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Prescribed defoliation strategies influence soil carbon dynamics and nitrous oxide emission
potential in West Virginia pastures

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Thesis submitted to the Davis College of Agriculture, Natural Resources and Design at West
Virginia University in partial fulfillment of the requirements for the degree of

Master of Science

In

Applied and Environmental Microbiology

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Abstract

Prescribed defoliation strategies influence soil carbon dynamics and nitrous oxide emission potential in West Virginia pastures

Jordan M.A. Koos

Growing interest in sustainable agriculture has driven inquiry into the impacts of grazing on cool-season perennial grasslands, which are commonly utilized as forage for ruminant livestock in West Virginia and throughout the Central Appalachian region. However, current understanding of the below-ground impacts of forage defoliation (*i.e.*, one selective pressure applied by grazing livestock) management strategies is nascent. The aim of this thesis is to investigate how defoliation management strategies affect above-ground (e.g., forage production) and below-ground processes (e.g., soil organic matter (SOM) accumulation, microbial carbon, nitrogen, and phosphorus (P) cycling capacity, and nitrous oxide (N₂O) emission potential). I hypothesized that the rotational (*i.e.*, moderate frequency) defoliation at low severity will increase SOM storage and the microbial capacity to cycle C, N, and P. However, I also hypothesize that the greatest N₂O emission capacity will also come from plots defoliated at a moderate frequency and low severity, due to greater below-ground C availability that will stimulate microbial activity. To address these hypotheses, data were collected from two West Virginia University Research Farms, one organically managed and one conventionally managed, each with experimental plots where forage defoliation treatments of increasing intensity and frequency have been implemented since May 2016. At the completion of two growing seasons, above- and below-ground parameters of interest were recorded, including leaf area index, forage utilization, stable and recalcitrant C pools, extracellular enzyme activities associated with microbial C, N, and P acquisition, and N₂O emission potential via quantification of microbial genes by qPCR. At the Organic farm, greater defoliation severity decreased labile carbon content in the soil (-21%). Further, at the Organic Farm, defoliation treatments at rotational frequency and low severity resulted in a 39% reduction in β -glucosidase activity, however β -glucosidase activity was not impacted by defoliation frequency or severity at the Reedsville Farm. In addition, N₂O emission potential was differentially affected by defoliation treatments at the two sites. At the Organic Farm, defoliation severity impacted N₂O emission potential, whereby low defoliation severity resulted in 82% decrease in potential N₂O emissions compared to high defoliation severity. At the Reedsville Farm, defoliation frequency impacted N₂O emission potential, whereby continuously defoliated plots experienced a 71% decrease in potential N₂O emissions compared to less frequent defoliation treatments. Taken together, these results suggest that defoliation management is capable of increasing soil C storage at the cost of increased nitrous oxide emission potential. However, management prescriptions must consider site specific nutrient dynamics to most accurately predict defoliation management impacts on soil C.

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Chapter 1: Defoliation management alters soil microbial enzyme activities in West Virginia pastures.

Abstract

Growing interest in sustainable agriculture has driven research into the impacts of grazing on cool-season perennial grasslands, which are commonly utilized as forage for ruminant livestock in West Virginia and throughout Central Appalachia. Soil carbon (C) dynamics and microbial activity are commonly utilized indicators of soil health, but how defoliation management may affect these parameters in Appalachia is still unknown. The aim of this study is to investigate how defoliation management strategies affect above-ground parameters (e.g., forage utilization and leaf-area index) as well as below-ground parameters such as soil C pools and soil microbial enzyme activities associated with C, nitrogen (N), and phosphorus (P) acquisition. Examination of defoliation impacts on soil C pools and microbial enzyme activities is important for predicting the environmental sustainability of defoliation management in these systems. I hypothesize that moderate (i.e., monthly) defoliation events at low severity will produce greater herbage utilization and light-capture efficiency, which will result in increased accumulation of labile C below-ground. I further hypothesize that such increases in soil C storage will reduce microbial nutrient demands for C and increase enzyme production associated with N and P acquisition. To address these hypotheses, I implemented experimental defoliation treatments at two West Virginia University Research Farms of increasing intensity and frequency, beginning in July 2016. After two full growing seasons, I collected data for several below- and above-ground parameters of interest such as, leaf area index, utilization, labile and recalcitrant soil C pools, as well as microbial extracellular enzyme activities associated with C, N, and P acquisition. Defoliation management resulted in decreased utilization and light capture in continuously defoliated treatments. In addition, enzyme activities related to microbial N and P acquisition were unaffected by the defoliation treatments, however β -glucosidase activity was affected by defoliation management. Variation in microbial activities were largely dependent on soil moisture and parameters associated with C. These data will develop our understanding of the below-ground impacts of grazing and inform more effective management plans for livestock grazing of perennial pastures in West Virginia and the Central Appalachian region.

Introduction

Sustainable management of agricultural lands is essential for the maintenance of soil quality and provides environmental and economic benefits for both society and growers. The accumulation of carbon (C) as soil organic matter (SOM) is a commonly used indicator of soil quality and it is also important for mitigating elevated atmospheric CO₂ and anthropogenic climate change (1, 2). Greater SOM increases yields, cation exchange capacity, water availability, water holding capacity and process rates of critical ecosystem functions (i.e., soil fertility (3–5). Through mineralization by decomposition, the organic C fraction of soil is the main source of C available to soil organisms (6), and the mean residence time (MRT) of soil organic C (SOC) is determined by physical, chemical, microbial, and ecological dynamics (7, 8). In Appalachia, a dominant land use type is pasture for livestock grazing, and in West Virginia, pasture represents the greatest single agricultural land use type in the state (11% of land in WV is currently in pasture; (9)), thus, proper management of pasture lands can have impacts on the cycling and storage of SOC, and ultimately, soil fertility across this region. The top-down ecological, physical, and chemical effects of livestock grazing on above-ground processes have been studied extensively (10–15); however, the current understanding of grazing impacts on belowground dynamics is nascent (16–23). By isolating pasture defoliation, which is a selective pressure applied by grazing livestock, this study aims to increase understanding of defoliation impacts on soil C dynamics and the soil microbial community's ability to cycle critical soil nutrients (e.g., C, nitrogen (N), and phosphorous (P)). Findings from this project will help guide agricultural land management strategies to increase stable soil organic C, which will increase soil

fertility and environmental sustainability of this environmentally and economically important land-use type.

Soil quality in pasture lands can be impacted by defoliation frequency and severity through defoliation-induced shifts in plant species composition, which can alter litterfall quality and quantity, plant rooting structures, and soil microenvironments (13, 14). For example, increasing defoliation frequency shifted the composition of grasses to shorter more defoliation-tolerant grasses, which have apical meristems closer to the ground that provides an ability to regrow – thus providing an advantage under severe defoliation (24). Further, increases in defoliation frequency and severity can shift pastures from C_3 grasses (e.g., *Stipa*) to C_4 grasses (e.g., *Bouteloua*), which are less suitable feedstock and whose biochemical composition may decrease decomposition rates compared to C_3 grasses (25, 26). Further, in a Chinese steppe under increased grazing frequency and severity, the dominant plant type in the community changed (e.g., from grasses to sedges or forbs) which can lead to a more pronounced change in feedstock suitability (27). Thus, grazing impacts on plant community composition and diversity can directly impact the decomposability of plant materials and SOC dynamics (28, 29). The direction and magnitude of changes to SOC pools and nutrient turnover rates may depend on the original and new pasture grass types, as well as the frequency and/or severity of defoliation.

Defoliation-induced changes in plant community composition can occur simultaneously with changes in plant physiology, which is one potential factor linking above- and belowground dynamics that mediate soil C storage. A plausible mechanism driving changes in plant physiology under increased defoliation frequency and severity is the differential physiological responses of forage species (14, 30). For example, compensatory regrowth (i.e., the accelerated growth of plant biomass following defoliation events) is an important process regulating the

productivity and biochemistry of foraged tissues (12, 31). Following defoliation, new leaf tissue may have lower C:N (32) and increased decomposability as compared to older leaf tissue (30, 33, 34). Belowground, plant residues, root turnover, and root exudates are all significant C inputs to SOC, which promote microbial growth, necromass, and metabolism (8, 35). Impacts of defoliation on root turnover are highly variable, as roots have been shown to increase or decrease in mass, as well as stop growth entirely under various defoliation management strategies (22, 23, 27, 36). Most importantly, plants roots are responsible for exuding low molecular weight (LMW) compounds, which provide the microbial community with labile sources of C that can increase microbial activity and growth (37, 38). Foliar defoliation has been shown to increase plant root exudation, subsequently increasing microbial abundance, and ultimately microbial necromass, which supports the accumulation of stable SOC (39). Thus, the timing and severity of pasture defoliation can have cascading impacts on rooting dynamics and soil microbial substrate availability, which can then feedback to affect long-term soil C storage.

