	125.95
Mean	736.0	7.3	82.14	124.24
3rd Quartile	840.0	8.2	104.24	149.2
Maximum	1183.1	13.7	168.67	234.84
Std. Dev.	158.0	1.7	36.03	38.72
CV	0.21	0.24	0.44	0.31
Skewness	0.27	1	0.26	-0.02
Kurtosis	-0.24	1.63	-0.49	0.1

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

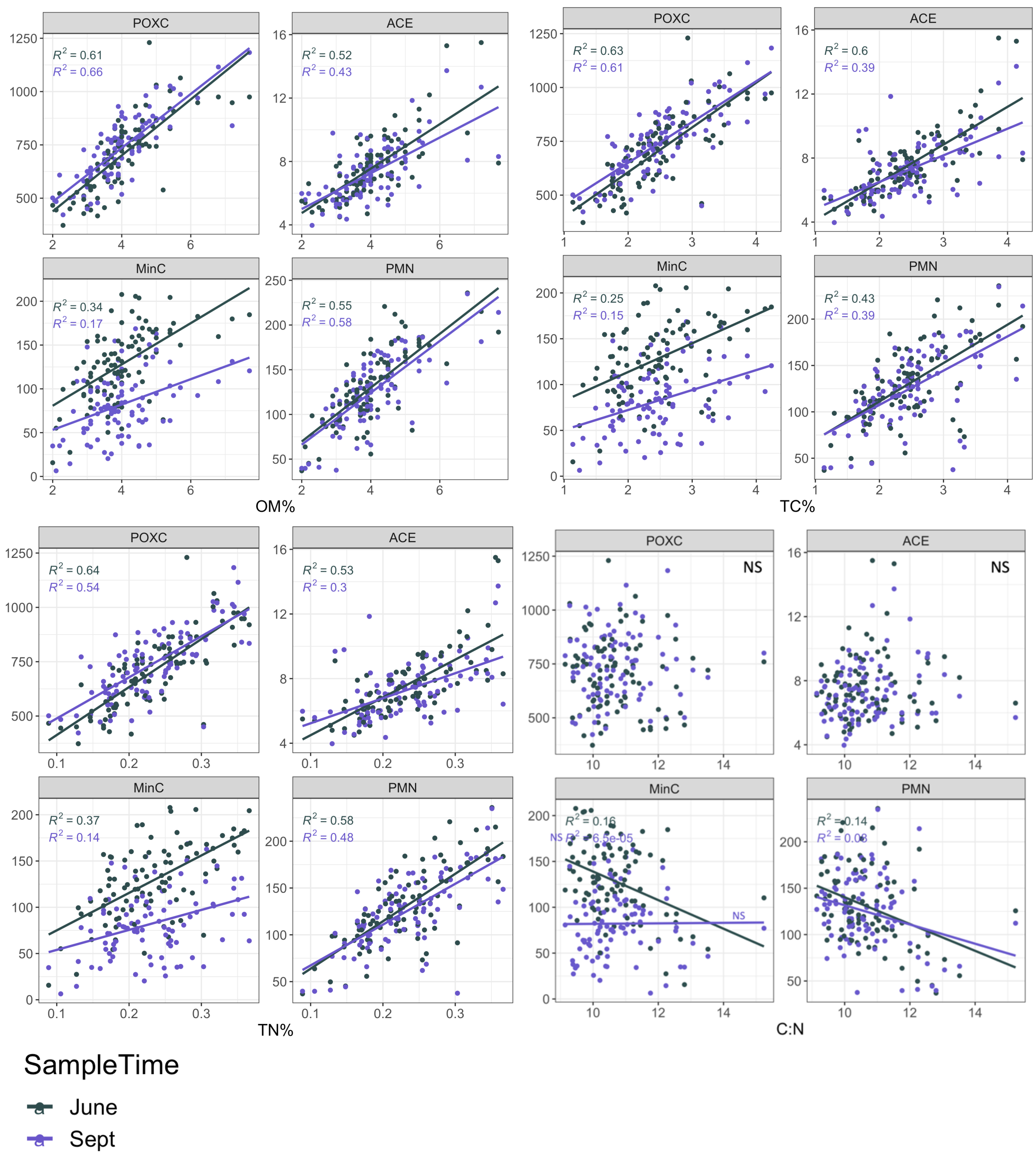

Figure S3.1: Scatterplots of biological soil health indicators (POXC, ACE, MinC and PMN) and organic matter (OM)%, total carbon (TC)%, total nitrogen (TN)%, and carbon-to-nitrogen ratio (C:N). June (green) and September (purple) values of POXC, ACE, MinC and PMN are graphed.

