SOIL NITROGEN STORAGE AND MINERALIZATION POTENTIAL IN A WINTER WHEAT SYSTEM UNDER LONG-TERM NITROGEN FERTILIZATION

By

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Bachelor of Science in Agroecology

West Virgnia University

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2020

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 2022

SOIL NITROGEN STORAGE AND

MINERALIZATION POTENTIAL IN A WINTER

WHEAT SYSTEM UNDER LONG-TERM NITROGEN

FERTILIZATION

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Date of Degree: MAY 2022

Title of Study: SOIL NITROGEN STORAGE AND MINERALIZATION POTENTIAL IN A WINTER WHEAT SYSTEM UNDER LONG-TERM NITROGEN FERTILIZATION

Major Field: SOIL SCIENCE

Abstract: In winter wheat systems, crop nitrogen(N) uptake is sourced primarily from inorganic N fertilization and N mineralization from soil organic matter(SOM). N mineralization is a dynamic N cycling process that relies on many different biological and chemical interactions within the soil. However, N fertilization potentially disrupts this process via changes in soil biological functioning. While N fertilization can increase total N in soil, the storage and availability of increased total N within SOM are unclear. Additionally, research has shown a direct suppression of N mineralization because of fertilization. The goal of this study was to understand how N fertilization alters the availability of N to be mineralized in soil and affects microbial groups and functioning related to N mineralization. Archived samples from a long-term NPK rate trial in a winter wheat system were used to evaluate changes in N storage between two distinct SOM fractions. Total N of both fractions increased with N fertilization rate, but a greater distribution of total N accumulated within available fractions as N fertilization increased. Fresh soils from the trial were sampled to measure a suite of microbial measurements including phospholipid fatty acid analysis(PLFA), CO_2 respiration, microbial biomass, and gross N mineralization rates. Results showed significant differences and trends among treatments. PLFA revealed decreased abundances of gram+/gram- ratios, fungi/bacteria ratios, and arbuscular mycorrhizal fungi. Microbial biomass C and N showed initial decreases in response to N fertilization, but then increased at higher rates. Data showed a potential for increased N availability as a result of fertilization, but decreases in important microbial groups and functions that may be involved in N mineralization.

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CHAPTER I

Introduction

Mineralization of soil nitrogen (N) is a crucial component of terrestrial N cycling, converting organic N from decaying plant and microbial biomass to inorganic, plant-available forms. It is well known that many interacting biological and chemical factors influence N mineralization. A challenge in sustainable soil management of cropping systems is that this dynamic N cycling process is routinely disturbed by N fertilizer inputs. Specifically, there is uncertainty as to how repeated fertilization alters availability of N to be mineralized and biological functioning in soil that drives N mineralization. N mineralization from soil organic matter (SOM) presents a viable N contribution and has even been suggested to support high N demand crops such as maize in fertilized systems(Osterholz et al., 2017). Other studies have illustrated the critical role of N mineralization even at high N application rates, showing that 54-83% of N uptake by the crop is still soil derived(Stevens et al., 2005) However, sole reliance on organic N sources without supplementation by inorganic fertilizer N has shown to increase the rate of SOM depletion in agricultural soils(Craine et al., 2007; Ladha et al., 2011). Because of this, inorganic fertilizers are relied upon to replace N deficits and maximize yields. While advancements in precision agriculture technology are enhancing producer capabilities to identify optimal N rates for a given year(Raun et al., 2002; Roberts et al., 2011), N is still commonly over-applied to ensure crop nutrient demand is met to maximize yield and profits.

Addition of N fertilizer at rates required for maximized yield or in excess has shown potential to maintain and possibly increase total soil C and N in both maize and wheat systems(Collier et al., 2017; Raun et al., 1998; Smith et al., 2019; Zhong et al., 2015). This has been typically attributed to increases in plant biomass returned to the soil from increased yield, however a growing body of evidence is supporting the fact that N fertilization may initiate shifts in soil microbial and chemical characteristics such as community structure and abundance(Du et al., 2019; Song et al., 2018; Sun et al., 2019) and N distribution across SOM fractions(Mahal et al., 2018; Xu et al., 2021; Zang et al., 2016). Suppression of rhizosphere priming, a key process enabling N mineralization, has also been reported in response to N fertilization(Feng and Zhu, 2021; Liu et al., 2018). These reports suggest that while N fertilization may increase plant biomass, it also may be inducing changes to the soil system related to N mineralizing capacity. Contrasting reports have also found inorganic N fertilizers to increase N mineralization and SOM decomposition(Liu et al., 2017; Qiu et al., 2016). Studies published on the relationship of N mineralization and N fertilization span a wide range of soil types, climates, cropping systems, and fertilizer rate and type, leading to highly variable results that make it had to generalize patterns on a larger scale. The goal of this study was to determine how inorganic N fertilization rate shifted storage of N within SOM fractions and altered biological parameters and functions directly or indirectly associated with N mineralization potential in a winter wheat cropping system. The use of a long-term N fertilization rate and yield trial for this study was useful in evaluating effects of nearly 50 years of repeated N application at the same rates.

CHAPTER II

Nitrogen Storage and Distribution within SOM Fractions

Literature Review

The dynamic role of SOM to serve both as a nutrient source and sink has implications for land management strategies targeting fertility management. In a sustainable soil system, it is essential to maintain both of these N storage and supply functions. Separating SOM based on particle size provides more nuanced insight into the potential for a soil to store and supply N. Particulate organic matter (POM), which is defined as sand-sized, and mineral-associated (i.e., silt and clay-sized) organic matter are often classified as an N source and sink, respectively (Lavallee et al., 2020). While MAOM is considered a passive N reservoir where turnover occurs on a centuries to-millennia timescale, emerging research suggests portions of it turnover on shorter time scales, a property typically associated with POM fractions (Jilling et al., 2018). MAOM in the rhizosphere may be vulnerable to decomposition due to the stimulatory effects of plant root exudates (Keiluweit et al., 2015). This mechanism destabilizes mineral-organic associations and releases bioavailable carbon (C) and N through a process known as priming (Jilling et al., 2021). The priming effect serves a critical role in unlocking a

vast organic N pool from SOM (which accounts for up to 95% of soil N) to microbes and plants via mineralization (Bingham and Cotrufo, 2016). Because mineralization of N bound within both SOM fractions is an important contributor to crop growth, it is important to understand how N fertilization impacts overall C and N storage and the relative distribution of C and N across SOM fraction.

Total C and N analysis via dry combustion is commonly considered an affordable, reliable soil health indicator, allowing for estimations of overall fertility status of a soil. The increase of total soil C and N in response to N fertilization described in the following studies clearly identifies positive impacts of fertilization to soil. Soil analyses conducted on four long-term winter wheat fertilization trials with varying N rates all between 0-269 kg ha^{-1} indicated significant increases in total C and N in surface soil (0-30cm)(Raun et al., 1998). For both C and N, the increases were most apparent when N rates exceeded requirements for maximum yield at all four sites. Interestingly, total N decreased at low to moderate N rates at two of the sites, possibly indicating that applied N had a "priming effect" on soil microbes, leading to increased net N mineralization. This information provides an argument for further considering not just the effect of fertilization in general, but the rate at which it is being applied. Analyzing changes in soil parameters across an increasing application gradient serves to reveal critical details that are possibly missed in some studies that only utilize a one or two treatments. More recently, an agricultural system simulation model was used to determine if fertilization can help maintain inorganic soil N in wheat systems. N fertilization served to maintain inorganic N stocks while increasing total soil C and N (Smith et al., 2019). N rates were applied in accordance to existing inorganic N reserves at the sites to maintain "soil N banks" between 0 and 175 kg N ha^{-1}. N fertilizer was determined to be critical for avoiding depletion of total C and N in soil. Depletion could only be avoided when a neutral or positive N balance was achieved, again indicating that there is a critical fertilization threshold in which fertilization leads to accumulation of C and N. In a continuous long-term corn fertilization

trial, N fertilization maintained soil C and N content, suggesting N fertilizer was a benefit to the soil C storage in the cropping system(Collier et al., 2017). Low N rates averaging 10 kg N ha^{-1} annually experienced significantly lower C and N stocks than both recommended N rates(56-168 kg N ha^{-1})and high N rates(112-252 kg N ha^{-1}). Recommended and high N rates led to 16 and 23% more carbon than the low N rate, respectively. For N, recommended and high N rates both led to a 5% increase relative to the low N rate. These studies emphasize the need to further investigate exactly why this increase in C and N occurs and how it is stored between SOM fractions.

The division of SOM pools provides insightful information into the mineralization potential of organic C and N. Analyzing N storage in different fractions may provide evidence as to the source of N and if buildup is due to decreased physiological capacity of microbes to mineralize N or possibly lacking accessibility to N. When evaluating C and N stored within SOM fractionated based on density, light fraction C and N, which is comparable to POM, significantly increased compared to zero N control treatments(Zhong et al., 2015). In a winter wheat fertilization trial with N rates between 0-60 kg N ha^{-1}, there were no significant differences between the N treatments, but sensitivity index calculations relative to the check plots revealed light fractions in the surface soil were more sensitive to N fertilization than heavy (i.e., mineral-associated) fractions. While it's inconclusive whether fertilizer has a consistent effect on C and N within fractions increased sensitivity of POM/light fractions reflect recent inputs from fertilizer and plant residues, and may mean that any observed increases in C and N are just yearly changes and have no impact on long-term(10+years) sequestration of C and N in soil. Another study looking at the response of SOM fractions to different forms of fertilizer in a winter wheat-corn rotation found clear effects of organic fertilization, but very limited effects of inorganic fertilization on SOM fractions(Tian et al., 2017). Both inorganic and organic fertilizer (manure) were used separately and in combination to evaluate their effects on four separate soil C fractions (non-protected,

chemically, physically, and biochemically protected). Out of the four fractions, only C content in the unprotected fine POM fraction experienced a significant increase in response to inorganic N applied at 267 kg ha^{-1}. However, organic fertilizers independently or in combination with inorganic fertilizers drove significant changes in C content of both protected and unprotected soil fractions. Increases in light/POM fraction C suggest an indirect effect of plant biomass as these fractions are primarily plant derived. More research to accurately describe allocations of C and N within SOM fractions in response to long-term fertilization is an important goal in better understanding well-documented increases of total C and N in response to fertilization.