Decomposition, which is a microbially-mediated process, is critical to the cycling and storage of nutrients in soil (40–43). For example, decomposition products make up the majority of stable SOM (6, 43–45). Microbes mediate decomposition through a suite of extracellular enzymes, which can serve as biological indicators of soil health and microbial nutrient demands (41, 46). These enzymes contribute to the breakdown of polyphenolic organic compounds such as lignin, cellulose, and hemicellulose, as well as to the acquisition of N and P from SOM (1, 2, 47). The activity of microbial exoenzymes can be affected by changes in environmental conditions. For example, in pasture lands, microbial enzyme activities associated with decomposition increased after introducing sheep, and further, rates of decomposition were greater under light defoliation compared to heavy defoliation by sheep (48). These results

demonstrate the need to identify and develop best management practices (BMPs) for pasture grazing that promote healthy soil microbial communities and optimal rates of decomposition, potentially increasing the C storage capacity of pasture soils.

Here, I investigate whether a top-down ecological cascade commencing with the defoliation of forage plants alters the availability of labile and recalcitrant C pools for microbial use, and further, whether differences in defoliation management strategy (i.e. frequency and severity) alter microbial enzyme activities and nutrient foraging (8, 49). My objective is to inform the development of best management practices for pasture managers by determining a treatment that increases SOC while maximizing forage production. I hypothesize that moderate (i.e., monthly) defoliation events at a low severity (i.e., 13 cm height) will produce greater herbage mass and productivity than continuous or hay defoliation frequencies resulting in increased below-ground C storage. I further hypothesize that the increases in soil C storage will reduce microbial extracellular enzyme production associated with C acquisition and increase enzyme production associated with N and P acquisition. To test these hypotheses, six defoliation treatments were implemented at an organically managed farm and a conventionally managed farm in West Virginia to examine how differences in defoliation frequency and severity affect SOC formation and microbial C, N, and P cycling potential.

Materials and Methods

Site description

This experiment was conducted at two West Virginia University (WVU) research farms: The Organic Agriculture Farm in Morgantown, WV (39°5N, -79°94W; hereafter “OF”) and the JW Ruby Research Farm in Reedsville, WV (39°1N, -79°82W; hereafter “RV”; Table 1). The OF was organically certified in 1998, and the RV site is conventionally managed. Soils at OF

and RV consist of Dormont silt loam (Fine-loamy, mixed, superactive, mesic Oxyaquic Hapludalfs) and Latham silt loam (Fine, mixed, semiactive, mesic Aquic Hapludults), respectively. Mean annual temperature (MAT) and mean annual precipitation (MAP) are 20.3°C and 125.5 cm, respectively, at OF and 12°C and 153 cm at RV. Experimental plots were established at each site in June 2016, plots measured 7m × 3m at OF and 8m × 3m at RV. On July 1, 2016, six experimental defoliation treatments consisting of three simulated frequency regimes at two levels of severity were implemented at each site (Table 2) in a randomized block design (3 frequency levels × 2 severity levels × 3 blocks × 2 sites = 36 total plots). The simulated grazing by defoliation frequency and severity treatments were selected to align with common pasture management strategies in the Central Appalachian region, representing continuous, rotational, and hay-stage pasture management as suggested by the United States Department of Agriculture Natural Resources Conservation Service (50). The continuous frequency treatment was defoliated every 7-10 days, rotational frequency plots were defoliated every 28-35 days, and hay-stage plots were defoliated every 42-45 days. Each simulated frequency treatment was also defoliated to two heights to represent low and high severity defoliation, respectively; the defoliation height was 6-8 cm for the high severity treatment and 13 cm for the low severity treatment. Simulated defoliation was achieved by clipping vegetation using a Monarch Sickle-Bar Mower outfit with a 36-inch serrated sickle bar (Jari USA, St. Peter, Minnesota).

Table 1: Summary characteristics of each experimental site in West Virginia.

	Organic Farm	Reedsville
Coordinates	39°5N, -79°94W	39°1N, -79°82W
Soil Series	Dormont Silt Loam	Latham Silt Loam
Management	Organic	Conventional
MAP (51)	125.5 cm	153.0 cm
MAT(51)	20.3°C	12°C
Relative N/P/K	High/Low/High	High/High/Low

Table 2: The six experimental treatments included in this experiment identified by defoliation frequency and severity (clipped stubble height).

	A	B	C	D	E	F
Frequency	Rotational	Rotational	Continuous	Continuous	Hay	Hay
Severity	6 cm	13 cm	8 cm	13 cm	6 cm	13 cm

Assessments of forage photosynthetic capacity and predicted utilization by grazing livestock

To determine differences in light capture due to defoliation treatment, an ACCUPAR LP-80 Ceptometer (Decagon Devices Inc. Pullman, WA) equipped with an external photosynthetically active radiation (PAR) sensor was used to measure light interception and estimate leaf area index (LAI), which is a dimensionless measure of plant canopy area per unit

ground area. LAI was collected from three different locations within a plot, with three replicate subsamples per location (n=9). When possible, LAI measurements were taken within one hour of solar noon.

I calculated “utilization” as the difference in pre-treatment and post-treatment dry mass of forage. Before and after each clipping event, the masses of forage was recorded, and the difference between before-and-after clip height represents the mass of forage available for grazing ruminants (hereafter referred to as “utilization”). To estimate forage utilization, a meter stick was used to measure the canopy height at ten random locations within a plot before and after clipping. The difference between the before and after canopy heights was then multiplied by 132 to determine estimated herbage utilization ($\text{kg DM}\cdot\text{ha}^{-1}$; *sensu* (52)).

Soil Sampling

Soil samples were collected following two seasons of defoliation treatments on November 7, 2017. Plots were randomly sampled 10 times using a soil probe (1.7 cm diameter) to a depth of 7.5 cm. The cores were then thoroughly homogenized by hand to produce one composite sample per plot. The homogenized soil samples were stored on ice in the field and returned to the lab within two hours of sampling. Upon return to the lab, each soil sample was sieved (2,000 μm) to remove coarse roots and rocks. Homogenized sieved soil was stored at 4°C for enzyme and physicochemical analysis.

Soil Physiochemical Characteristics

To determine the impact of defoliation on soil properties, soil physiochemical characteristics were assayed that have been shown to impact plant and microbial function. These are pH, soil moisture, total C, total N, soil organic matter content, and permanganate oxidizable C content (i.e., labile C; (2, 32, 42, 53)). Soil pH was measured using an Accumet AE 150 probe (Fisher Scientific, Hampton, NH) in a 1:5 soil to 0.01M CaCl₂ slurry (54). The moisture content of soil samples was determined gravimetrically, whereby 10 g of field-moist soil was dried at 105°C for 24 hours. Following drying, samples were re-weighted to determine dry mass and then combusted at 505 °C for 6 hours to determine percent soil organic matter (SOM) content by loss on ignition (LOI). The amount of labile C was estimated by the permanganate oxidizable C method (55), whereby four analytical replicates of each sample were assayed using clear 96-well plates (Corning, Corning, NY) on a Synergy HTX plate reader (Biotek, Winooski, VT). Absorbance was recorded at 550 nm. The total C and N content of the soil was determined by combustion analysis in a Carlo Erba 1500 NCS (Carlo Erba, St. Joseph, MI) using 12 mg of dry ground soil.