Linear regressions between bulk and labile C and N measurements were conducted separately for each sampling time. Coefficients of determination (R^2) and the equation estimated through linear regression are included in each figure (p-value <0.05).

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

Table S3.2: Linear regression results for all soil properties and pasture age and September POXC, ACE, MinC and PMN for all sampled pastures (n=92).

Table includes p-values, and if the relationship was significant (p-value <0.05), coefficient of determination (R2) and direction of relationship (+ or -). ▲signifies that explanatory variables were transformed into a polynomial to fit assumptions of linear regression; for the trendline direction, the first sign corresponds to "x" and the second sign corresponds to "x^2".

	POXC			ACE			MinC			PMN		
	p-value	R^2	Slope	p-value	R^2	Slope	p-value	R^2	Slope	p-value	R^2	Slope
pH	<0.001	0.206	+	0.553			0.244			0.057	0.040	+
sand (%)	0.059			0.059			0.005	0.083	-	<0.001	0.385	-
silt (%)	0.045	0.044	+	0.128			0.002	0.101	+	<0.001	0.378	+
clay (%)	0.347			0.012	0.067	-	0.032▲	0.074	+, -	<0.001▲	0.375	+,-
Pasture age	<0.001	0.161	+	<0.001	0.174	+	0.085			<0.001	0.165	+

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

Table S3.3: Analysis of Variance (ANOVA) results for drainage class (top) and previous land use (bottom) for September POXC, ACE, MinC and PMN. Analysis was on all sampled pastures (n=92).
Table includes p-values and, when p-value < 0.05, letters to indicate significant differences according to Fisher's LSD.

		Drainage Class					
		poorly drained	somewhat poorly drained	moderately well drained	well drained	somewhat excessively drained	excessively drained
n		2	17	18	52	1	2
		ANOVA					
	p-value						
POXC (mg kg^{-1})	0.001	a	b	b	b	b	b
ACE (g kg^{-1})	0.111						
MinC (mg kg^{-1})	0.337						
PMN (mg kg^{-1})	0.001	a	b	bc	b	bc	c

		Previous Land Use				
		Fallow	Hay	Row Crops	Row crops & Hay	Unknown
n		8	14	29	30	8
		ANOVA				
	p-value					
POXC (mg kg^{-1})	0.039	ab	c	bc	abc	a
ACE (g kg^{-1})	<0.001	a	c	bc	b	bc
MinC (mg kg^{-1})	0.268					
PMN (mg kg^{-1})	0.013	ab	bc	c	c	a

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

Table S3.4: Analysis of Variance (ANOVA) results for texture class and drainage class for September POXC, ACE, MinC and PMN. Analysis was for rotationally grazed pastures (n=78).
Table includes p-values and, when p-value < 0.05, letters to indicate significant differences according to Fisher's LSD.

	Texture Class						Drainage Class					
		loamy sand	sandy loam	loam	silt loam	silty clay loam		somewhat poorly drained	moderately drained	well drained	somewhat excessively drained	excessively drained
n		1	8	8	58	3		16	14	45	1	2
	ANOVA p-value						ANOVA p-value					
POXC (mg kg^{-1})	0.068						0.035	a	a	ab	ab	a
ACE (g kg^{-1})	<0.001	b	a	b	b	b	0.027	ab	a	ab	ab	a
MinC (mg kg^{-1})	0.162						0.663					
PMN (mg kg^{-1})	<0.001	c	bc	ab	a	ab	0.127					

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

Table S3.5: Analysis of Variance (ANOVA) results for region and land ownership for OM, TC, TN, C:N, POXC, ACE, MinC and PMN. Analysis was for rotationally grazed pastures (n=78).
Table includes p-values and, when p-value < 0.05, letters to indicate significant differences according to Fisher's LSD.