Only focusing on total soil C and N overlooks the multifunctionality of SOM and different SOM fractions. POM and MAOM cycle on vastly different timescales and do not equally contribute to N availability in soil. Addressing this knowledge gap in N cycling research, especially the effects of inorganic fertilization, will inform understanding of plant and microbial accessibility to the soil N pool and potentially improve our ability to estimate mineralizable N. The objective of this study was to determine how SOM fractions responded to between 25 and 50 years of repeated N fertilization.

Methods

Site and Soil Information- The Oklahoma State University North Central Research Station is home to the 502 experiment (E502) and is located on a well-drained, deep, and moderately permeable Grant silt loam (fine-silty, mixed, thermic Udic Argiustoll) in Lahoma, Oklahoma (36.388267, -98.108654). The 502 experiment is a continuously managed NPK (nitrogen-phosphorus-potassium) rate and grain yield trial established in 1971 using a winter wheat crop. It is a randomized complete block design with 14 treatments x 4 replicates for a total of 56 plots. In this study, treatments 1-7 and their four respective replicates (28 total) were of interest as N increases in equal intervals from 0-112 kg, while P and K remained constant except for treatment

1, which served as the control(table 1). N is applied as urea (46-0-0), P is applied as triple superphosphate (0-46-0), and K is applied and potassium chloride (0-0-60) all prior to fall planting of winter wheat. The system was under conventional tillage since establishment until 2010 when no till practices began.

Table 1. NPK fertilization rates at E502

Treatment	N	P	K
1	0	0	0
2	0	20	56
3	22	20	56
4	45	20	56
5	67	20	56
6	90	20	56
7	112	20	56

Archived samples from E502 were air dried and stored in a dry, dark location until ready for analysis. 1995 and 2010 samples were sampled in July of their respective years, and 2020 samples were sampled in August, prior to any fertilization.

Soil Physical Fractionation- 10g of air dried, 2mm sieved soil was added to 30ml of 0.5% sodium hexametaphosphate, a solution used to disperse soil aggregates and particles. After 8 hours of shaking, fractionation was performed using a Fritsch automatic wet sieve shaker (Fritsch, Idar-Oberstein, Germany). Soil that passed through the 53µm sieve was defined as MAOM and collected in a 500 ml centrifuge bottle and subject to 30 minutes of centrifugation at 7,500 rpm until a pellet formed on the bottom. The supernatant was gently poured off and the pellet was washed out into a pre-weighed metal tin. Soil that did not pass through the sieve was defined as POM and gently washed off into a separate pre-weighed metal tin. Upon recovery of both fractions, they were dried in a forced heat oven at 105C until all liquid was evaporated and reweighed to calculate the actual soil mass in each fraction. Soil was stored in plastic scintillation vials until needed for further analysis.

Total C and N Analysis of Isolated SOM Fractions- 200mg of dried and finely ground soil from the particulate and mineral-associated fractions were packed into foil tins to be analyzed in a LECO combustion analyzer (LECO, St. Joseph, MI).

Statistical Analysis- Data analysis was performed in R studio (version 2022.02.0). One-way analysis of variance (ANOVA) was used to test the effect of N treatment on SOM fractions and N storage with treatments coded as factors. Assumptions of homoscedasticity and normality were assessed using a Levene's test and Shapiro wilks test. Log and reciprocal transformations were used as necessary to normalize data. A Tukey honest significant difference (HSD) test was done to determine if the differences between pairs of treatment means were statistically significant at a P-value of <0.05. Regression analyses were also performed on individual years to identify significant linear and/or quadratic trends along an increasing N treatment gradient. Because of a change in management practice from conventional to no till in 2010, the effect of N fertilization on fractions and N storage was not compared across years.

Results

SOM fractionation of archived soil from three years (1995, 2010, and 2020) showed no statistically significant differences in mass proportions between treatments or overall trends in any of the years (Table 2). POM and MAOM are displayed on a per gram of recovered soil basis due to variability in sample recovery from the fractionation (90-95% of initial 10g recovered). POM accounted from anywhere between 20-36% of recovered soil, while MAOM made up between 64-80% of the recovered soil.

Table 2. POM and MAOM recovered (g fraction g^{-1} recovered soil)

Trt	N-P-K (kg ha^{-1})	1995 POM	2010 POM	2020 POM	1995 MAOM	2010 MAOM	2020 MAOM
2	0-20-56	0.24(0.05)	0.21(0.01)	0.36(0.03)	0.76(0.05)	0.79(0.01)	0.64(0.03)
3	22-20-56	0.26(0.07)	0.20(0.01)	0.34(0.02)	0.74(0.07)	0.80(0.01)	0.66(0.02)
4	45-20-56	0.24(0.02)	0.21(0.03)	0.35(0.03)	0.76(0.02)	0.79(0.03)	0.65(0.03)
5	67-20-56	0.23(0.02)	0.21(0.04)	0.34(0.05)	0.77(0.02)	0.79(0.04)	0.66(0.05)
6	90-20-56	0.22(0.02)	0.22(0.04)	0.33(0.04)	0.78(0.02)	0.78(0.04)	0.67(0.04)
7	112-20-56	0.22(0.02)	0.21(0.02)	0.32(0.02)	0.78(0.02)	0.79(0.02)	0.68(0.02)

*Parentheses indicate standard error of averages. (n=4)

Distribution of total N was calculated as the proportion of fraction N (mg N g^{-1} fraction) out of total N summed from both fractions. No statistically significant differences between groups were identified based on ANOVA, however many significant trends were identified(table 3)(fig.1). The 1995 samples showed significant ($P<0.01$) linear trends for POMN% and MAOMN%. POMN% increased with fertilization, while MAOMN% decreased. 2020 samples exhibited the exact same trend between POMN% and MAOMN% but was only deemed marginally significant ($P<0.1$).

Table 3. N distribution between POM and MAOM (% of total soil N)

Trt	N-P-K (kg ha^{-1})	1995 POMN%	2010 POMN%	2020 POMN%	1995 MAOMN%	2010 MAOMN%	2020 MAOMN%
2	0-20-56	34.3	37.1	23.9	65.6	62.9	76.1
3	22-20-56	32.6	35.2	30.2	67.4	64.8	69.8
4	45-20-56	32.1	31.8	29.9	67.9	68.2	70.1
5	67-20-56	33.7	37.2	33.1	66.3	62.8	66.9
6	90-20-56	40.6	39.3	32.0	59.4	60.7	68.0
7	112-20-56	40.5	37.0	32.7	59.5	63.0	67.3
L		**	ns	.	**	ns	.
Q		.	ns	ns	.	ns	ns

Symbols indicate linear(L) or quadratic(Q) trends at specified significance levels (***=0.001,**=0.01,*=0.05,'.'=0.1)

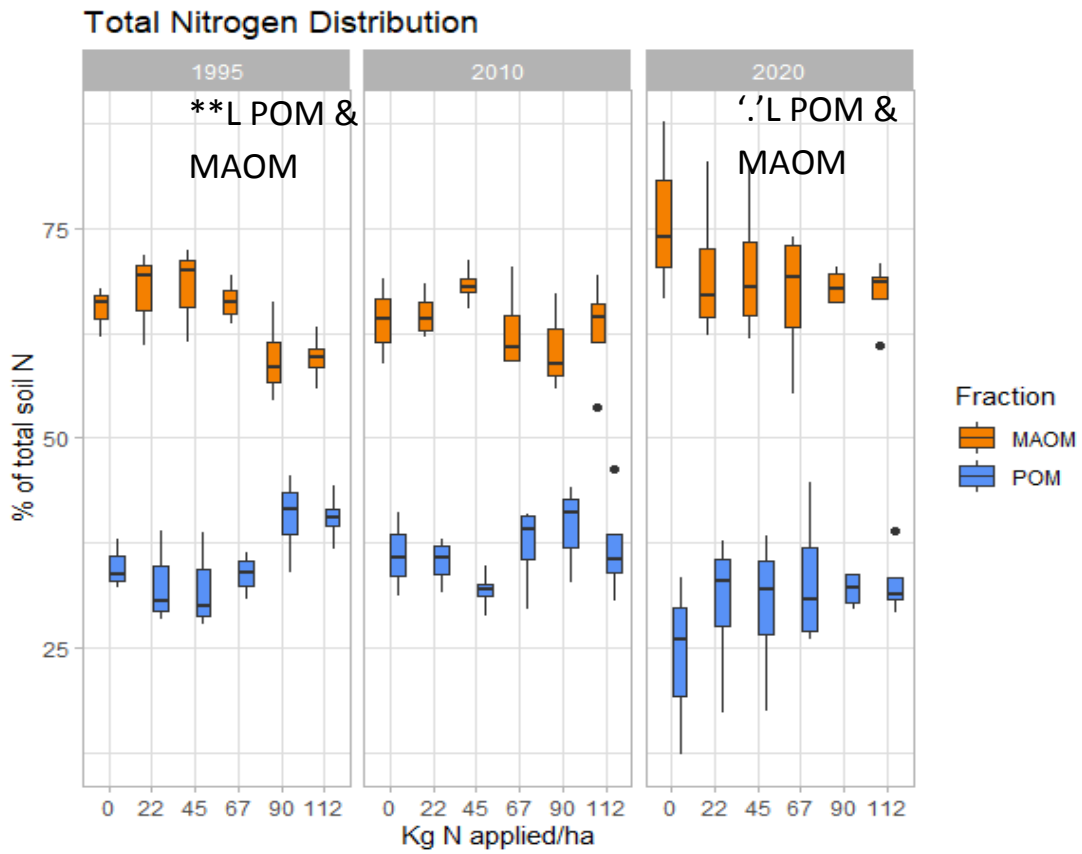

Fig 1. % of total soil N distributed within POM and MAOM fractions at six different N rates, separated by year. Symbols indicate linear(L) or quadratic(Q) trends at specified significance levels (***=0.001,**=0.01,*=0.05,'.'=0.1)

fraction), showed two different statistically significant trends(table 4)(fig.2). In soils collected in 1995, we observed a significant($P<0.05$) linear decreasing trend in the C/N ratio of MAOM with increasing fertilization rate. In 2020 samples, we observed a significant ($P<0.01$) quadratic trend where values initially increased with fertilization rate, but then began to decrease at high rates.