Extracellular Enzyme Activity

To determine the impact of prescribed defoliation (the management of harvest) on the microbial capacity to mineralize C, N, and P from SOM, I assessed the activity of microbially-derived extracellular enzymes that are associated with the cycling and storage of C, N, and P in soil, namely, β -glucosidase (BG), β -N-acetylglucosaminidase (NAG), and acid phosphatase (AP, Table 3). Acid phosphatase is an enzyme that hydrolyzes nucleic acids and phospholipids making them biologically available (56). NAG binds to chitin and breaks down chitin into N-

acetylglucosamine increasing the availability of organic N, which is important for microbial N acquisition (2, 56). Enzyme assays were completed within 48 hours of sampling with previously established methods (57) on a Synergy HTX plate reader (Biotek, Winooski, VT). Enzyme activity assays were performed on opaque plates (Corning, Corning, NY) and read using 365 nm excitation and 450 nm emission. Eight analytical replicates were used per soil sample for all assays.

Table 3: Soil microbial enzymes assayed for potential activity. (EC, Enzyme commission classification; MUB, methylumbelliferyl)

Enzyme	Common Abbreviation	EC	Experimental Substrate	Environmental Substrate
β -glucosidase	BG	EC 3.2.1.21	4-MUB- β -D-glucoside	Cellulose
β -N-acetylglucosaminidase	NAG	EC 3.2.1.14	4-MUB-N-acetyl- β -D-glucosaminide	Chitin
Acid Phosphatase	AP	EC 3.1.3.1	4-MUB-phosphate	Phosphomonoesters/ phosphodiester

Effects of Defoliation on Microbial Activity

To determine whether defoliation treatments altered the enzymatic capacity of the microbial community to acquire C, N, and P, we considered “microbial activity” as the normalized profile of C-, N-, and P-acquiring enzymes (58). Here, enzyme activities were combined in to a single matrix and normalized using the “standardise” function for C-, N- and P-acquiring enzyme activities to contribute equal weight in down-stream analyses using Primer (version 6, Primer-E Ltd., Plymouth, UK).

Statistical Analysis

Univariate statistical analysis was conducted using the general linear model function in SAS JMP (Version 13, SAS Institute Inc., Cary, NC). A two-way analysis of variance (ANOVA) was performed on each parameter and tested for two main effects (frequency and severity) and their interaction (frequency $\times$ severity). For all statistical tests, results were considered significant at $\alpha < 0.05$ and marginally significant at $\alpha < 0.10$. If a significant interaction was detected a Student's T-test was used to determine differences among means.

To test the hypothesis that defoliation treatments impact microbial activity (as defined above), a Bray-Curtis dissimilarity matrix was used in a two-way permutational multivariate analysis of variance (PerMANOVA; 58) with defoliation frequency and severity as factors for both study sites. Further, the impact of soil parameters (i.e., soil pH, moisture, C:N, SOM, OXC) on microbial activity was assessed by Distance-based Linear Models (DistLM), wherein the adjusted r^2 criterion was used in the “best” model selection (60). Prior to running the *best* model selection procedure, a marginal DistLM was performed to determine which environmental parameters, when considered individually, accounted for a significant proportion of the variance in microbial activity. Only significant parameters were included in *best* model selection. To visualize the impacts of defoliation treatments on microbial activity, NMDS ordinations were generated using the vegan package (61) in R (62). Environmental vectors were included as vector overlays of significant environmental parameters from marginal DistLM tests when a defoliation effect was detected.

Results

Differences between sites following defoliation implementation

The OF and RV farms were chosen as sites for this experiment due to their unique soil physico-chemical properties and management practices (i.e., organic vs. conventional). For example, average oxidizable C, soil organic matter content, soil C:N, acid phosphatase activity, NAG activity, pH, and % soil moisture were all significantly different between the two sites (Table 4). Soils at RV exhibited greater C:N, soil organic matter and oxidizable C content, as well as reduced activities of microbial enzyme as compared to soils at OF.

Table 4: Soil and microbial enzyme parameters at each site. (a) and (b) represent differences between the two sites at $p < 0.05$.

Parameter	OF	RV	SE
Soil moisture (%)	29.3	29.0	0.4
pH	6.0(a)	6.4(b)	0.06
Total C	2.5	3.3	.09
Total N	0.27	0.34	.007
Soil C:N	8.83(a)	9.9(b)	0.2
SOM (%)	8.00(a)	9.8(b)	0.1
Oxidizable C ($\text{mg} \cdot \text{kg}^{-1}$)	782.9(a)	1175.5(b)	48.5
β -glucosidase ($\mu\text{M} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$)	23.5(a)	16.7(b)	1.2
β -N-acetylglucosaminidase	17.2(a)	7.3(b)	1.3
Acid phosphatase	23.5(a)	49.7(b)	2.8

Herbage production and light capture

To determine the effect of defoliation on canopy development and light capture, LAI was measured prior to each defoliation event. Effects of defoliation frequency and severity on LAI

were significant at both OF and RV, however each site responded differently. At OF LAI was highest in the plots that were defoliated at a lower frequency (Figure 1, ANOVA frequency effect; $p = 0.06$). At RV, there was a moderately significant frequency $\times$ severity interaction (Figure 2, $p = 0.09$). Post-hoc tests indicate that at RV, LAI was greater under less severe defoliation (29% greater than high severity). In addition, LAI was greatest in the hay stocking frequency and lowest in the continuous stocking treatment.

Figure 1: Leaf area index at Organic Farm through the second season of pasture defoliation treatments. LAI was moderately significantly impacted by grazing frequency ($p = 0.06$). Bars represent means $\pm$ 1SE ($n = 6$). Letters indicate significant differences between treatment means. The severity effect was not significant, thus only frequency treatments were included in the figure.

Figure 2: Leaf area index at the Reedsville farm through the second season of pasture defoliation treatments. A significant frequency $\times$ severity interaction was detected. Hollow bars represent high severity clipping. Solid bars represent low severity clipping. Bars are means $\pm$ 1 SE ($n = 3$). Letters separate means.

To determine the impact of defoliation frequency and severity on potential forage utilization by livestock, I calculated utilization as described above. At OF, defoliation frequency significantly influenced forage utilization (Figure 3, $p = 0.01$), wherein utilization was 30% lower in rotational stocking plots compared to continuous or hay stocking plots. At RV, both a significant frequency ($p < 0.01$) and severity ($p = 0.04$) effect were detected; the interaction term was not significant (Figure 4). Here, low defoliation severity resulted in reduced utilization (-13% lower than high-severity). Low defoliation frequency also increased utilization, as hay resulted in the highest utilization (9,241 kg Dm ha⁻¹) while continuous resulted in the lowest utilization (6,348 kg Dm ha⁻¹).

Figure 3: Utilization at OF through the second season of pasture defoliation treatments. Utilization was different across defoliation frequencies ($p = 0.01$) No significant defoliation severity effect nor a frequency $\times$ severity interaction was detected. Bars represent means $\pm$ 1SE ($n = 6$).

Figure 4: Utilization at RV through the second season of pasture defoliation treatments. Defoliation frequency and severity treatments were significantly different. Bars represent means $\pm$ 1SE ($n = 3$). Letters separate means.

Defoliation effects on soil C stocks

To test for differences in soil C stocks due to defoliation frequency and severity, total C, total N, SOM, as well as permanganate oxidizable C (OXC; i.e., labile C) were measured. At both OF and RV, total C, total N, C:N and % SOM were not significantly different due to any defoliation effect or interaction. However, at OF, OXC was significantly influenced by defoliation severity, as OXC activity was 25, 45, and 47% greater in high defoliation severity plots compared to low severity plots under continuous, rotational, and hay stocking rates, respectively. Overall at OF, OXC was 30 % greater in low severity plots as compared to high severity plots (Figure 5, severity effect $p = 0.06$). At RV, there was no impact of defoliation frequency or severity on OXC (Figure 6).

Figure 5: Oxidizable carbon (OXC), 0-7.62 cm depth at OF following two seasons of defoliation treatment. No significant defoliation frequency effect nor a frequency $\times$ severity interaction was exhibited. Severity effect $P < 0.05$. Bars represent means $\pm$ 1SE ($n = 3$).

Figure 6: Oxidizable carbon (OXC) at RV following two years of defoliation frequency and severity treatments. Bars represent ± 1 SE ($n = 9$).

Defoliation effects on microbial enzyme activity

To determine the impact of pasture defoliation frequency and severity on the microbial potential to degrade cellulose, the activity of the microbial extracellular enzyme β -glucosidase (BG) was measured. At OF, BG exhibited a significant defoliation frequency $\times$ severity interaction (Figure 7, $p = 0.04$). Student's t -test determined simulated rotational stocking at low severity exhibited the lowest BG activity ($p < 0.10$), whereas continuous stocking low severity, rotational high severity, and hay high severity all exhibited the greatest BG activity. At RV, BG activity was not affected by defoliation frequency nor severity (Figure 7, $p = 0.96$).