		Region			Land ownership			Operation Type			
		Kickapoo	Marathon		Owned	Rented		Dairy	Heifers	Beef	Multispecies
n		38	40		68	10		15	7	44	12
		ANOVA			ANOVA			ANOVA			
	p-value			p-value			p-value				
POXC (mg kg^{-1})	0.004	b	a	0.722			0.039	a	ab	b	ab
ACE (g kg^{-1})	<0.001	b	a	0.719			0.963				
MinC (mg kg^{-1})	0.092			0.830			0.358				
PMN (mg kg^{-1})	0.860			0.609			0.018	a	ab	b	ab

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

Table S3.6: Analysis of Variance (ANOVA) results for previous land use, outwintering, and hay frequency for September POXC, ACE, MinC and PMN. Analysis was for rotationally grazed pastures (n=78).
Table includes p-values and, when p-value < 0.05, letters to indicate significant differences according to Fisher's LSD.

	Previous Land Use						Outwinter			Hay frequency			
		Fallow	Hay	Row Crops	Row crops & hay	Unknown		No	Yes		Never	Sometimes	Often
n		5	14	28	27	4		57	21		55	13	10
	ANOVA p-value						ANOVA p-value			ANOVA p-value			
POXC (mg kg^{-1})	0.273						0.004	b	a	0.021	a	b	ab
ACE (g kg^{-1})	0.006	a	b	b	b	ab	<0.001	b	a	0.004	a	b	a
MinC (mg kg^{-1})	0.819						0.050	b	a	0.274			
PMN (mg kg^{-1})	0.210						0.242			0.240			

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

Table S3.7: Analysis of Variance (ANOVA) results for fertilizer use, fertilizer applied this year, manure use, manure applied this year, and lime use for September POXC, ACE, MinC and PMN. Analysis was for rotationally grazed pastures (n=78).
Table includes p-values and, when p-value < 0.05, letters to indicate significant differences according to Fisher's LSD.

	Fertilizer applied (5yrs)			Fertilizer applied this year			Manure applied (5yrs)			Manure applied this year			Lime applied (5yrs)		
		No	Yes		No	Yes		No	Yes		No	Yes		No	Yes
n		40	38		55	23					67	8		53	25
	ANOVA p-value			ANOVA p-value			ANOVA p-value			ANOVA p-value			ANOVA p-value		
POXC (mg kg^{-1})	0.239			0.072			0.386			0.008	b	a	0.001	a	a
ACE (g kg^{-1})	0.884			0.124			0.271			0.315			0.492		
MinC (mg kg^{-1})	0.002	a	b	0.003	a	b	0.067			0.855			0.830		
PMN (mg kg^{-1})	0.010	a	b	0.630			0.226			0.135			0.001	a	b

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

Table S3.8: Linear regression results for all continuous variables and September permanganate oxidizable carbon (POXC), autoclaved-citrate extractable protein (ACE), mineralizable carbon (MinC), and potentially mineralizable nitrogen (PMN) of rotationally grazed pastures (n=78).

Table includes p-values, and if the relationship was significant (p-value <0.05), coefficient of determination (R2) and direction of relationship (+ or -). ▲signifies that explanatory variables were transformed into a polynomial to fit assumptions of linear regression; for the trendline direction, the first sign corresponds to "x" and the second sign corresponds to "x^2".

	POXC			ACE			MinC			PMN		
	p-value	R^2	Slope	p-value	R^2	Slope	p-value	R^2	Slope	p-value	R^2	Slope
Soil Properties												
pH	0.000	0.25	+	0.642			0.450			0.059		
Sand (%)	0.668			0.002	0.12	+	0.195			0.000	0.32	-
Silt (%)	0.360			0.009	0.09	-	0.098	0.04	+	0.000	0.35	+
Clay (%)	0.170			0.000	0.18	-	0.851			0.000	0.31	+, -
Pasture management												
Pasture age	0.013	0.08	+	0.000	0.17	+	0.310			0.035	0.06	+
Fertilizer frequency (past 5 years)	0.230			0.183			0.002	0.12	-	0.073		
Manure frequency (past 5 years)	0.051	0.05	+	0.778			0.456			0.071		
Legume %	0.592			0.775			0.725			0.769		
Grazing management												
Days between grazing and sampling	0.378			0.001	0.14	+	0.706			0.900		
Stocking rate	0.469			0.784			0.294			0.252		
Stocking density	0.001▲	0.18	+, -	0.044▲	0.08	+, -	0.418			0.431		
Seasonal grazing pressure	0.027▲	0.09	+, -	0.089▲	0.06	+, -	0.520▲			0.176▲		
Number of rotations	0.193▲			0.056			0.033▲	0.09	+, -	0.017▲	0.10	+, -
Time in paddock	0.307			0.677			0.476			0.978		
Rest period	0.943			0.005	0.10	+	0.806			0.031	0.06	-
Residual pasture height	0.657			0.899			0.010	0.08	+	0.324		