Table 4. CN ratios of each fraction

Trt	N-P-K (kg ha^{-1})	1995 POMCN	2010 POMCN	2020 POMCN	1995 MAOMCN	2010 MAOMCN	2020 MAOMCN
2	0-20-56	14.3(1.28)	13.9(1.71)	15.5(8.54)	8.54(0.21)	8.52(0.40)	8.64(0.12)
3	22-20-56	14.6(3.71)	13.0(0.88)	13.2(5.92)	8.08(0.56)	8.83(0.16)	9.26(0.48)
4	45-20-56	15.7(0.89)	13.1(1.11)	11.8(1.77)	8.31(0.34)	8.47(0.26)	9.34(0.66)
5	67-20-56	15.6(0.84)	13.8(1.37)	12.7(0.74)	8.10(0.20)	8.76(0.32)	9.26(0.76)
6	90-20-56	15.7(1.72)	14.5(0.16)	10.9(0.75)	8.28(0.23)	8.62(0.39)	8.80(0.54)
7	112-20-56	15.8(1.05)	14.2(2.08)	11.0(3.09)	7.90(0.22)	8.63(0.40)	8.32(0.28)
L		ns	ns	ns	*	ns	ns
Q		ns	ns	ns	ns	ns	**

*Parentheses indicate standard error of averages. (n=4) Symbols indicate linear(L) or quadratic(Q) trends at specified significance levels (***=0.001,**=0.01,*=0.05,'.'=0.1)

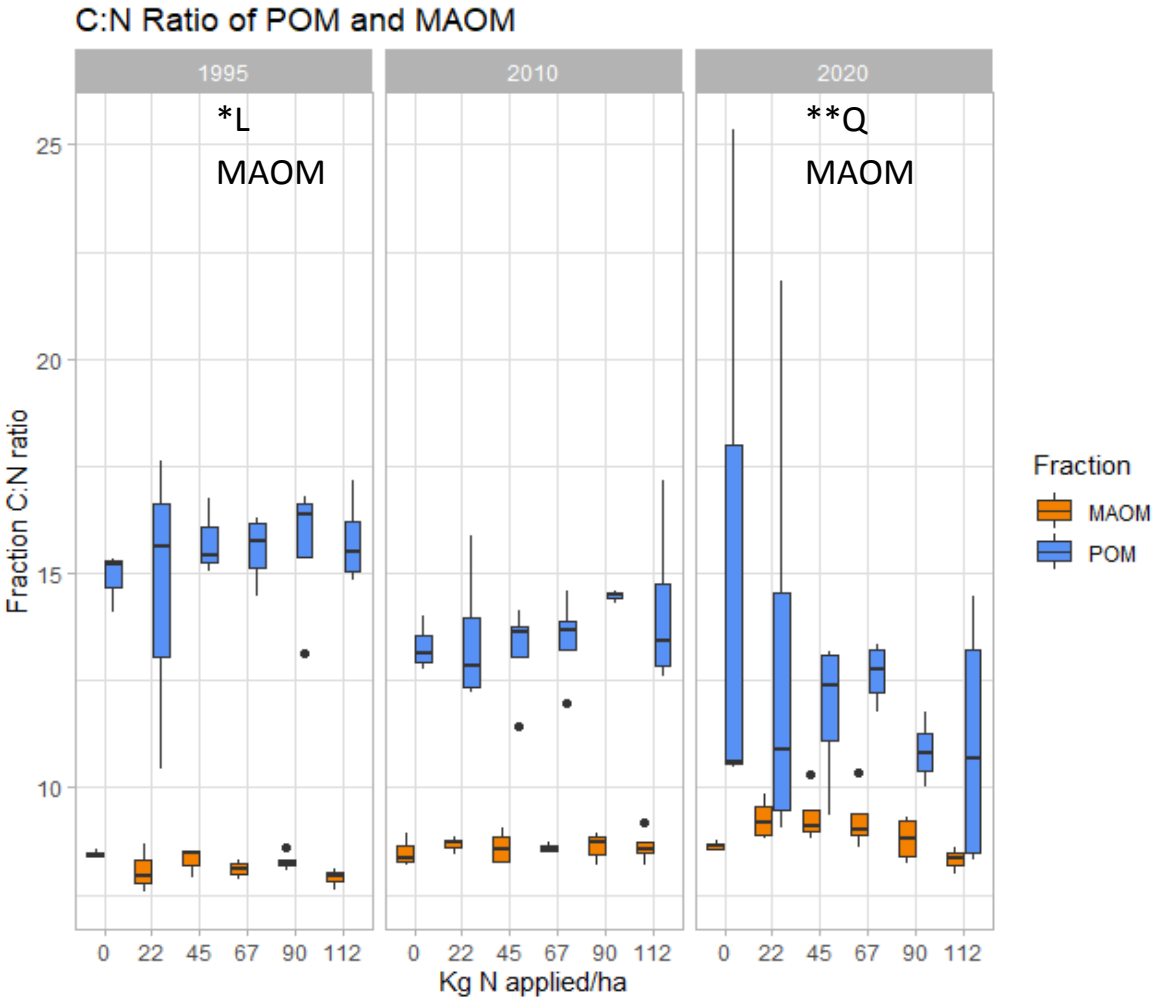

Fig 2. C:N ratio of POM and MAOM fractions at six different N rates, separated by year. Symbols indicate linear(L) or quadratic(Q) trends at specified significance levels (***=0.001,**=0.01,*=0.05,'.'=0.1)

Final fraction N concentrations were calculated with g of recovered soil from each fraction (mg fraction N g^{-1} recovered soil). Marginally significant ($P<0.1$) linear and quadratic trends were identified in the 1995 POM N fraction concentration (table 5)(fig.3). In 2020 MAOM N fraction concentration had a significant ($P<0.05$) linear increasing trend with fertilization rate. While not deemed statistically significant, numerical increases in the mg of fraction N g^{-1} recovered soil were observed in both fractions across all three years.

Table 5. N concentration of POM and MAOM (mg fraction N g^{-1} recovered soil)

Trt	N-P-K (kg ha^{-1})	1995 POMN	2010 POMN	2020 POMN	1995 MAOMN	2010 MAOMN	2020 MAOMN
2	0-20-56	1.22(0.20)	1.12(0.21)	1.22(0.59)	7.30(0.57)	7.26(0.37)	6.53(0.31)
3	22-20-56	1.32(0.33)	1.00(0.11)	1.49(0.59)	7.67(0.39)	7.18(0.64)	6.27(0.33)
4	45-20-56	1.07(0.17)	0.92(0.12)	1.39(0.35)	7.60(0.29)	7.30(0.48)	6.47(0.53)
5	67-20-56	1.13(0.10)	1.13(0.12)	1.57(0.31)	7.66(0.28)	7.10(0.45)	6.68(0.72)
6	90-20-56	1.45(0.21)	1.22(0.25)	1.50(0.14)	7.44(0.49)	7.26(0.12)	6.82(0.72)
7	112-20-56	1.47(0.13	1.21(0.37)	1.50(0.10)	7.81(0.50)	7.62(0.40)	7.16(0.37)
L		.	ns	ns	ns	ns	*
Q		.	ns	ns	ns	ns	ns

*Parentheses indicate standard error of averages. (n=4) Symbols indicate linear(L) or quadratic(Q) trends at specified significance levels (***=0.001,**=0.01,*=0.05,'.'=0.1)