Figure 7: Mean β -glucosidase (BG) activity by defoliation treatment at OF (A) and RV (B) following two years of defoliation treatments. Open bars represent high severity clipping. Solid bars represent low severity clipping. There was a significant frequency $\times$ severity interaction at OF, but no significant frequency effect, severity effect or frequency $\times$ severity interaction at RV. Bars represent means $\pm$ 1SE ($n = 3$). Letters separate means.

To identify differences in microbial N acquisition potential due to defoliation frequency and severity, β -*N*-acetylglucosaminidase (NAG) activity was quantified. NAG activity was not impacted at either site due to defoliation frequency (OF $p = 0.52$; RV $p = 0.92$) nor severity (OF $p = 0.78$; RV $p = 0.22$), and there was not a significant frequency $\times$ severity interaction (OF $p = 0.67$; RV $p = 0.60$). Though non-significant, at OF, hay stocking frequency at low severity did exhibit a 48% greater NAG activity compared to all other treatments. At RV, the continuous

frequency high severity treatment had approximately 45% greater NAG activity compared to all other treatments, though this response was not significant.

To identify differences in microbial P acquisition due to defoliation strategy, AP activity was quantified. At both OF and RV, AP activity was not impacted by defoliation frequency (OF $p = 0.48$; RV $p = 0.60$) nor severity (OF $p = 0.93$; RV $p = 0.88$), and there was no significant frequency $\times$ severity interactions (OF $p = 0.21$, RV $p = 0.24$). Though non-significant, at OF AP activity was greatest in rotational frequency high severity plots, which exhibited a 20% greater activity than the next highest treatment (hay stage frequency high severity). At RV, rotational low severity exhibited the greatest AP activity, which though non-significant, was 24% greater than the average of all other treatments.

Defoliation impacts on microbial activity

Microbial activity, as defined by the combined activity of C-, N-, and P- acquiring enzymes, was significantly impacted by the defoliation treatments at OF, wherein the response was moderately significant for severity (PerMANOVA; severity effect; $p = 0.06$; Figure 8), and not significant for defoliation frequency. Distance-based linear models were then used to test the impact of measured soil environmental parameters on microbial activity. At OF, marginal DistLM revealed that total C (19% of variation explained), C:N (19%), SOM content (17%) and percent moisture (15%) all accounted for a significant proportion of variation in microbial activity. The influence of pH and OXC were not significant. At OF, *best* model selection revealed that a model including both SOM and C:N most parsimoniously accounted for the greatest variance in microbial activity across the treatments (Table 5, $R^2 = 0.32$, adjusted $R^2 = 0.22$). At RV, there was no response of microbial activity to defoliation frequency nor severity

(PerMANOVA main tests $P > 0.10$). However, despite there being no significant impact of defoliation frequency nor severity on microbial activity at RV, marginal DistLM revealed that total C (35% of variation explained), percent moisture (18%), C:N (15%) and OXC (12%) all accounted for a significant proportion of variation.

Table 5: Marginal Distance based linear model (DistLM) of microbial activity against environmental factors in OF and RV. Including the results of *best* model, which determines the maximum proportion of variance explained with minimum parameters.

	Organic Farm		Reedsville Farm	
	Pseudo-F	Prop. of Variation (%)	Pseudo-F	Prop. of Variation (%)
pH	1.4	8	0.9	5
% Moisture	2.7*	15	3.4*	18 ^{&}
% SOM	3.2*	17 ^{&}	1.1	6
Total C	3.7*	19	8.6*	35 ^{&}
C:N	3.6*	19 ^{&}	2.9*	15
OXC	1.5	9	2.3*	12 ^{&}
Best Model Power (R^2)		0.31		0.49

* $P < 0.10$

[&]parameter included in “best” model selection

Figure 8: Non-metric multidimensional scaling plots of microbial activity at OF (A) and RV (B). At OF, microbial activity was significantly impacted by defoliation severity ($p < 0.05$), but not defoliation frequency. No effect of defoliation was apparent at RV. Vectors in plot A indicate influence of environmental parameters deemed significant by marginal DistLM on microbial activity. Parameter vectors were not displayed for panel B, because no significant defoliation effect was detected.

Legacy effects on pasture canopy due to defoliation

To determine whether legacy effects exist due to defoliation treatment on forage, LAI was recorded at the beginning of the following growing season in May 2018. Indeed, significant differences due to defoliation frequency at OF and RV were observed (Figure 9, $p < 0.01$ and $= 0.05$, respectively). At OF, continuous frequency plots had ~50% greater LAI than either rotational or hay frequency plots, but at RV continuous stocking had ~25 % lower LAI than either rotational or hay.

Figure 9: OF (A) and RV (B) LAI at the beginning of the third year of pasture defoliation treatments. At OF and RV, a defoliation frequency effect was detected ($p < 0.01$ and $p = 0.05$, respectively) no significant defoliation severity effect nor a frequency $\times$ severity interaction was exhibited at either site. Bars represent means $\pm$ 1SE ($p = 0.05$, $n = 6$). Letters separate means.

Discussion

My hypothesis that moderate defoliation management strategy would significantly affect soil C stocks and the activity of microbial extracellular enzyme activity associated with microbial C acquisition was partially supported, as BG activity was reduced in the rotational-low severity and C stocks were only significantly altered by defoliation severity (Figure 5) at OF, but not RV. In addition, my prediction that enzymes associated with N and P would be increased

with greater soil C was not supported. For example, at OF, BG activity (a C-obtaining enzyme) was significantly reduced (-38%) in rotational low severity treatments, suggesting that the microbial capacity to mineralize C from SOM is reduced in this moderate-to-low frequency and severity treatment combination. Extracellular enzyme activities related to N and P were not different across defoliation treatments. Indeed, it is well established that grazing can impact microbial C dynamics (63, 64), however this is the first assessment of such changes in Central Appalachian pastures. These results demonstrate that defoliation frequency and severity can significantly impact C-associated extracellular enzyme activities in relatively short time scales, in this case only two years. This is shorter to other assessments of grazing impacts on soil enzyme activity, wherein significant effects were observed after 5 years of treatments (65).

Results from this study indicate that pasture defoliation can lead to changes in soil C availability (35, 39). This response has also been shown in other studies, for example, in grasslands, defoliation resulted in greater exudation of low molecular weight C molecules from roots (8, 66–69). At OF, greater oxidizable C availability when defoliation events were less severe, however at RV there was no discernible pattern in OXC in relation to defoliation management strategy. Thus, it is possible that organically managed systems are more amenable to management-induced changes in soil nutrient dynamics. A meta-analysis found that organically managed farms sequester more soil C compared to conventionally managed farms (70). Quantitatively, organic management was shown to increase mineral-associated organic matter as much as 40% compared to conventional management (71, 72). The findings here and the results of others shown above might suggest greater microbial nutrient mineralization in organic farms, however further comparisons of defoliation impact in organic and conventional

systems using replicated experimental designs are needed to properly test whether this observation is indicative of a larger phenomenon.

In this study, less severe pasture defoliation management strategies led to significant differences in labile soil C pools at both OF and RV, but not more temporally stable C pools as indicated by no significant differences in total soil C and SOM content due to defoliation treatments. Three mechanisms by which defoliation increases SOC have been identified: changes in the allocation of net primary production (NPP) to soil (i.e. root exudates), changes in soil organic N stocks, and the alteration of SOM decomposition (73). For example, grazing can increase SOC via increased NPP allocation to root exudates (8, 63, 66) and by facilitating the mechanical breakdown of plant materials prior to decomposition by soil microbes (43, 74). It is apparent that differences in defoliation management could induce changes in total SOC via small changes in root exudation or alteration of decomposition, however these changes often take many years or even decades to manifest (43, 74, 75). In this study, greater OXC was exhibited in less severely defoliated plots after two growing seasons, which suggests that defoliation management differences could result in greater SOC in our study sites.