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

Table S3.9: September POXC random forest analysis results.
"VIMP rank", "Importance" and "relative importance" correspond with variables of importance (VIMP) analysis. Variables are ranked in order of variable importance (VIMP), which is calculated based on how important the variable was in explaining variation in the indicator value. "Minimal depth rank" and "minimal depth value" refers to the average minimal depth the factor appears in the regression trees. A minimal depth closer to 1 signifies that the factor was selected closer to the root node in the regression trees, and is more predictive of variation in the indicator as a single split.

POXC Random Forest: Factor Importance					
	VIMP Analysis			Minimal Depth Analysis	
	Rank	Variable Importance	Relative Importance	Rank	Average Minimal Depth
pH	1	8764	1	1	2.37
Time in paddock	2	5402	0.62	4	4.06
Stocking density	3	5213	0.59	2	3.63
Pasture age	4	3343	0.38	3	3.89
Residual pasture height	5	2524	0.29	5	4.39
Clay (%)	6	1409	0.16	9	5.10
Previous land use	7	1301	0.15	7	4.84
Seasonal grazing pressure	8	1103	0.13	6	4.80
Rest period	9	1002	0.11	11	5.19
Hay frequency	10	949	0.11		
Outwinter	11	909	0.10		
Drainage class	12	898	0.10		
Lime	13	875	0.10		
Region	14	872	0.10		
Operation type	15	718	0.08		
Legume (%)	16	705	0.08	8	4.96
Stocking rate	17	702	0.08		
Silt (%)	18	683	0.08	10	5.19
Number of rotations	19	532	0.06		
Manure frequency	20	511	0.06		
Sand (%)	21	346	0.04	12	5.23
Fertilizer frequency	22	262	0.03		
Days between grazing and sampling	23	137	0.02		
Fertilizer this year	24	131	0.02		
Manure	25	120	0.01		
Fertilizer	26	-18	0.00		
(OOB) R squared: 0.37					
(OOB) Requested performance error: 12608					

Table S3.10: September ACE random forest analysis results.
"VIMP rank", "Importance" and "relative importance" correspond with variables of importance (VIMP) analysis. Variables are ranked in order of variable importance (VIMP), which is calculated based on how important the variable was in explaining variation in the indicator value. "Minimal depth rank" and "minimal depth value" refers to the average minimal depth the factor appears in the regression trees. A minimal depth closer to 1 signifies that the factor was selected closer to the root node in the regression trees, and is more predictive of variation in the indicator as a single split.

ACE Random Forest: Factor Importance					
	VIMP Analysis			Minimal Depth Analysis	
	Rank	Variable Importance	Relative Importance	Rank	Average Minimal Depth
Pasture age	1	2.63	1	1	2.92
Rest period	2	0.80	0.30	3	4.34
Clay (%)	3	0.61	0.23	2	4.00
Outwinter	4	0.57	0.22	5	4.95
Region	5	0.52	0.20		
Time in paddock	6	0.44	0.17	8	5.15
Previous land use	7	0.41	0.16		
Residual pasture height	8	0.30	0.12		
Silt (%)	9	0.29	0.11	7	5.00
Hay frequency	10	0.28	0.10	4	4.75
Sand (%)	11	0.26	0.10	6	5.00
Seasonal grazing pressure	12	0.14	0.05		
Stocking density	13	0.12	0.05		
Stocking rate	14	0.11	0.04		
Number of rotations	15	0.11	0.04		
Drainage class	16	0.06	0.02		
pH	17	0.04	0.02		
Lime	18	0.04	0.01		
Legume (%)	19	0.04	0.01		
Fertilizer frequency	20	0.04	0.01		
Manure frequency	21	0.03	0.01		
Days between grazing and sampling	22	0.01	0.00		
Operation type	23	0.01	0.00		
Fertilizer this year	24	0.01	0.00		
Manure	25	0.00	0.00		
Fertilizer	26	0.00	0.00		
(OOB) R squared: 0.37					
(OOB) Requested performance error: 1.82					

Table S3.11: September MinC random forest analysis results.
"VIMP rank", "Importance" and "relative importance" correspond with variables of importance (VIMP) analysis. Variables are ranked in order of variable importance (VIMP), which is calculated based on how important the variable was in explaining variation in the indicator value. "Minimal depth rank" and "minimal depth value" refers to the average minimal depth the factor appears in the regression trees. A minimal depth closer to 1 signifies that the factor was selected closer to the root node in the regression trees, and is more predictive of variation in the indicator as a single split.