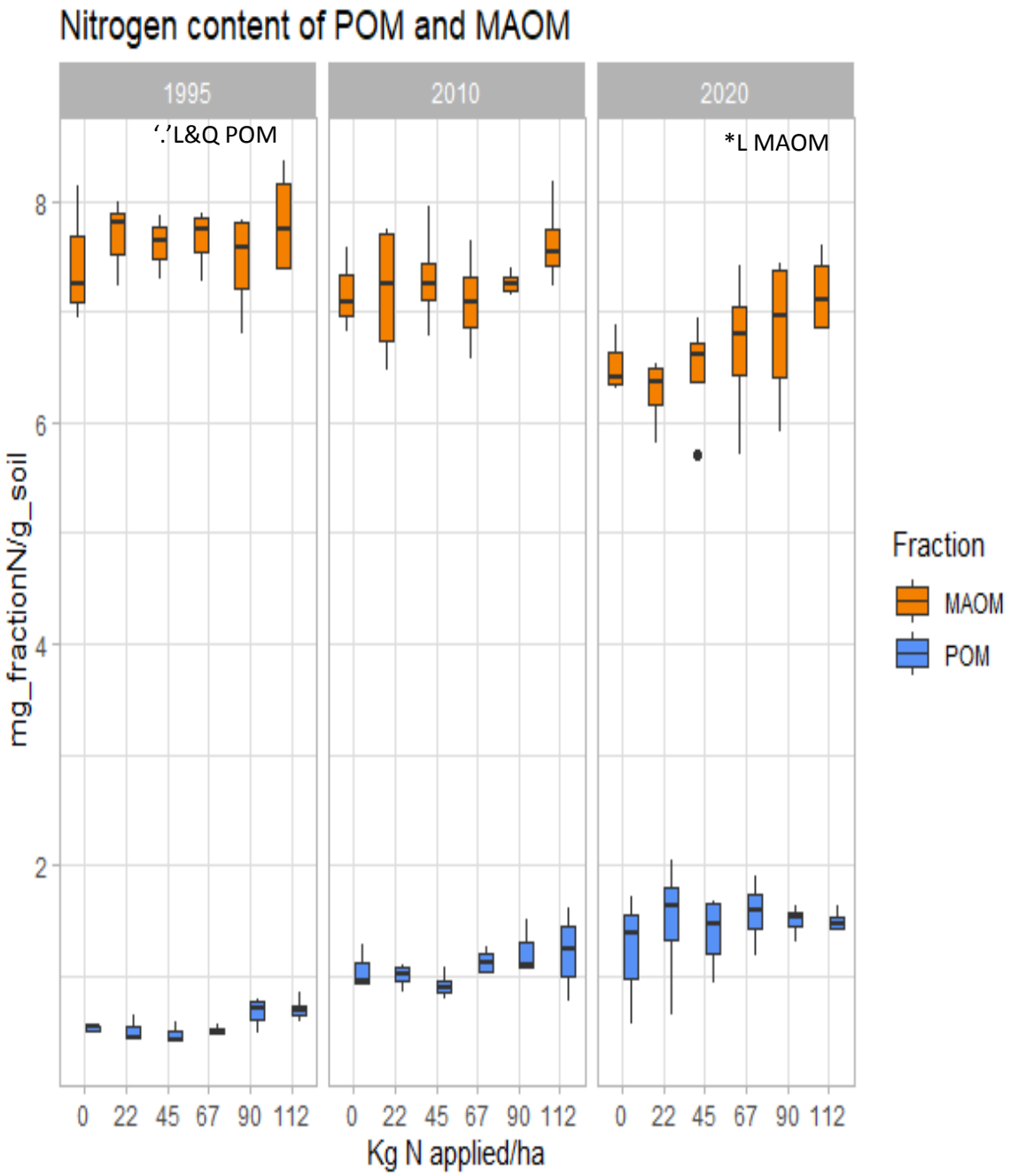

Fig 3. N Content of POM and MAOM based on amount of recovered soil fraction at six different N rates, separated by year. Symbols indicate linear(L) or quadratic(Q) trends at specified significance levels (***=0.001,**=0.01,*=0.05,'.'=0.1)

Discussion

The primary purpose of this study was to identify how N fertilization rate impacts SOM fractions and N storage within fractions. This data provides insight into potential N supply potential, even though N supply was not directly measured.

The proportions of POM and MAOM fractions did not change significantly between treatments in any given year, but there was a noticeable increase in POM in the 2020 samples compared to both 1995 and 2010. While it was outside the scope of this study to investigate the effect of changes in tillage practice from conventional to no till, conversion likely served to increase plant-derived biomass, especially in the surface soil, thereby increasing proportions of POM.

Even though the proportions of POM did not increase across treatments, significant trends indicating an increase in the storage of N within POM may be due to higher N content of plant derived inputs with increasing fertilization rates. This is consistent with our finding that the C/N ratio of POM tends to decrease with increasing fertilization, especially in 2020. Such noticeable shifts of N into the POM pool could suggest that increased fertilization has a positive effect on soil in terms of N that is potentially available to be mineralized.

Significant increasing trends in MAOMN content are a clear result of nearly 50 years of repeated fertilization. It is less surprising to see increases in POMN content, as it is a direct reflection of increased plant biomass, but nonetheless an important result for understanding N availability among treatments. Increase of MAOMN are critical to the long-term storage of N in soil, and while it has historically been considered unavailable, emerging research is suggesting that plants and microbes do have access to N within MAOM.

These results provide evidence that N fertilization is increasing availability of total soil N by shifting greater proportions into readily available fractions. In addition, fertilization is still providing a positive effect in increasing the N content of both fractions. Future studies should

continue evaluating valuable soil archives from long-term trials such as E502 in addition to continued future sampling. Fractionation and total CN analysis are ideal analyses on archived soils, as it provides informative data while limiting time-sensitive concerns typically associated with biological analyses performed on fresh soils.

CHAPTER III

Biological Parameters Related N Mineralization Potential

Literature Review

Phospholipid fatty acid analysis (PLFA) provides a snapshot of soil microbial communities by linking specific fatty acids to certain functional groups such as gram negative/positive, bacteria and fungi, among others. While PLFA does not necessarily have specific interpretations for soil health, groups and ratios of groups can be correlated to certain soil functions such as nutrient cycling, availability, and SOM turnover. The USDA recently adopted PLFA as a recommended soil health indicator, chosen as a coarse indicator of community structure. Results are only representative of the soil community at time of sampling due to microbial sensitivity to environmental conditions and management practices. While this potentially presents a challenge of evaluating changes over time and between sites, it makes this method ideal for comparing differences between treatments at the same or similar sites. PLFA has its limitations and does not provide the taxonomic resolution of DNA sequencing and metagenomics, but it is cost effective and sensitive enough to detect changes in management practices (Frostegård et al., 2011). Because microbial communities are so sensitive to factors like geographical location, soil type, and climate, there are conflicting conclusions on how N fertilization affects community composition and function. Additionally, variance in N fertilization rate and fertilizer type add another level of complexity in understanding and comparing results.

N fertilization has known direct and indirect effects on measurements like soil pH, inorganic N pools, and carbon availability, all of which potentially can alter microbial community structure and abundance. PLFA analysis could help determine if the fertilization rate was detrimental or not to a soils biological functioning. PLFA determination in a maize-wheat cropping system with six fertilization treatments ranging from 0-350 kg N ha^{-1} revealed an increasing trend in fungal abundance and fungi to bacteria ratios as fertilization increased in both cropping seasons (Zhao et al., 2014). Fungi also had a significant negative correlation with soil pH, a likely result of the fertilization itself. Total PLFA biomass and abundance of bacteria and actinomycetes experienced no variance because of N fertilization rate. Bacterial and fungal abundance are both correlated positively with soil NH_4^+-N. In this scenario, increases in fungal abundance could be driven by the pH decrease because of fertilization, as fungal communities can thrive in low pH environments.

It also has been demonstrated that N fertilization can be beneficial to many microbial groups and ratios until certain thresholds where it becomes detrimental to many groups. In a two year perennial grass system N addition study, N was applied at four different rates between 0-10 g N m^{-2} $year^{-1}$ (0-100 kg N ha^{-1})(Zhang et al., 2017). N rates at 25 kg N ha^{-1} were beneficial to increasing microbial biomass, and PLFA's for bacteria, fungi, and actinomycetes compared to check plots with no N. Interestingly, rates at or above 50 kg N ha^{-1} served as a threshold where N became detrimental to abundance and diversity the microbial community. Fungi:bacteria ratios never significantly responded to any of the N treatments. If more research clearly identified such a trend, it would build a more tangible argument of the indirect costs to applying fertilizer beyond recommended rates. Unfortunately, literature does not reflect such a scenario and in some cases N fertilization causes no alterations in microbial community structure until a certain threshold. A 7+ year perennial grass system N addition study found no significant differences in any PLFA groups or ratios until N rates in excess of 224 kg N ha^{-1}(Shi et al., 2016). Six N treatments ranged

between 0-560 kg N ha^{-1}. These examples suggest that while N fertilization may not always be the controlling factor in microbial community structure and abundance, there are many instances where the controlling factor is undoubtedly N fertilization and could be suppressing a soil biological functions related to nutrient cycling.

Measurements of gross N mineralization by use of a ^{15}N isotope pool dilution take in to account the complex soil processes in which consumption and production reactions are constantly occurring. In contrast, net mineralization simply measures change in NH_4^+ concentration over time. While application of the pool dilution method relies on multiple assumptions and can be a tedious procedure, it offers an advantage in understanding N cycling processes and coupled interactions between soil properties and management practices(Barrett and Burke, 2000; Bengtsson et al., 2003; Murphy et al., 2003). The capacity of a soil to mineralize N is an important consideration as it provides a secondary nutrient source in addition to fertilization. The accurate quantification of gross N mineralization in agricultural systems has been of interest to researchers as it's consideration in management strategies may serve to reduce crop reliance on fertilizers(Osterholz et al., 2017). Studies evaluating the relationship between N fertilizer applied and gross N mineralization have had varied conclusions and cannot be generalized due to differences in cropping systems, amount of fertilizer and yearly repetitions of applied N.

It is unknown how quickly significant changes in the soil environment to limit gross N mineralization will occur in response to N fertilization. The results of a short-term (three-year) N rate experiment indicated that applied N at rates of 100 and 200 kg N ha^{-1} did not significantly affect gross N mineralization rates compared to the control (Ouyang and Norton, 2020). It is possible that the suppressive effect of fertilizer could be altered over many years, but the direct and immediate interaction of fertilizer with soil is still the controlling factor. A study found that N fertilizer did significantly suppress gross N mineralization rates, but the effect of N was most pronounced directly after N application, and became insignificant as soil inorganic N pools

returned to levels before fertilization (Mahal et al., 2019). The suppressive effect of fertilizer on gross N mineralization was much greater in historically under fertilized soils than in soils that had been receiving N fertilizer for nearly 15 years, suggesting microbial adaptation to overcome any effect of the N fertilizer. Additionally, a negative correlation found between NH_4^+ pool size and gross N mineralization rates indicated a direct effect of NH_4^+ on gross N mineralization. It is possible that identification of such direct effects on gross N mineralization rates could be more comparable across soil types and cropping systems, more so than changes to biological soil features that have increased sensitivity to other environmental variables.