Counter to my hypothesis, despite significant difference in OXC across defoliation management treatments, there were no significant differences in AP and NAG activity. NAG and AP are N and P acquiring enzymes and considered to be important indicators of microbial nutrient demands (2, 56). This may be because neither nitrogen nor phosphorus were limiting due to the use of fertilizers at OF (2). In addition, defoliation has been shown to increase belowground N and P allocation, which may increase cycling and availability of N and P to the microbial community reducing microbial nutrient foraging (76). Finally, the results shown here do not indicate that reduced C demands resulted in increased nutrient foraging.

Microbial activity, as indicated by the combined activities of extracellular enzymes associated with C, N, and P acquisition, was responsive to defoliation severity but not frequency at OF, and it was not responsive to severity nor frequency at RV. Environmental parameters that parsimoniously accounted for the greatest variation in microbial activity differed between sites, but each had significant proportions of variation due to parameters related to C. These data demonstrate that microbial activity is dependent on soil parameters, particularly those related to soil C and can vary with management strategy. These results are in concordance with other findings. For example, microbial activity positively correlated with soil C pools in grazed pastures and further, increased grazing severity reduced soil C (24). In addition, microbial activity and important soil parameters (i.e. pH, soil moisture, and total C) were greater under less severe grazing in an Indian pasture (77). In this study, microbial activity responded differently to defoliation treatments and soil parameters in an organically and conventionally, this has been exhibited previously (78). However, because of inherent differences between sites, accurate assessments of organic versus conventional standards cannot be made and would be an interesting topic of future research. Nonetheless, results shown here suggest that defoliation management is capable of inducing changes in microbial activity.

In this study, pasture defoliation increased legacy light capture at both sites (Figure 9), which aligns with the findings of previous studies (12, 30, 79, 80). At OF, utilization was greatest in the continuously defoliated plots, whereas at RV, utilization was lowest in the continuously defoliated plots. Given that mean annual precipitation and temperature are higher at OF as compared to RV (Table 1), these differences may be due to forage experiencing greater stress at RV where precipitation and temperature were more limiting and possibly prevented luxuriant compensatory regrowth following frequent defoliation (12). At OF, the increased

oxidizable C content in plots experiencing lower defoliation severity may be associated with greater light capture following defoliation events or it may be due to reduced stress on the plant to recover lost leaf area (12). In addition, the differences in LAI due to defoliation frequency were more pronounced at the beginning of the third year of treatments, which suggests the differences in canopy development may become more pronounced with time. While treatment differences in LAI were observed, all LAI measurements recorded were within the suggested acceptable range of LAI for pasture (~3-5, 81).

Conclusion

Overall, the results of this study demonstrated that defoliation management strategy can induce a top-down ecological effect on forage utilization and light-capture efficiency, soil C availability and extracellular enzyme activity, which may have long-lasting impacts on soil C stocks. Enzyme activities related to microbial N and P acquisition were unaffected by the defoliation treatments, which is plausibly due to the use of N- and P-rich fertilizer. These results do not necessarily conform to previous research on the effects of greater C availability to soil, but this is most likely because neither N nor P were limiting in our experimental plots. In future studies, examining the impacts of defoliation management with and without fertilizer addition may reveal novel microbial responses to this selective pressure. For example, if defoliation increases root exudates and reduces the need to acquire C, more resources may be devoted to acquiring N or P resulting in greater NAG and AP activities if N and P are indeed limiting. In addition, because grazing introduces environmental fluxes that were not introduced in this study (e.g. nutrient hot spots and soil compaction from animals) our study is limited in its explanatory

power and future studies should include the presence of either grazing livestock or simulated excreta and soil compaction.

Chapter 2: Greater soil carbon increases nitrous oxide emission potential in two West Virginia pastures

Abstract

Nitrous oxide (N_2O) is a potent greenhouse gas and its emission from soil is largely controlled by biogeochemical processes, which can be altered via land management. Proper management of agricultural soils, specifically those used for grazing which account for over 600 million acres of land in the U.S., can significantly reduce N_2O emissions. The aim of this study is to investigate how defoliation management strategies affect below-ground parameters such as soil organic matter (SOM) production, as well as the abundance of microbial functional guilds associated with the nitrogen cycle, which I use to predict potential emissions of a pervasive greenhouse gas, namely, N_2O . Soil microbial activity is mainly dependent on environmental factors such as pH, soil moisture, and carbon (C) availability. If defoliation alters these soil parameters, then greater potential for N_2O emissions could occur. I hypothesize that the greatest N_2O emission potential will come from experimental plots that are defoliated in a rotational frequency (i.e. monthly) at a high severity (i.e., low forage height), due to greater below-ground C availability that will stimulate microbial activity and incomplete denitrification. To address this hypothesis, I implemented forage defoliation treatments of increasing intensity and frequency in experimental plots at two West Virginia University Research Farms since July 2016. After two full growing seasons, I assessed soil physical and chemical parameters, as well as N_2O emission potential as estimated by the quantifying the abundance of the bacterial genes *nirK* and *nosZ*, the former being associated with nitrite reduction and the latter associated with N_2O reduction. At the Reedsville site, continuous grazing reduced potential N_2O emissions to only 28% of N_2O emission potential from either rotational or hay defoliation frequencies. While at the Organic Farm, N_2O emissions were reduced by 45% under high severity defoliation. These data will inform our understanding of the below-ground impacts of grazing and allow us to develop a more effective management plan for livestock grazing of C_3 perennial grasses to reduce N_2O emissions from Central Appalachian pastures.

Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas with a climate warming potential 298 times that of carbon dioxide (CO_2), it is also associated with ozone depletion (82).

Anthropogenic N_2O emissions mainly come from agricultural land, which are estimated to account for 54% of global N_2O emissions (83). Further, N_2O emissions are increasing at a rate of 0.3% annually (83). This increase in N_2O emissions is at least partially due to land management practices aimed to increase crop yields (84), while neglecting to manage for environmental sustainability (85, 86). Proper management of agricultural soils, specifically the 242 million hectares of land in the U.S. that are currently used as pasture for grazing livestock, can significantly reduce N_2O emissions (9, 87–89). For example, no-till pastures have reduced N_2O emissions and greater soil carbon (C) storage, but it also reduces yield (90, 91). To inform the development of best management practices (BMP), it is necessary to understand how forage management strategies may affect microbial functional guilds that are associated with the production of N_2O , a potent greenhouse gas, which is the focus of this study.

N_2O emissions are mainly the result of two microbial processes: nitrification and denitrification (92). Nitrification is an aerobic process that oxidizes ammonia (NH_3) to nitrate (NO_3^-) and denitrification is an anaerobic process that reduces nitrate (NO_3^-) or nitrite (NO_2^-) to gaseous N products (NO , N_2O , and N_2). However, only denitrification is recognized as a significant biological consumer of N_2O , because N_2O emissions from nitrification are often $< 1\%$ of total N_2O emissions in agricultural systems, but values as high as 30% have been demonstrated (92–94). Current conceptual frameworks postulate that both distal and proximal controls affect N_2O emissions (92, 95, 96). For example, distal controls, including C substrate availability, pH, temperature, O_2 availability, plant community dynamics, and disturbance can influence microbial functional guilds associated with denitrification (95, 96). However, the rate

and final products of denitrification are ultimately constrained by proximal controls that affect the metabolism of the denitrifying community and include: O₂ and C availability, pH, and temperature (95, 96). Therefore, N₂O emissions can be affected by defoliation management via increases in organic C, changes in pH, and O₂ availability, which alter the structure of the denitrifying community (8, 53, 92, 97–100). This conceptual framework, which links the C and nitrogen (N) cycles, is known as the “hole-in-the-pipe model” (92, 101). Because the C and N cycles are inherently linked it is necessary to incorporate both cycles to effectively estimate the N₂O emission potential associated with environmental changes (i.e. by pasture defoliation). Failure to recognize the coupled N and C cycles in agricultural land management could result in net increases of total GHG emissions and reduced environmental sustainability (102). For example, land management practices aimed to increase SOC are desirable to reduce atmospheric CO₂ concentrations (103), however those increases in SOC could result in an unintended consequence of greater emissions of N₂O (104).