MinC Random Forest: Factor Importance					
	VIMP Analysis			Minimal Depth Analysis	
	Rank	Variable Importance	Relative Importance	Rank	Average Minimal Depth
Sand (%)	1	290	1	1	3.38
Fertilizer frequency	2	221	0.76	2	3.79
Clay (%)	3	136	0.47	5	4.34
Fertilizer this year	4	126	0.44		
Fertilizer use	5	113	0.39		
Residual pasture height	6	105	0.36	6	4.39
Manure frequency	7	91	0.31		
Number of rotations	8	89	0.31	4	4.24
Silt (%)	9	74	0.25	7	4.40
Rest period	10	62	0.21		
pH	11	57	0.20	3	4.15
Time in paddock	12	56	0.19		
Stocking density	13	55	0.19		
Outwinter	14	47	0.16		
Pasture age	15	44	0.15		
Operation type	16	36	0.12		
Previous land use	17	32	0.11		
Region	18	27	0.09		
Hay frequency	19	23	0.08		
Legume (%)	20	20	0.07		
Manure	21	19	0.07		
Stocking rate	22	7	0.02		
Seasonal grazing pressure	23	6	0.02		
Days between grazing and sampling	24	3	0.01		
Drainage class	25	0	0.00		
Lime	26	0	0.00		
(OOB) R squared: 0.25					
(OOB) Requested performance error: 886					

Table S3.12: September PMN random forest analysis results.
"VIMP rank", "Importance" and "relative importance" correspond with variables of importance (VIMP) analysis. Variables are ranked in order of variable importance (VIMP), which is calculated based on how important the variable was in explaining variation in the indicator value. "Minimal depth rank" and "minimal depth value" refers to the average minimal depth the factor appears in the regression trees. A minimal depth closer to 1 signifies that the factor was selected closer to the root node in the regression trees, and is more predictive of variation in the indicator as a single split.

PMN Random Forest: Factor Importance					
	VIMP Analysis			Minimal Depth Analysis	
	Rank	Variable Importance	Relative Importance	Rank	Average Minimal Depth
Clay (%)	1	708	1	3	4.10
Sand (%)	2	534	0.76	4	4.30
Silt	3	484	0.68	5	4.34
Rest period	4	321	0.45	2	3.76
Pasture age	5	288	0.41	1	3.58
Operation type	6	79	0.11		
Time in paddock	7	68	0.10		
Seasonal grazing pressure	8	64	0.09	6	5.26
Number of rotations	9	61	0.09		
pH	10	58	0.08	7	5.29
Residual pasture height	11	49	0.07		
Stocking rate	12	43	0.06		
Outwinter	13	37	0.05		
Lime	14	34	0.05		
Stocking density	15	32	0.04		
Hay frequency	16	26	0.04		
Legume (%)	17	24	0.03		
Drainage class	18	16	0.02		
Manure frequency	19	15	0.02		
Previous land use	20	11	0.02		
Fertilizer frequency	21	6	0.01		
Days between grazing and sampling	22	4	0.01		
Manure	23	2	0.00		
Fertilizer	24	1	0.00		
Region	25	0	0.00		
Fertilizer this year	26	0	0.00		
(OOB) R squared: 0.49					
(OOB) Requested performance error: 544					