Soil CO_2 respiration, otherwise known as C mineralization, is a relatively reliable indicator of microbial activity and available SOC in soils (Franzluebbers et al., 2000). Correlations between respiration and N mineralization have been documented, but results varied in literature based on soil type, management practices, and methodology relating to soil processing, incubation time, and measurement tools (Castro Bustamante and Hartz, 2016; Haney et al., 2008; Wade et al., 2018). In recent years the use of Infrared gas analyzers(IRGA) have become desired due to simplicity, cost, dual measurements and decreased waste product (Sherrod et al., 2012). N cycling processes are a direct reflection of microbial activity, making measures of CO_2 respiration an important tool to evaluate effects of management practices such as fertilization. CO_2 respiration is not just a baseline process to be measured but can also be stimulated in soil by the release of plant root rhizodeposits that stimulates microbial activity to decompose SOM and mineralize nutrients. This inducing of microbial decomposition is referred to as the priming effect and is commonly measured by CO_2 respiration methods. While methods of simulating root exudation are challenging, many studies focus on evaluating the respiration response of soil to additions of fresh plant C compounds that are exuded by roots (glucose, oxalic acid, etc.). Overall, CO_2 respiration and priming response is an important tool in gauging the

response of microbial activity to external inputs, especially as it correlates to N mineralization in many cases.

The suppression or stimulation of the priming effect by fertilization requires specificity as to the rate and type of fertilization. A meta-analysis suggested that priming effects in response to N additions on a global scale are controlled by the N limitation of microbial respiration, but in general N addition almost always suppresses the priming effect (Feng and Zhu, 2021). Many factors could contribute to the varied results seen in literature, but there are likely thresholds where N fertilization causes an abundant supply of N that slows activity, or accumulation of inorganic salts become toxic to microbes. It is possible that longer length incubations also blur clear effects of fertilization occurring within hours to days. Soil from an agricultural ecosystem treated with urea-N applied at 200 kg N ha^{-1} respired only 9% more cumulative CO_2-C respiration in a 190 day lab incubation study than the control (Qiu et al., 2016). The addition of N caused a positive priming effect resulting in a 9.1% increase in native SOC decomposition during the study. If this were to be true in other soils, this would contrast with findings of accumulated C and N with fertilization. Another study found the opposite in that N fertilization strongly decreased cumulative respiration by 27-42% and resulted in negative priming effects from all three N treatments relative to the control (Zang et al., 2016). Three N application rates were used between 0-208 ug N/ g soil in addition to a control treatment with no N applied. It was concluded that increased N availability led to negative priming effects.

Biological indicators are key for understanding a soil's capacity to supply N to plants. Limited studies have evaluated the long-term effects of repeated N fertilization on such soil functions, and long-term and short-term studies alike have found varied results possibly due to variations in cropping system, fertilizer type and amount, and even soil type. The purpose of this study is to evaluate how long-term N fertilization influenced biological measures related to microbial activity and soil N supply.

Methods

Sampling Information- Site information is described in Chapter 2. Fresh soil samples were taken in June 2021 from E502 by randomly sampling fifteen 15cm soil cores throughout each plot and homogenizing the cores in a plastic bag. Bags of soil were kept in a cooler on ice until they were returned to the lab to be processed and kept in the refrigerator. Processing of samples involved picking out any large rocks and plant material, then sieving through a 2mm sieve.

Gravimetric Water Content (GWC)- GWC was measured by placing 10g of fresh soil in a baking tin and drying for 24hrs at 105C. The difference in soil weight before and after drying accounts for water which was then divided by the initial soil weight to get GWC as a percentage. ((fresh soil weight-oven dried soil weight)/(fresh soil weight)*100=GWC %)

Water Holding Capacity (WHC)- 10g of oven dried soil from the GWC procedure was added to a Whatmann 40 lined funnel with a beaker underneath. Soil was then saturated with DI water until it pooled on top of the soil. Once soil was completely covered in water, the funnel was covered with foil to prevent evaporation and left to drain for 24 hours. After 24 hours, as much as possible of the saturated soil was removed from the filter and placed onto a dry, pre-weighed filter and the soil weight was taken. The soil and the filter were dried in an oven at 105C for 24 hours then reweighed. The calculated GWC represented 100% of the soils water holding capacity.

pH- pH was conducted on all 28 field samples with a 1:1 soil: water suspension using a Mettler-Toledo SevenCompact pH meter (Mettler-Toledo, Columbus, OH). Treatment replicate pH values were first converted to the -log of H+ ion concentration, averaged, then converted back to pH.

Chloroform Fumigation for Microbial Biomass- 10g of fresh soil was weighed into a 50mL glass beaker and placed in a desiccation chamber with 40ml of chloroform in a separate beaker. Another 10g was also weighed out and placed into a desiccation chamber with no chloroform to

serve as a blank. Air was evacuated from both chambers initially and the blank is left under vacuum conditions for 24hrs in the dark. In the fumigated chamber, air was evacuated until chloroform boiled for approximately 15 seconds. That chamber was then opened to fill with air and the process was repeated three times until the chloroform boiled for two minutes on the fourth and final time. The chamber was left under vacuum conditions and placed in the dark for 24 hours with the blank set. After 24 hours both chambers were reopened and filled with air. Residual chloroform is evacuated using a vacuum several times for safety. Soil was transferred to specimen cups with lids and shaken for 30 minutes with 50ml of 1M potassium chloride (KCl). After shaking, soil solution was drip filtered through Whatman #40 filters to obtain clear extracts. Extracts were kept frozen until ready to be ran through a Elementar TOC/TN analyzer (Elementar, Ronkonkoma, NY). Organic C and total N values from blank unfumigated samples were subtracted from values obtained in the fumigated samples under the principle that the additional C and N in fumigated samples accounts for biomass C and N.

Phospholipid fatty acid analysis (PLFA)- 15g of fresh soil were weighed out into 50ml falcon tubes and first sent to the OSU Microscopy Laboratory for freeze drying of samples. Next samples were sent to Microbial ID (Now MIDI Labs) (Newark, DE) for full PLFA analysis. Individual fatty acids were summed to produce total plfa biomass (nmol/g) then calculated into % fatty acid type and microbial type categories by Microbial ID for analysis.

Incubations for Carbon Mineralization - 50g of fresh soil was weighed into 1-pint (473mL) mason jars with lids altered with an air-tight septa ring, then adjusted to 40% WHC. All 28 field samples were replicated 3x for a total of 84 samples in two sets (T0 and T1). The 84 T0 jars served as a baseline and were harvested for selected soil analyses following a one-week pre-incubation period at 25˚C. The 84 T1 jars went through the pre-incubation period as well, then 5mL gas samples were taken with a syringe and analyzed using a LICOR LI-850 CO_2/H_2O gas analyzer at hour 0, 2, 8, and 24 on the first day, then sampled every 24 hours for the next 10 days

to track soil CO_2 respiration. All jars were monitored to maintain soil moisture at 40% WHC and opened each day following gas sampling to allow equilibration to atmospheric conditions before being resealed and placed back in the temperature-controlled chamber at 25˚C. Following incubation, the jars were harvested for selected soil analysis.

An additional experiment was conducted to test CO_2 respiration in response to glucose additions. Treatments 1, 4, and 7 and their 4 field replicates were replicated 3x for a total of 36 jars. Jars were set-up, incubated, and maintained as referenced above, with a glucose-water solution being added at a rate of 0.2 mg glucose-C g^{-1} soil.

Plate-Based Ammonium Assay- 1M KCL extracts from ^{15}N incubations described below were used to determine soil extractable ammonium via colorimetric analysis (Nelson, 2008). 80 µL of KCl extract from each sample was added to a 96 well plate. Next, 60 µL of a sodium salicylate solution and of a bleach solution was added to each well using a multi-channel pipette. Plates were left to incubate for approximately 60min and then read at 650nm.

^{15}N Pool Dilution for Gross N Mineralization- From all 28 samples, 4g was weighed out twice into separate specimen cups with lids and labeled as T4 or T24. Using ^{15}N labeled ammonium sulfate at 10 atom%, 500µL of 0.25 mM ^{15}N tracer solution was added to soil. Following the tracer addition, soils incubated for either 4 or 24 hours (T4 and T24) at which point the incubation was terminated by adding 30mL of 1M KCl. The soil solution then shook for 30 minutes before drip filtration through a Whatmann #40 filter. Clear extracts were frozen until the start of microdiffusion. Microdiffusion occurred by adding 100mg of Magnesium Oxide to clean specimen cups, then adding a 10mL aliquot of KCl extract to the cup with an acid trap, closing the lid and letting it shake for 48 hours. Acid traps were made by placing a hole punching Whatmann 40 filters and placing filter pieces on top of a 20-25cm piece white Teflon tape, spaced out 1-2cm apart. Next, 4µL of 2.5M $KHSO_4$ was placed on each filter piece and another piece of Teflon tape of similar length was laid on top. The top side of a pipette tip that was

slightly larger in circumference than the filter piece was used to press down and seal the two pieces of tape until it became translucent but did not break. Each trap was cut apart with a fresh razor blade. Following the 48-hour microdiffusion, acid traps were taken out and placed in 2mL reaction tubes and allowed to dry in a desiccation chamber under vacuum conditions with a beaker of concentrated sulfuric acid. Upon drying, acid traps were handled with clean tweezers to separate the Teflon tape and carefully remove the filter piece that was then placed in a foil tin and sent off to the Cornell Stable Isotope Lab (Ithaca, NY) for isotope ratio mass spectrometer (IRMS) analysis. Total %N and isotopic analysis of the filters were measured with a Thermo Delta V IRMS interfaced to a NC2500 elemental analyzer after the 4- and 24-hour incubation periods to calculate gross N mineralization rates.

$$M = \frac{([NH_4^+]_0 - [NH_4^+]_t)}{t} \cdot \frac{\log(APE_0/APE_t)}{\log([NH_4^+]_0/[NH_4^+]_t)}$$

M = Gross-mineralization in mg $kg^{-1}d^{-1}$

$[NH_4^+]_0$, $[NH_4^+]_t$ = Concentration of ammonium at time 0 (=4h) and t (=24h) in mg kg^{-1}

APE_0, APE_t = Atom percent-^{15}N of NH_4^+ - Pools at time 0 and t

Statistical analysis- Data analysis was performed in R studio (version 2022.02.0). One-way analysis of variance (ANOVA) was used to test the effect of N treatment on variables with treatments coded as factors. Assumptions of homoscedasticity and normality were assessed using a Levene's test and Shapiro wilks test. Log and reciprocal transformations were used as necessary to normalize data. A Tukey honest significant difference (HSD) test was done to determine if the differences between pairs of treatment means were statistically significant at a P-value of <0.05. Regression analyses were also performed on all variables to identify significant linear and/or quadratic trends along an increasing N treatment gradient.