It is plausible that pasture management strategies can alter the abundance of microbial functional guilds associated with denitrification, which in turn could increase or decrease the potential for N₂O emissions. Differences in pH, soil texture, and organic C can influence the composition and diversity of denitrifying communities in a variety of agricultural settings (86, 87, 100, 105, 106). Further, defoliation and grazing can alter the soil microbial community via a top-down ecological cascade, whereby defoliation selects a grazing- tolerant plant community, which may increase root exudation of low molecular weight C molecules following defoliation events, fueling microbial activity (8, 17, 35, 48). However, how this cascade may affect the abundance of denitrifying bacteria and N₂O emission potential is still unknown. Denitrification can be stimulated by the addition of labile root exudates, which can stimulate microbial activity and denitrification

(20). In addition, organic farm standards can increase N₂O emissions as well as the abundance of genes associated with denitrification as compared to conventional management standards (107). Importantly, N₂O can be produced through an incomplete denitrification process. Under sub-optimal conditions, an under-abundance of microbes that possess the *nosZ* gene, which encodes an enzyme that reduces N₂O to benign N₂ gas, cannot sustain N₂O reduction rates equal to rates of N₂O production by bacteria harboring the *nirK* gene. Hence, the microbial population may either serve as a sink (if N₂O is reduced to N₂) or a source of N₂O emissions from soil (108).

The use of molecular techniques provides a means by which N₂O emissions can be estimated. For example, the gene *nirK* encodes a nitrite reductase, which reduces NO₂⁻ to N₂O (101, 109). In addition, the gene *nosZ* encodes a nitrous oxide reductase (N₂O → N₂; 6), which reduces the pollutant gas to an inert form. In a recent meta-analysis, the abundances of *nirK* and *nosZ* significantly correlated to their process rate in agricultural soils, suggesting that it is possible to estimate N₂O emission rates from *nirK* and *nosZ* gene abundances (111). Quantifying the genes most closely associated with N₂O production and their relation to available C may provide valuable information to estimate the potential for N₂O emission from pastures.

The objective of this study was to determine the impact of defoliation frequency and severity on the N₂O emission potential of agricultural soils. I hypothesize that plots defoliated at a moderate frequency (rotational stocking) and low severity (clipped to 13 cm) will have greater C bioavailability and consequently greater N₂O emission potential. To address my hypothesis, six defoliation treatments were implemented at two West Virginia farms and qPCR was used to quantify the abundance of the genes *nirK* and *nosZ* to estimate the potential N₂O emissions due to defoliation management strategy (*sensu* 41, 42).

Methods

Site description

This experiment was conducted at two West Virginia University (WVU) research farms: The Organic Research Farm in Morgantown, WV (39°5N, -79°94W; hereafter “OF”) and the JW Ruby Research Farm in Reedsville, WV (39°1N, -79°82W; hereafter “RV”; Table 6). The OF was organically certified in 1998, and the RV site is conventionally managed. Soils at OF and RV consist of Dormont silt loam and Latham silt loam, respectively. Mean annual temperature (MAT) and mean annual precipitation (MAP) are 20.3°C and 125.5 cm, respectively, at OF and 12°C and 153 cm at RV. Experimental plots were established at each site in June 2016, plots measured 7m × 3m at OF and 8m × 3m at RV. On July 1, 2016, six experimental defoliation treatments consisting of three simulated frequency regimes at two levels of severity were implemented at each site (Table 7) in a randomized block design (3 frequency levels × 2 severity levels × 3 blocks × 2 sites = 36 total plots). The simulated frequency and severity treatments were selected to align with common pasture management strategies in the Central Appalachian region, representing continuous, rotational, and hay-stage pasture management as suggested by the United States Department of Agriculture Natural Resources Conservation Service (50). The continuous frequency treatment was defoliated every 7-10 days, rotational frequency plots were defoliated every 28-35 days, and hay-stage plots were defoliated every 42-45 days. Each simulated frequency treatment was also defoliated to two heights to represent low and high severity defoliation, respectively; the defoliation height was 6-8 cm for the high severity treatment and 13 cm for the low severity treatment. Simulated defoliation was achieved by clipping vegetation using a Monarch Sickle-Bar Mower outfit with a 36-inch serrated sickle bar (Jari USA, St. Peter, Minnesota).

Table 6: Summary characteristics of each experimental site in West Virginia.

	Organic Farm	Reedsville
Coordinates	39°5N, -79°94W	39°1N, -79°82W
Soil Series	Dormont Silt Loam	Latham Silt Loam
Management	Organic	Conventional
MAP (51)	125.5 cm	153.0 cm
MAT(51)	20.3°C	12°C
Relative N/P/K	High/Low/High	High/High/Low

Table 7: The six experimental treatments included in this experiment by stocking rate and clipped stubble height.

	A	B	C	D	E	F
Stocking	Rotational	Rotational	Continuous	Continuous	Hay	Hay
Clip Height	6 cm	13 cm	8 cm	13 cm	6 cm	13 cm

Soil Sampling

Soil samples were collected following two years of defoliation treatments on November 7, 2017. Plots were randomly sampled 10 times using a soil probe (1.7 cm diameter) to a depth of 7.5 cm. The cores were then thoroughly homogenized by hand to produce one composite sample per plot. The homogenized soil samples were stored on ice in the field and returned to the lab within two hours of sampling. Upon return to the lab, each soil sample was sieved (2,000

µm) to remove coarse roots and rocks. Homogenized sieved soil was stored at -20 °C for molecular analysis.

Soil Physiochemical Characteristics

To determine the impact of defoliation on soil properties, soil physiochemical characteristics were assayed that have been shown to impact both plant and microbial function were pH, soil moisture, total C, total N, soil organic matter content, and permanganate oxidizable C content (i.e., labile C; (2, 32, 42, 53)). Soil pH was measured using an Accumet AE 150 probe (Fisher Scientific, Hampton, NH) in a 1:5 soil:0.01M CaCl₂ slurry (54). The moisture content of soil samples was determined gravimetrically, whereby 10 g of field-moist soil was dried at 105°C for 24 hours. Following drying, samples were re-weighed to determine dry mass and then combusted at 505 °C for 6 hours to determine percent soil organic matter (SOM) content by loss on ignition (LOI). The amount of labile C was estimated by the permanganate oxidizable C method (55), whereby four analytical replicates of each sample were assayed using clear 96-well plates (Corning, Corning, NY) on a Synergy HTX plate reader (Biotek, Winooski, VT). Absorbance was recorded at 550 nm. The total C and N content of the soil was determined by combustion analysis in a Carlo Erba 1500 NCS (Carlo Erba, St. Joseph, MI) using 12 mg of dry ground soil.

DNA Extraction

Total DNA was extracted from 1.0 g of field weight homogenized soil using the DNeasy Powerlyzer Powersoil® kit (QIAGEN, Hilden, Germany) according to the manufacturer's

protocol. Each sample was extracted in duplicate and combined after elution. DNA extraction quality and quantity were assessed on a NanoDrop One (Thermo Fisher, Scientific, Waltham, MA) and QuBit (Life Technologies, Carlsbad, CA) spectrophotometers, respectively, following manufacturer's protocol.

Quantitative Polymerase Chain Reaction (qPCR)

To determine how pasture management impacts soil N₂O emission potential, I quantified the abundance of microbial genes associated with nitrification (*nirK* and *nosZ*) by quantitative polymerase chain reaction (qPCR). These genes are relevant for estimating N₂O emission potential, as the *nirK* (copper-containing nitrite reductase) encodes an enzyme that reduces NO₂⁻ to N₂O (101), whereas the enzyme encoded by *nosZ* (nitrous oxide reductase) reduces N₂O to N₂ (11, 101, 112). Thus, a greater ratio of *nirK/nosZ* would indicate a greater N₂O emission potential by the soil microbial community. All qPCR reactions were performed on a QuantStudio5 real-time PCR machine (Applied Biosystems, Foster City, CA) using TemPlate® 384-well PCR plates and TemPlate® RT Select Optical film (USA Scientific, Orlando, FL).