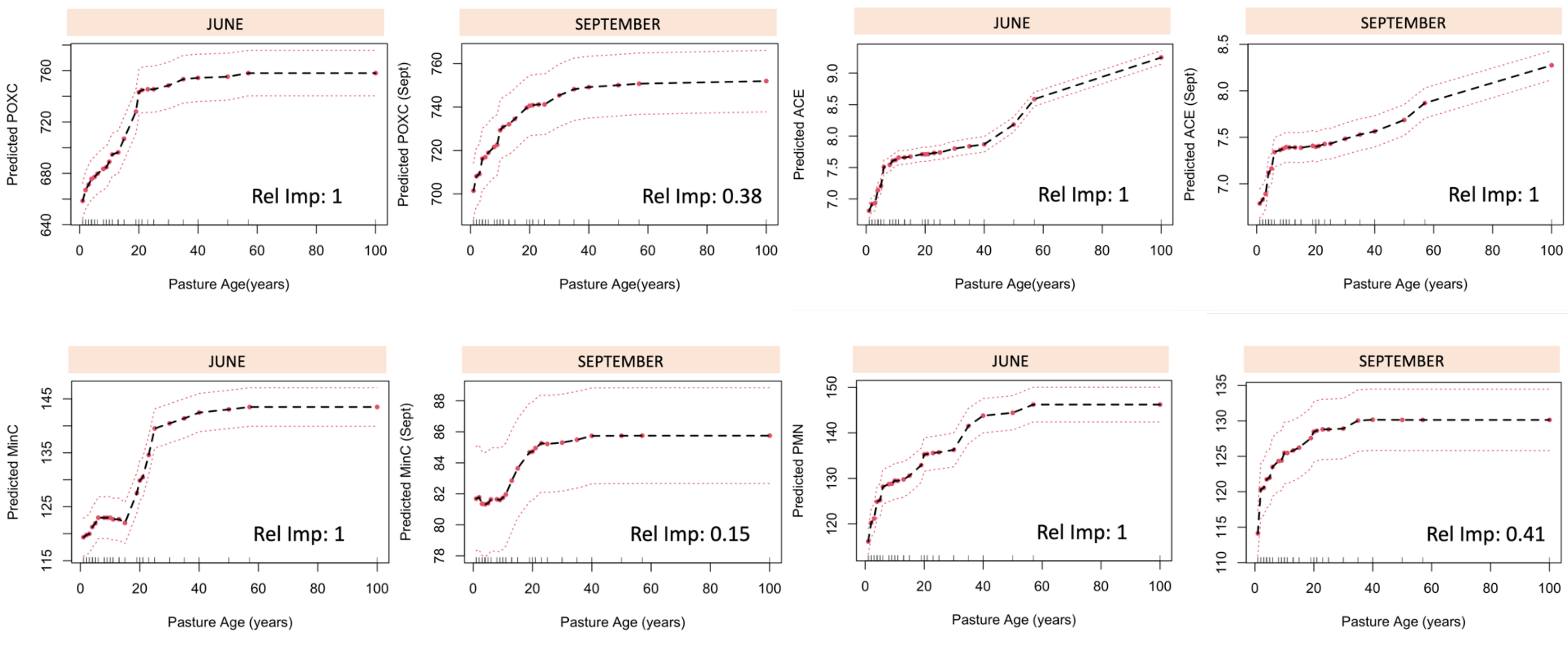

Figure S3.2: Partial effect of pasture age on June & September POXC, ACE, MinC and PMN
Predicted values are calculated as pasture age varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

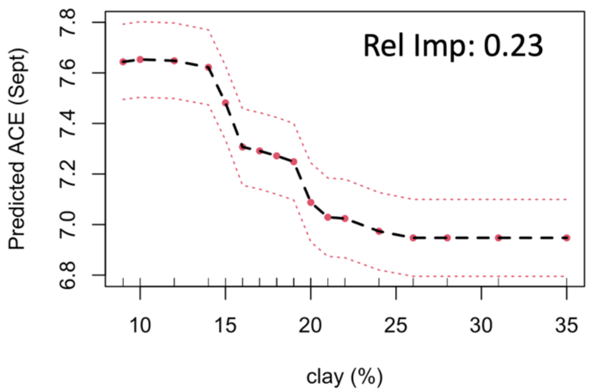

Figure S3.3: Partial effect of clay (%) on September ACE
Predicted values are calculated as clay (%) varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
ACE = autoclaved-citrate extractable protein (g kg^{-1})