Results

Soil properties tested on fresh soil including WHC, pH, total C and N, and C/N ratio are presented in table 6. WHC, expressed as g water g^{-1} dry soil at 100% WHC, ranged from 57.1-65.7%. pH values ranged from 4.77-6.33, with a significant ($P<0.001$) linear decrease as N fertilization increased. Total C values ranged between 8.07-9.26 mg C g^{-1} soil. There was significantly($P<0.05$) more C in treatments 4, 5, and 7 than in treatments 1 and 2 and a significant($P<0.01$) linear trend indicating that C increased with N fertilization. Total N values ranged between 0.99-1.06 mg N g^{-1} soil, and while no significant differences existed between specific treatments, total N had an expected positive correlation with total C. C:N ratios ranged from 8.37-8.77 and saw a marginally statistically significant($P<0.1$) increasing trend with fertilization rate.

Table 6. Physicochemical properties of fresh soils sampled at E502 in June 2021

Trt	N-P-K (kg ha^{-1})	GWC at 100% WHC (g water g^{-1} dry soil)	pH (1:1 soil:water)	Total C (mg C g^{-1} soil)	Total N (mg N/ g^{-1} soil)	C/N Ratio
2	0-20-56	61.2(0.02)^{ab}	6.33(0.22)^{a}	8.20(0.29)^{b}	0.98(0.07)	8.37(0.54)
3	22-20-56	63.3(0.02)^{ab}	5.88(0.22)^{ab}	8.78(0.28)^{ab}	1.04(0.06)	8.43(0.22)
4	45-20-56	57.1(0.02)^{b}	5.61(0.29)^{bc}	9.00(0.60)^{ab}	1.05(0.10)	8.61(0.31)
5	67-20-56	61.0(0.04)^{ab}	5.37(0.23)^{bcd}	8.97(0.32)^{ab}	1.05(0.05)	8.53(0.29)
6	90-20-56	65.7(0.02)^{a}	5.16(0.32)^{cd}	8.69(0.21)^{ab}	1.01(0.03)	8.61(0.14)
7	112-20-56	61.8(0.07)^{ab}	4.77(0.09)^{d}	9.26(0.35)^{a}	1.06(0.06)	8.77(0.17)
L		Ns	***	**	ns	.
Q		Ns	ns	ns	ns	ns

*Different letters indicate significantly different means between treatments according to HSD test at $P<0.05$. parentheses indicate standard error of averages. (n=4) Symbols indicate linear(L) or quadratic(Q) trends at specified significance levels (***=0.001,**=0.01,*=0.05,'.'=0.1)

Microbial Biomass C and N values are expressed as mg of chloroform extractable CN g soil^{-1} and values are displayed in table#. Biomass C values ranged from 0.14-0.20 mg C g^{-1} soil and N ranged from 0.005-0.013 mg N g^{-1} soil(fig. 4). No significant differences were identified among treatment groups, but regression analysis revealed statistically significant($P<0.05$) quadratic trends for both biomass C and N, where values initially decrease with N rate, but then start increasing at higher fertilization levels.

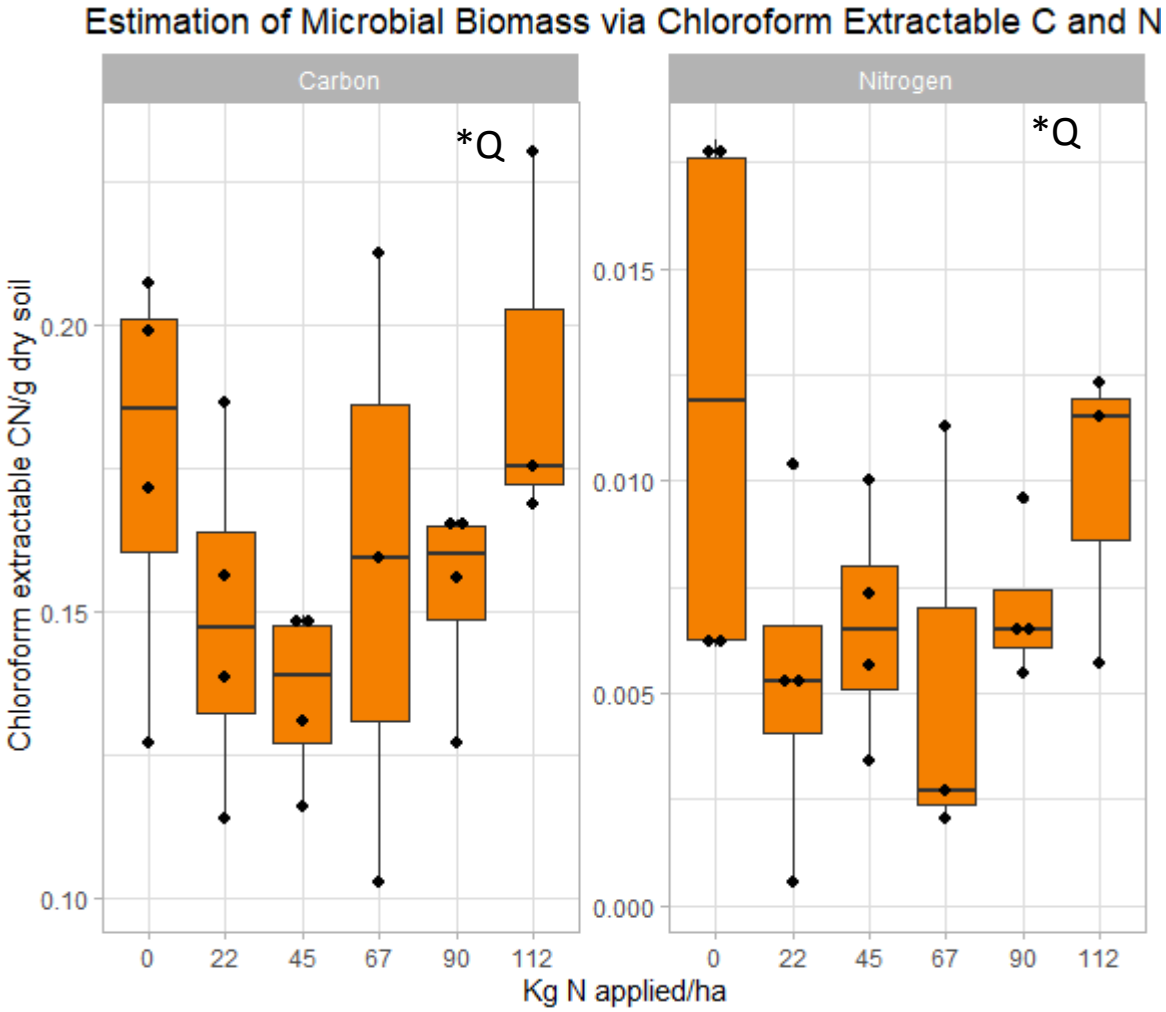

Fig 4. Microbial biomass C and N at six different N rates. Dots represent individual points. Symbols indicate linear(L) or quadratic(Q) trends at specified significance levels (***=0.001,**=0.01,*=0.05,'.'=0.1)

In the soil incubation study for CO_2 respiration, we found no significant differences between any of the seven treatments in cumulative respiration over the 11-day period. Treatments cumulatively respired between 24,321-30,663 µg CO_2-C g^{-1} soil over the 11-day period. In the separate experiment where three treatments received a glucose addition, cumulative respiration ranged from 79,934-107,285 µg CO2-C g^{-1} soil and was significantly higher in treatment 4 than in both treatments 1 and 7($P<0.05$)(fig.5). Treatment 4 respired 26% and 34% more CO_2 than treatments 1 and 7, respectively.