Primers nirK876 and nirK1040 (101) were used for the quantitation of the *nirK* gene and the primers nosZ2R and nosZ2F were used for the quantification of the *nosZ* gene (Figure 10 and Table 8, (110)). For standard curve generation, PCRs were performed using each primer set on an environmental soil sample, and the resulting products were purified using ExoSAP-IT (Thermo Fisher, Waltham, MA) and used template DNA in qPCR reactions. The copy number of each PCR product in the standard was calculated based on the DNA concentration (ng · μL⁻¹) of the sample as determined by Qubit, divided by the mass of the PCR product assuming a mass of 325 Daltons per nucleotide (114). For qPCR, a 10 μL final reaction volume was used, containing

2 μ L of molecular grade water (Mediatech Inc., Manassas, VA), 0.5 μ L of each primer (total primer concentration = 500 nM), 5 μ L of LUNA qPCR 2X master mix (New England Biolabs, Ipswich, MA), and 2 μ L of template DNA. For all functional genes, real time amplification was performed using the following program: 1 cycle at 95 °C for 5 minutes, 35 cycles at 95 °C for 20 s and 60 °C for 45 s, 1 cycle at 95 °C for 15 s. To assess the quality of the PCR reaction a melt-curve was performed.

Statistical Analysis

All statistical analyses were performed in SAS JMP (Version 13, SAS Institute Inc., Cary, NC). An ANOVA was first used to test for differences across sampling locations. A two-way analysis of variance (ANOVA) was performed on each parameter and tested two main effects (severity and frequency) and their interaction (frequency $\times$ severity). If a significant interaction was detected, a Student's T-test was used to determine differences among treatment means. Results were considered significant at $\alpha < 0.05$ and marginally significant at $\alpha < 0.10$. Associations between denitrification-associated functional genes and soil C parameters were determined using the general linear model in StatPlus (115)

Figure 10: Genes associated with the production of enzymes facilitating nitrogen cycle transformations. This study focused on two genes: *nirK* and *nosZ*.

Table 8: Target genes and corresponding primer sequences with reference.

Target Gene	Primer	Sequence	Reference
<i>nirK</i>	nirK.876	ATYGGCGGVAYGGCGA	(101)
<i>nirK</i>	nirK.1040	GCCTCGATCAGRTTTRTGGTT	(101)
<i>nosZ</i>	NosZ2F	CGCRACGGCAASAAGGTSMSSGT	(110)
<i>nosZ</i>	NosZ2R	CAKRTGCAKSGCRTGGCAGAA	(110)

Results

Differences between sites following defoliation implementation

The OF and RV farms were chosen as sites for this experiment due to their unique management practices (i.e., organic vs. conventional) as well as soil physico-chemical properties. For example, average oxidizable C, soil organic matter content, soil C:N, acid phosphatase activity, NAG activity, pH, and % soil moisture were all significantly different between the two sites (Table 9). Soils at RV exhibited greater C:N soil organic matter and oxidizable C content, as well as reduced activities of microbial enzyme as compared to soils at OF.

Table 9: Differences in microbial enzyme and soil parameters at each site. (a) and (b) represent differences between the two sites at $p < 0.05$.

Parameter	OF	RV	SE
Soil moisture (%)	29.3	29.0	0.4
pH	6.0(a)	6.4(b)	0.06
Total C	2.5(a)	3.3(b)	.09
Total N	0.27(a)	0.34(b)	.007
Soil C:N	8.83(a)	9.9(b)	0.2
SOM (%)	8.00(a)	9.8(b)	0.1
Oxidizable C (mg kg ⁻¹)	782.9(a)	1175.5(b)	48.5
β -glucosidase	23.5(a)	16.7(b)	1.2
β -N-acetylglucosaminidase	17.2(a)	7.3(b)	1.3
Acid Phosphatase	23.5(a)	49.7(b)	2.8

To determine differences in soil C storage and availability SOM, C:N, and OXC were measured. SOM was not significantly different due to defoliation treatment at either site SOM averaged 8.0% at OF and 9.8% at RV. Soil C:N was not different across all defoliation treatments. Soil C:N average 8.8 at OF and 9.9 at RV. Oxidizable C averaged 820.1 and 1175.5

mg kg⁻¹ soil at OF and RV, respectively. Moderately significant defoliation frequency and intensity effects on OXC were detected at OF ($p = 0.06$ and 0.09 , respectively), whereas no significant defoliation effect was detected at RV. At OF, oxidizable C values were always greater under low defoliation severity. In addition, rotational frequency had the lowest oxidizable C while hay stage stocking treatment had the greatest amount of oxidizable C.

Abundance of genes associated with N₂O production by denitrification

To determine microbial NO₂⁻ reduction potential, I quantified the *nirK* gene using qPCR. On average, the *nirK* gene abundance was significantly lower at OF than RV (1.075×10^5 and 2.150×10^5 *nirK* copies g⁻¹ of dry soil at OF and RV, respectively; $p = 0.03$). At OF, there was no defoliation frequency nor severity effect on *nirK* gene abundance (Figure 11). However, at RV there was both a moderately significant frequency and severity effect on *nirK* gene number (Figure 11, frequency and severity effect $p = 0.09$), and the frequency $\times$ severity interaction was not significant. At RV, rotational frequency at low severity exhibited the highest *nirK* abundance, which was on average 58% greater than all other defoliation treatments.

Figure 11: Mean *nirK* abundance at OF (A) and RV (B) on November 7, 2018. Open bars represent high severity clipping. Solid bars represent low severity clipping. There was no significant main effect or interaction at OF, but at RV both frequency ($p = 0.09$) and severity ($p = 0.09$) were effect *nirK* abundance. Letters indicate significant differences between treatment means. All data are presented as means $\pm$ 1 SE.

To determine the impact of defoliation frequency and severity on microbial nitrous oxide reduction potential, I quantified the *nosZ* gene abundance using qPCR. The mean values for *nosZ* gene number were not significantly different between OF and RV, with average values of 3.887×10^5 and $4.147 \times 10^5 \text{ g}^{-1}$ of dry soil at OF and RV, respectively. At OF, a marginally significant frequency $\times$ severity interaction was observed (Figure 12, $p = 0.09$), wherein post-hoc tests revealed that continuously defoliated, high severity treatments exhibited the greatest *nosZ*

abundance (+38% greater than the mean rotational and hay frequency plots $p = 0.02$). The continuously defoliated low severity treatments exhibited the lowest *nosZ* abundance (-38% lower than the mean rotational and hay plots; $p = 0.07$). At RV, plots exposed to continuous frequency exhibited the greatest abundance of *nosZ*, which was 40% greater than plots that experienced rotational or hay-stage defoliation frequency (Figure 13, frequency effect $p = 0.07$). There was no effect of defoliation severity on *nosZ* abundance at RV.

Figure 12: *nosZ* gene abundance at OF on November 7, 2018. A significant defoliation frequency severity $\times$ interaction was observed. Different letters indicate differences between treatment means using a Student's T-test. All data are presented as means $\pm$ 1 SE.

Figure 13: *nosZ* gene abundance at RV following two years of defoliation treatments. A significant defoliation frequency effect was observed ($p < 0.05$), but not a severity nor frequency $\times$ severity interaction. Different letters indicate differences between treatment means using a Student's T-test. All data are presented as means $\pm$ 1 SE.

To quantify the impact of defoliation treatments on N₂O emission potential, I calculated the *nirK/nosZ* ratio (*sensu* 42, 44) wherein higher values are indicative of a greater potential for N₂O emissions, whereas lower values indicate a lower N₂O emission potential. The mean values of *nirK/nosZ* were significantly different by site, whereas abundance equaled 2.807 at OF and 5.937 at RV ($p = 0.03$). At OF, a marginally significant severity effect was detected (Figure 14, $p = 0.09$), wherein low defoliation severity increased the ratio of *nir/nosZ* (+45% increase), which indicates greater N₂O emissions compared to high defoliation severity. At RV, a marginally significant frequency effect was detected (Figure 15, $p = 0.08$), wherein continuous stocking was only 28% of the *nirK/nosZ* ratio of either rotational or hay treatments, indicating a lower N₂O emission potential.

Figure 14: The ratio of gene copies *nirK* g⁻¹ of dry soil to *nosZ* g⁻¹ of dry soil following two years of defoliation treatments at OF. A significant defoliation severity effect was observed ($p < 0.05$), but not a frequency nor frequency $\times$ severity interaction. All data are presented as means $\pm$ 1SE. Letters separate means ($p < 0.05$).

Figure 15: The ratio of *nirK* g⁻¹ of dry soil to *nosZ* g⁻¹ of dry soil following two years of defoliation treatments at RV. A significant defoliation frequency effect was observed ($p < 0.05$), but not a severity or frequency $\times$ severity interaction. All data are presented as means $\pm$ 1SE ($p < 0.05$). Letters indicate significant differences between treatment means.