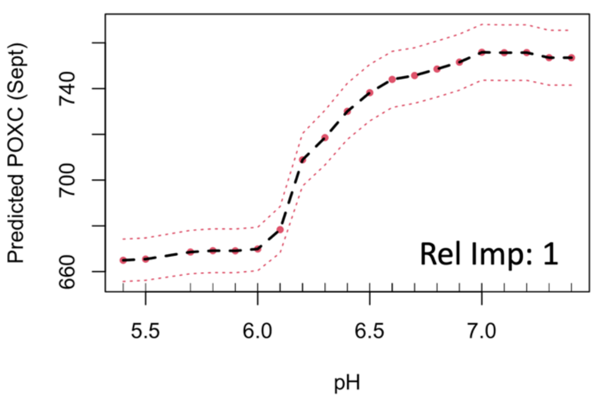

Figure S3.4: Partial effect of pH on September POXC
Predicted values are calculated as pH varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
POXC = permanganate oxidizable carbon (mg kg^{-1})

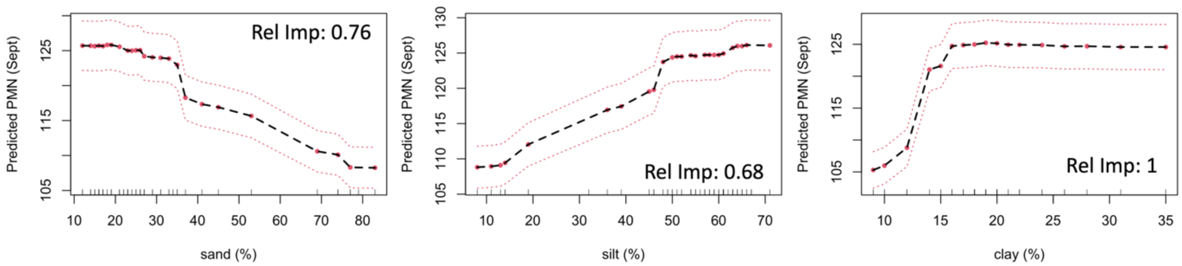

Figure S3.5: Partial effect of sand (%), silt (%), clay (%) on September PMN
Predicted values are calculated as sand, silt, or clay (%) varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
ACE = autoclaved-citrate extractable protein (g kg^{-1})

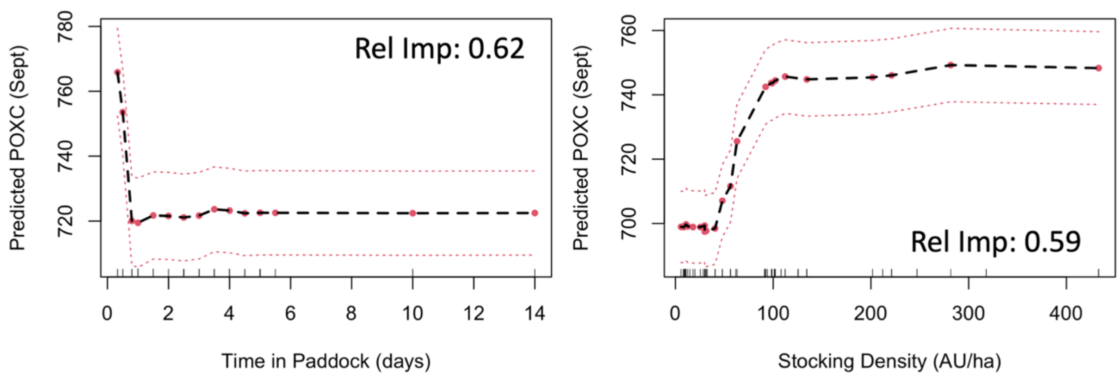

Figure S3.6: Time in paddock (left) and stocking density (right) on September POXC
Predicted values are calculated as clay (%) varies over observed values. Values are calculated over all remaining covariates, averaged, and plotted. Dashed red lines indicate +/- two standard deviations.
Rel Imp= relative importance according to VIMP
POXC = permanganate oxidizable carbon (mg kg^{-1})