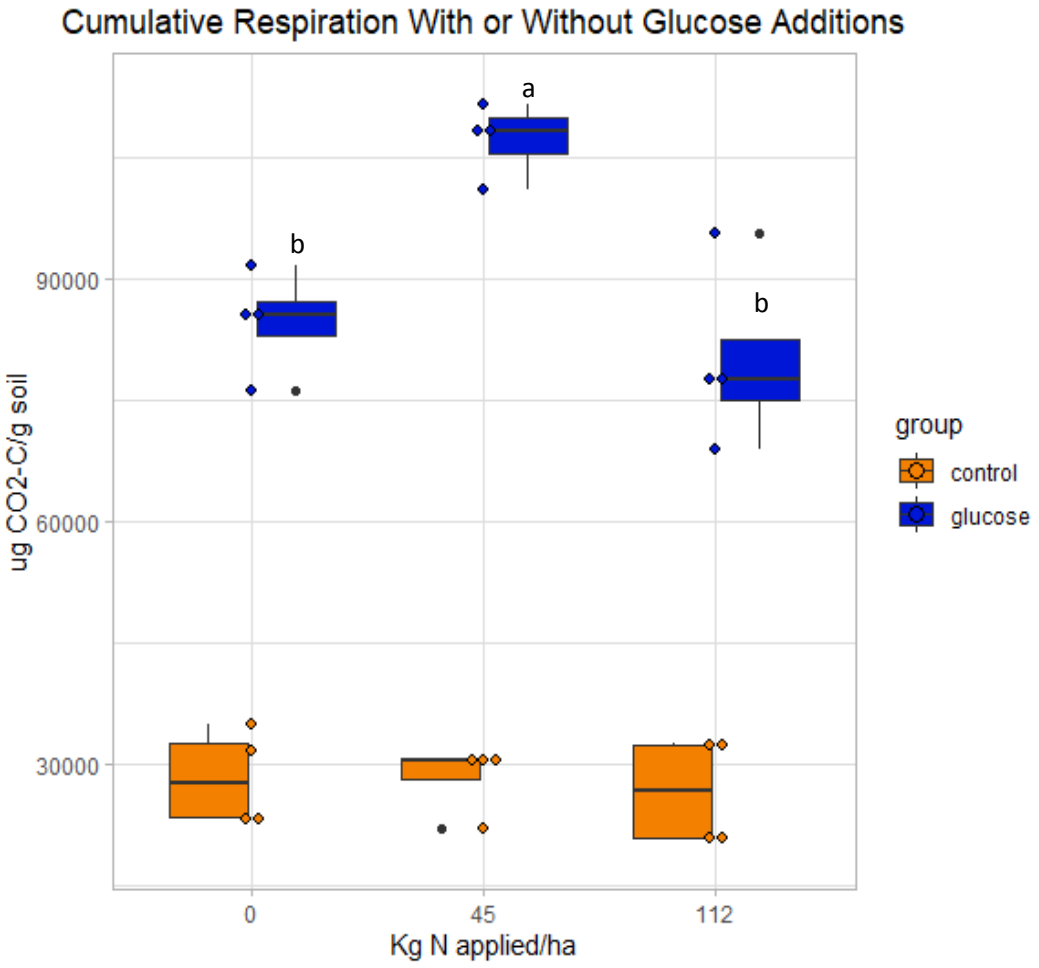

Fig 5. Cumulative CO_2 respiration over an 11-day incubation from soil groups that did or did not receive a glucose addition prior to measurements. Selected soils are from field treatments receiving three different N rates. Dots represent individual points. Letters indicate significantly different means within each group according to a Tukey's HSD test at $P<0.05$)

PLFA microbial group indicators for actinomycetes and arbuscular mycorrhizal fungi (AM fungi) showed significant($P<0.05$) linear decreasing trends in abundance as N rate increased(Table 7). Additionally, AM fungi showed significant($P<0.05$) differences between groups(fig. 6). Indicators for gram positive bacteria significantly increased with N rate, and while not deemed significant, there was a clear inverse effect on gram negative bacteria, decreasing with N rate. Regression analysis confirmed significant ($P<0.001$) linear trends for both groups(table 8). Reported PLFA ratios for fungi/bacteria(F/B) and gram negative/gram positive(G+/G-), showed both significant differences between groups($P<0.05$) and linear decreasing trends($P<0.01$)(fig.7).

Table 7. Relative abundances of PLFA microbial groups(%) and ratios

Trt	Actinomycetes	AM Fungi	Fungi/Bacteria Ratio	Gram Positive Bacteria	Gram Negative Bacteria	G+/G- ratio
2	19.37(1.51)	3.49(0.62)^{ab}	0.070(0.017)^{ab}	46.24(2.88)	30.41(1.54)^{c}	1.86(0.06)^{a}
3	17.8(1.03)	3.92(0.18)^{a}	0.076(0.008)^{a}	46.15(0.96)	32.48(1.26)^{bc}	1.67(0.10)^{ab}
4	17.71(1.82)	3.39(0.13)^{abc}	0.062(0.004)^{ab}	42.93(0.28)	34.02(1.07)^{ab}	1.58(0.13)^{bc}
5	17.76(0.92)	3.09(0.26)^{bc}	0.061(0.005)^{ab}	42.19(0.61)	34.81(0.95)^{ab}	1.52(0.07)^{bc}
6	17.6(0.89)	2.74(0.48)^{bc}	0.056(0.007)^{b}	42.69(0.69)	35.22(0.89)^{a}	1.53(0.04)^{bc}
7	16.8(0.46)	2.66(0.21)^{c}	0.056(0.002)^{b}	42.09(0.82)	35.58(1.18)^{a}	1.44(0.08)^{c}
L	*	***	**	***	***	***
Q	Ns	ns	ns	*	*	.

*Different letters indicate significantly different means between treatments according to HSD test at $P<0.05$. parentheses indicate standard error of averages. (n=4) Symbols indicate linear(L) or quadratic(Q) trends at specified significance levels (***=0.001,**=0.01,*=0.05,'.'=0.1)

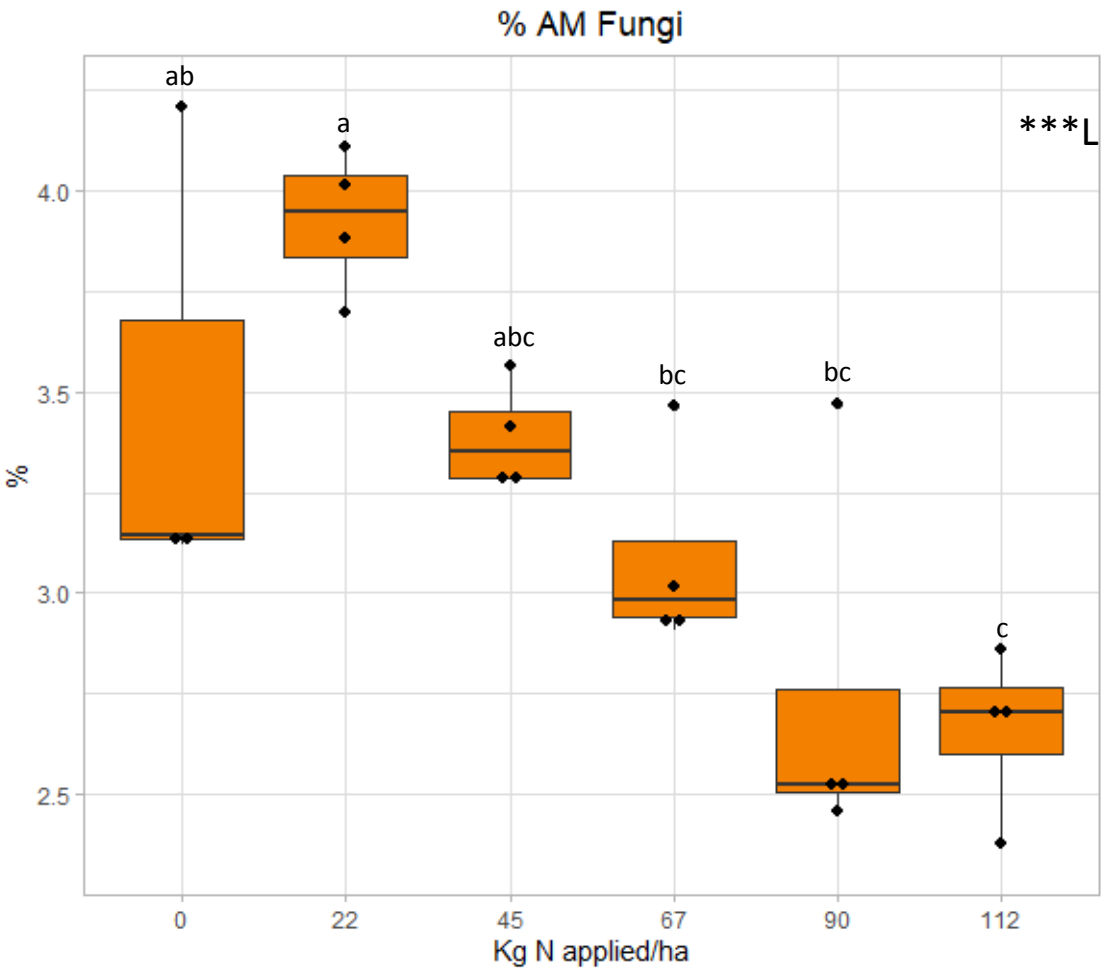

Fig 6. PLFA relative abundance of AM fungi at six different N rates. Dots represent individual points. Letters indicate significantly different means according to a Tukey's HSD test at P<0.05). Symbols indicate linear(L) or quadratic(Q) trends at specified significance levels (***=0.001,**=0.01,*=0.05,'.'=0.1)