Connecting the C and N cycle

To determine the association of soil C stocks with N₂O emission potential, I regressed the C:N, % SOM, and OXC data against *nirK/nosZ*. C:N was not related to *nirK/nosZ* at either site or when sites were combined. With OF and RV data combined, % SOM and OXC were significantly, but weakly correlated to the ratio of *nirK/nosZ* (Figure 16, SOM $r^2 = 0.12$, $p = 0.04$; OXC $r^2 = 0.13$, $p = 0.04$). However, when separated by site, at OF, % SOM was significantly associated with the ratio *nirK/nosZ* (Figure 17, $r^2 = 0.38$, $p = 0.006$), but not OXC ($r^2 = 0.01$, $p = 0.66$). At RV, % SOM was not significantly related to the ratio of *nirK/nosZ* ($r^2 = 0.02$, $p = 0.54$), nor was OXC ($r^2 = 0.07$, $p = 0.30$).

Figure 16: Linear regression relating the ratio *nirK/nosZ* to % SOM (A) and OXC (B) including both OF and RV data (A, $p = 0.04$). Linear regression relating OXC to the ratio *nirK/nosZ* including both OF and RV data (B, $p = 0.04$).

Figure 17: Linear regression relating % SOM to the ratio *nirK/nosZ* at OF ($p = 0.006$).

Discussion

In this study, I determined that greater defoliation severity decreased N_2O emission potential via reduced SOM and oxidizable C availability in two West Virginia managed pastures. Further, the association between N_2O emission potential and soil C stock (i.e, SOM) was greatest at OF, not at RV. Across all experimental plots in both OF and RV, SOM ($r^2 = 0.12$, $p = 0.04$) and oxidizable C was associated ($r^2 = 0.13$, $p = 0.04$) with greater N_2O emission potential, however both associations were weak. This suggests that while common environmental parameters may influence N_2O emissions across sites, the same environmental may constrain N_2O emissions differently under different soil physico-chemical conditions. Further, it is plausible that other unmeasured soil physiochemical factors may be impacting N_2O emission potential in this experiment. There are four major requirements for denitrification to occur: the presence of denitrifying bacteria, the availability of organic C, O_2 restriction, and presence of N-oxides (92, 95). In addition, the denitrifying community may be shaped by a variety of distal controls including: C availability, pH, and soil moisture. Further, soil moisture and soil pH are

major determinants of microbial abundance and diversity (116). In this study, soil pH and soil moisture did not differ across defoliation management treatments for the sampling period, thus, observed changes in N₂O emission potential are most likely linked to defoliation-induced changes in soil C stocks. However, the availability of nitrate (NO₃⁻), the substrate in the first reaction in denitrification, can influence N₂O emissions ((92, 95)), further study should include assessments of soil NO₃ in relation to N₂O emissions. These results support my hypothesis that the soil microbial potential for N₂O emissions will be increased with increasing availability of soil C, attributable to indirect effects caused by defoliation management strategy (8, 20, 35, 92, 117).

Data generated in this study begin to illuminate a possible mechanism by which defoliation intensity and severity may increase the *nirK:nosZ* ratio, and hence the N₂O emission potential of soils through changes in both above- and below-ground dynamics. Firstly, defoliation can alter belowground C dynamics via changes in plant physiology, namely, C allocation to root tissues and exudation of low molecular weight C compounds from roots at less frequent defoliation events (8, 39, 118). Secondly, variations in defoliation management may enhance or reduce C allocation to roots, which supply the microbial community with important C substrate fueling metabolism (119). In addition, grazing tolerant plants have a greater ability to allocate C to roots following defoliation events (120). As a result, if N₂O emissions are constrained by labile C availability, grazing-tolerant plants would harbor microbial communities with increased N₂O emission potential due to the plant's greater capacity to supply soil microbial communities with C. However, grazing-tolerant plants can also reduce N₂O emissions *in situ* (121), thus further investigations are needed to resolve plant-soil feedbacks between grazing tolerant plant and microbial functional guilds associated with N₂O emissions.

In this study, I quantified the genes responsible for N₂O production and reduction by denitrification; further study should also include N₂O emission potential by nitrification. In defoliated grasslands, a nitrification inhibitor reduced NO₂⁻ and consequently N₂O emissions by 57 % (122). In future studies, it is important to consider nitrification-based N₂O emission because it is plausible that soil parameters associated with increased denitrification-based N₂O emission may also be associated with decreased nitrification-based N₂O emissions and vice-versa (123, 124). For example, if the abundance of nitrifying bacteria is decreased, but the abundance of nitrite reducers remains the same nitrite/nitrate concentrations could be reduced, as well as, N₂O emissions. For example, N fertilization can increase the abundance of nitrifying and denitrifying bacteria with no change in the diversity of nitrifying communities (125). In future studies, including assessments of nitrification-based N₂O emissions would provide important information for more accurately assessing N₂O emission potential across defoliation management strategies.

To successfully implement progressive defoliation management strategies, plant productivity must be satisfactory to the land owner. In this study, the treatment exhibiting the lowest potential for N₂O emissions also exhibited the lowest utilization at OF, which could prevent it from being implemented by land managers. This pattern was repeated at RV where continuous defoliation resulted in both the lowest N₂O emission potential and lowest utilization. Selection of low-GHG associated plant species may help improve the environmental sustainability of increased productivity, as highly-productive plant mixtures had the lowest N₂O emissions in a recent mesocosm study (121). Also, C additions to pastures decreased N₂O emissions through adsorption of urine-derived ammonia without sacrificing productivity (126). Although the data presented here do not indicate N₂O emissions can be limited while providing

comparable productivity rates, other studies have shown promising results. Further study into environmentally sustainable management strategies will help to together optimize land productivity with the minimization of harmful GHG emissions.

Currently, organic agricultural management is being utilized to improve above-and below-ground biodiversity and increase soil C (70, 127). For example, a recent meta-analysis found that organically managed farms exhibited greater soil C storage compared to conventionally managed farms (70). However, given what I and others (20, 92, 95) have shown, increased soil C can lead to greater increases in N₂O emission potential in organically managed pasture than conventionally managed pasture. However, in this study, N₂O emission potential was overall greater in conventionally managed pastures as compared to organically managed pasture, but it did not respond to any defoliation treatment. This finding contrasts other research indicating greater N₂O emissions from organic standards (107), and is possibly due to a 24% greater SOM content at the RV farm (i.e., conventionally managed) versus the OF (i.e., organically managed). In this study, OF N₂O emission potential was more closely associated with soil C stocks (Fig 11) than RV soils. Thus, organically managed pasture lands may be more amenable to defoliation management than conventional management, regarding soil C and GHG emissions. In future studies, differences in organic farming and conventional farming responses to management and environmental changes should be properly investigated to understand how the different land management strategies impact GHG emissions.

Future research should include not only impacts of pasture defoliation, but also other selective pressures associated with livestock grazing, e.g., excreta and soil compaction, to better understand the impact pasture grazing has on the capacity for soil microbes to produce N₂O. For example, livestock excreta produce hot spots of high nutrients, which could increase microbial

activity and increase the availability of N for nitrification and denitrification, which may ultimately result in greater N₂O emission (128). In addition, soil compaction from cattle grazing increases the amount of soil under anoxic conditions, thus increasing denitrification rates and N₂O production (129). Future studies should also examine temporal changes across growing season and years, as N₂O emissions can vary dramatically over time (130, 131).

Conclusion

Overall, these results suggest a mechanism for the microbial response to defoliation management through the increased availability of soil C, as two years of differential defoliation management strategies produced changes in the N₂O emission potential of C₃ pastures via differences in SOM and labile C. In addition, organically managed pasture had the least N₂O emission potential when managed in a rotational fashion, while conventional management had the least N₂O emission potential when continuously defoliated. To limit N₂O emissions from organically managed WV pastures, defoliation should occur at moderate (i.e., 28-35 day) intervals at a high severity (i.e., height of 6 cm), however defoliation at frequent (i.e., 7-10 day intervals) and a low severity (i.e., height of either 8 or 12 cm) would be appropriate in conventionally managed pastures. Overall, these findings improve understanding of how changes in environmental parameters associated with pasture land management may alter the N₂O emission potential in pasture soils and will aid in developing best management practices (BMPs) to minimize N₂O emissions from pasture land.

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