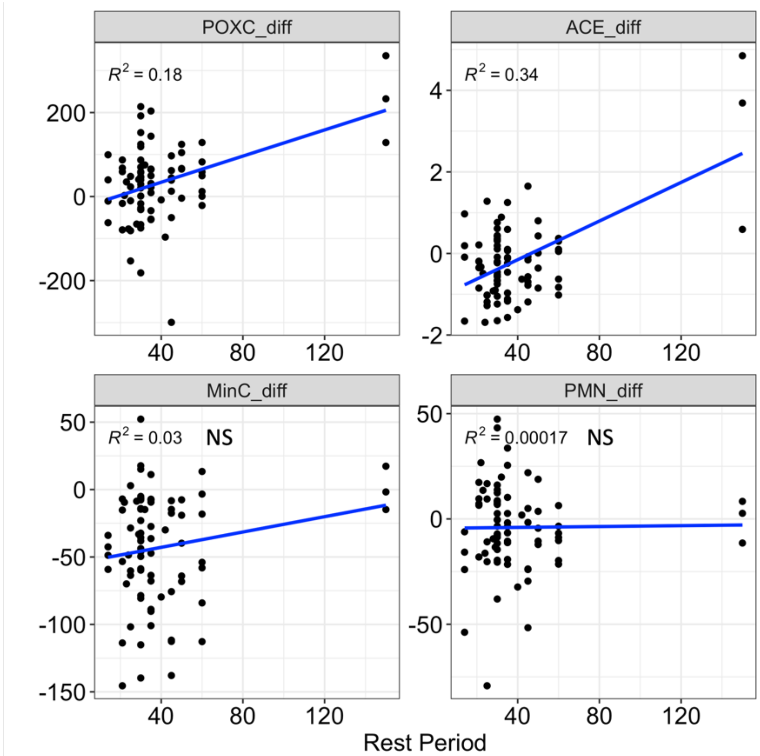

Figure S3.7: Scatterplots of rest period and the difference in biological soil health indicators (defined as September-June).

Linear regression analysis was run to determine coefficients of determination (R^2) and the equation estimated through linear regression are included in each figure (p-value <0.05). Difference in MinC and PMN were not significant.

POXC = permanganate oxidizable carbon (mg kg^{-1}); ACE = autoclaved-citrate extractable protein (g kg^{-1}); MinC = mineralizable carbon (mg kg^{-1}); PMN = potentially mineralizable nitrogen (mg kg^{-1})

R Code

Univariate Statistics:

```
#group soil C and N indicators
library(dplyr)
Indicators <- SH_da %>%
  dplyr:: select(POXC_Sept, ACE_Sept, MinC_Sept, PMN_Sept)

#analysis
library(pastecs)
library(psych)
summary(Indicators)
describe(Indicators)
stat.desc(Indicators)
```

Linear Regression:

```
m <- lm(POXC_Sept~OM, data=SH_da)
plot(m) #check assumptions of linear model
summary(m)
```

Correlation matrix:

```
library(GGally)
library(ggpubr)
library(ggplot2)
ggpairs(Indicators)
```

Paired t-test:

```
t.test(SH_da$POXC_June, SH_da2$POXC_Sept, paired = TRUE, alternative = "two.sided")
```

ANOVA

```
library(agricolae)
model <- aov(POXC_Sept ~ Texture, data= SH_da)
summary(model)
lsd=LSD.test(model, c("Texture"), group=T)
lsd
```

Random Forest

```
#load packages
library(randomForestSRC)
library(ggRandomForests)

#set seed & make random forest
set.seed(220603)
POXC_S_RF <- rfsrc(POXC_Sept~ Region+ pH + sand + clay + silt +
        DrainageClass+ OperationType_General + PastureYears + PreviousLandUse_cat +
        Legume_Sept+
        Fertilizer_thisYear + Fertilizer + Fertilizer_freq + Manure +
        Manure_freq + Lime + Overwinter  + HayFreq + DaysSinceGrazing_Sept +
                    StockingRate_ha + StockingDensity_ha +
            SeasonStockingDensity_ha + NumberRotations +
        TimeInPaddock + RestPeriod + ResidualPastureHeight_cm,
       data = Rotation, ntree = 5000,
       na.action = "na.impute", nimpute = 1,
       importance = "anti",
       forest=TRUE)

#retrieve random forest results
POXC_S_RF
#plot random forest results
```

```
plot(POXC_S_RF)
plot(gg_vimp(POXC_S_RF))

#minimal depth analysis
var.select(object = POXC_S_RF, conservative = "low")

#plot partial effects
plot.variable(POXC_S_RF, xvar.names = "PastureYears", partial = TRUE,
        xlab="Pasture Age(years)", ylab="Predicted POXC (Sept)")
```