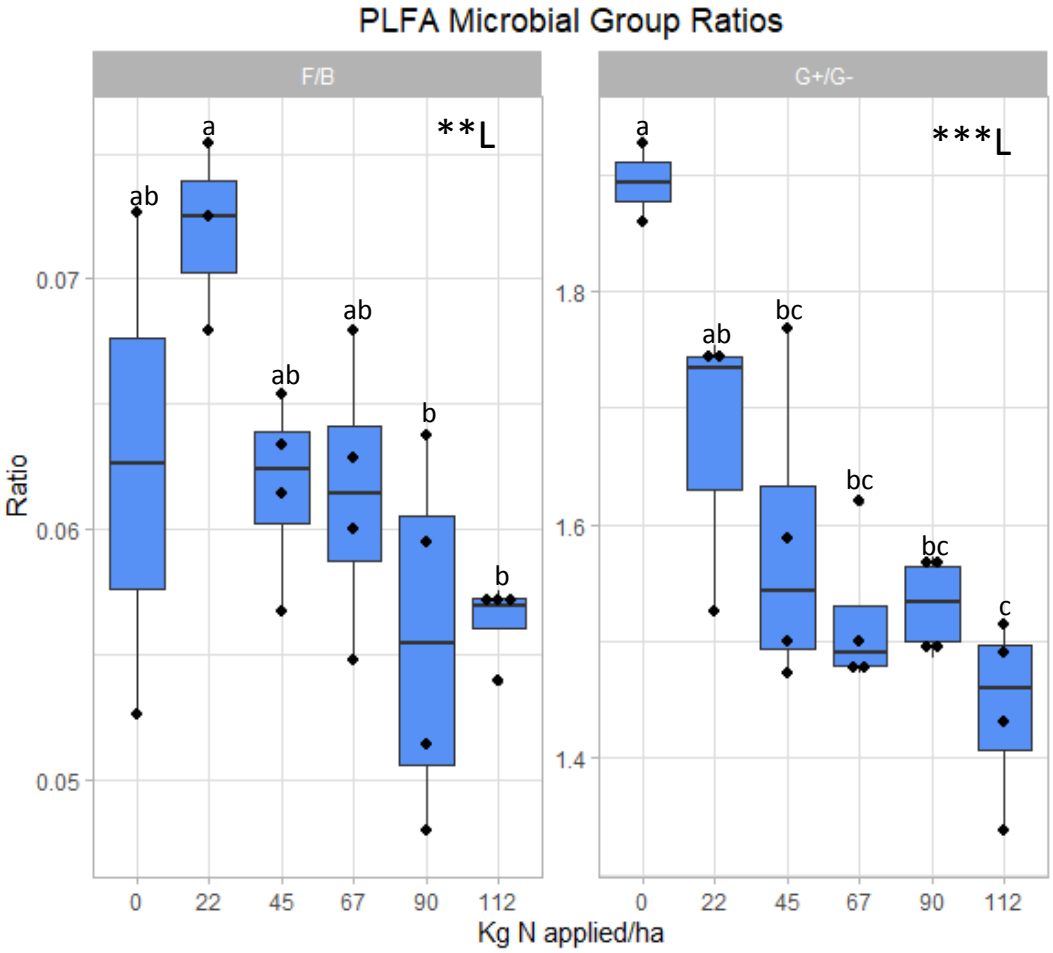

Fig 7. Selected PLFA microbial ratios at six different N rates. Dots represent individual points. Letters indicate significantly different means according to a Tukey's HSD test at P<0.05). Symbols indicate linear(L) or quadratic(Q) trends at specified significance levels (***=0.001,**=0.01,*=0.05,'.'=0.1)

Gross N mineralization ranged between 2.08-2.88 mg NH_4^+-N kg^{-1} soil day^{-1}, but no significant differences were observed between any of the treatments nor any significant trends(Table 8). Treatments with 67 and 90 kg N ha^{-1}, experienced numerical decreases of 19% and 20% relative to the next lowest rate of 2.72 mg NH_4^+-N kg^{-1} soil day^{-1}.

Table 8. Gross N mineralization rates(mg NH_4^+-N kg^{-1} soil day^{-1})

Trt	N-P-K (kg ha^{-1})	gross N mineralization
2	0-20-56	2.82(0.48)
3	22-20-56	2.72(0.34)
4	45-20-56	2.88(0.37)
5	67-20-56	2.18(0.38)
6	90-20-56	2.21(0.41)
7	112-20-56	2.82(0.57)

*Parentheses indicate standard error of averages (n=4)

Discussion

The primary purpose of this study was to identify relationships between N fertilization rate and biological parameters that are potentially related to N mineralization, such as soil microbial composition and functioning.

Increases in total N with increasing N rate is consistent with previous studies at the 502 experiment (Aula et al., 2016; Raun et al., 1998). While this finding reveals an important relationship between soil total N accumulation and N fertilizer, it does not reveal underlying mechanisms of increased total N, nor reveal implications for N mineralization potential. The mentioned studies also reported similar results and trends for pH at E502.

Despite not being able to obtain statistically significant differences between groups, the significant quadratic trends for chloroform extractable biomass C and N are worth noting where treatments that received no N and treatment 7 with 112 kg N ha^{-1} produced higher values for extractable chloroform C and N than the other treatments. This trend could be due to plant

competition for N at low N rates. As N rates increase, more N is available for increasing microbial biomass. The C:N ratio of MAOM exhibits the exact opposite quadratic trend where low rates increase the C:N ratio at first, but then begins to decrease again. It's possible that these trends are connected due to the fact microbial biomass can be a direct addition to MAOM fractions. Effects of N fertilization on microbial biomass vary across literature, with one paper finding that a N threshold of 50 kg N ha^{-1} will begin to suppress microbial biomass C and N(Song et al., 2018), while another found almost linear increases up until 250 kg N ha^{-1}(Babur et al., 2021). A seasonal effect on microbial abundance and other measured parameters should not be ruled out, but requires further research specific to winter wheat systems in Oklahoma(Huang et al., 2018).

Even though differences in cumulative CO_2 respiration were not deemed statistically significant between treatments over and 11-day period, it was a critical baseline measurement of microbial activity. The significant increase in respiration between treatment 4 and treatments 1 and 7 could indicate a higher capacity to respond to C inputs and thereby a higher capacity to mineralize and release N. A relatively moderate N rate where crop demand is not fully satisfied, could be stimulating microbes to mineralize more N. Measuring inorganic N pools before and after glucose additions would help better evaluate what is occurring between these treatments. The first 48-72 hours showed the most pronounced differences between treatments in the glucose added jars. This is no surprise with a simple C compound like glucose that can be quickly consumed and processed by microbes, but also points to the short time scales that priming effects occur on in soil and how quickly it may cause fluxes in soil N pools.

PLFA analysis revealed significant differences in relative abundances of certain microbial groups and ratios of groups. The decrease in AM fungi in response to N fertilization is consistent with other studies citing decreases in AM fungal growth and overall PLFA abundance of AM fungi(Babalola et al., 2022; van Diepen et al., 2010). This finding, while important, does not

come as a surprise as there is evidence of AM fungi playing substantial roles in N acquisition from organic sources in soil to meet their own demand and demand of plants they share symbiotic relationships with(Hodge and Fitter, 2010; Saia et al., 2020). If no there is not a demand for microbes to access nutrients in SOM pools, then it would help explain a buildup of C and N in the soil. Actinomycetes are not as heavily studied as AM fungi in regard to nutrient cycling, but are known for their versatile enzyme production capacities and degrade complex organic compounds in soil(Javed et al., 2021). The decreasing trend in actinomycetes with fertilization rate could also help explain suppressed decomposition of SOM pools. The relative amounts of gram-positive and gram-negative bacteria has limited interpretations in literature to this point, and the two classification groups historically have not been considered as indicators of soil function. Despite this, the decreasing trend in gram positive bacteria could be important in light of more recent research suggesting that gram-positive bacteria strongly influence decomposition of organic N compounds in soil(Enggrob et al., 2020). It has been suggested that gram-negative bacteria are dependent on availability of simple C compounds derived from plant biomass(Fanin et al., 2019). This would hold true with the results of this research as gram-negative bacteria significantly increase in relative abundance with N fertilization and total C and N availability.

The effect of N fertilizer of gross N mineralization was minimal at this time of soil sampling after harvest of winter wheat. Its highly believable that results taken much sooner to the direct application of N fertilizer or during certain growth stages would produce much different results, as Mahal et al. (2019) observed. In that study, the negative correlation between NH_4^+ pools and gross N mineralization is indicative of a direct effect of fertilizer on many microbial processes. While urea itself doesn't contain NH_4^+, it quickly transforms and adds to the inorganic N pool. The CO_2 and glucose addition study is another example of how sensitive a soil can be to nutrient additions. Just as cumulative respiration showed no differences, glucose additions show very different responses between treatments that could otherwise not be seen.

Many of the measured soil biological parameters measured in this experiment are subject to change with time, environmental conditions, and management. The effect of time in relation to nutrient additions and wheat growth should be considered in future studies to best understand effects of gross N mineralization when it plays a significant role in crop growth. Additionally, the ^{15}N pool dilution procedure itself is rather technical and small errors like incomplete homogenization of ^{15}N in soil and incomplete diffusion of NH_4^+ can lead to inconsistent results.

CHAPTER IV

Conclusion

The results of this study demonstrate that while long-term N fertilization may increase total soil N, and especially allocation of N within readily accessible POM, N fertilization also significantly disrupts soil microbial communities and functions. A decreased abundance and capacity of microbial groups involved in N mineralization and other nutrient cycling processes informs us that the soil is more dependent on external N sources. It remains inconclusive as to the direct impact of fertilization on gross N mineralization. It seems unlikely that any decreases in gross N mineralization that could be seen would be due to insufficient N to be mineralized, rather a decreased microbial capacity to respond to rhizodeposits and seek N to be mineralized. Such increases of N within both SOM fractions show that N fertilizer could both directly and indirectly contribute to the increases through crop and microbial consumption. It would not be appropriate to disrupt the management of these long-term sites, therefore future studies should use lab-based stable isotope tracing techniques to directly evaluate the response of these soils to differing C and N inputs to simulate management changes in the field and response to rhizodeposits. Continued efforts should be put towards evaluating relationships between fertilization and N cycling processes. The research produced at long-term agronomic sites will benefit from further collaborations between agronomists and soil scientists, both seeking to solve similar problems, but at much different scales of research. Data similar to that compiled in this study, correlated to agronomic data collected at long-term sites could reveal more predictor variables that can be used to accurately estimate yearly crop N supply from mineralization. Raun et al. (2019) describes

the increasing entropy of biological systems with time, exemplified by yearly variations in crop yield levels and N mineralization supply, regardless of management. Promoting self-sustaining soil systems requires a continued search to identify trends in data that span beyond a single year. As with most management practices in agriculture, N fertilization comes with clear costs and benefits, but overall, it has continued potential to contribute to long-term sustainability when managed responsibly.

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