Nitrogen fixation as a nutrient input in a local permaculture livestock farm

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Abstract

Biological nitrogen fixation (BNF) is the process by which atmospheric nitrogen (N_2) is converted into ammonium (NH_4^+), a form of nitrogen that plants can readily use. The process of BNF is performed by very specific groups of bacteria, some of which form symbiotic relationships with plant roots. BNF can be an important input to agricultural systems and an alternative to mineral fertilizer use, in order to minimize environmental impacts of fertilizer production and application. This study examined the pattern and contribution of BNF as an input to the overall nitrogen budget at Timbercreek Farm, an approaching permaculture livestock farm, located in Albemarle County, Virginia. The contribution of BNF to the farm was estimated using two approaches: 1) by applying linear relationships from Anglade et al. (2015), using plant shoot N yield and dry matter (DM) values of the legumes surveyed on the farm, and 2) from ^{15}N isotopic analysis of the plant materials. Total BNF for the six pasture fields at Timbercreek Farm was estimated to be 621 kgN/yr based on DM and 633 kgN/yr based on N yield. The average N_2 fixation rate based on shoot DM yield was 57 kgN/ha/yr for clover and 49 kgN/ha/yr for alfalfa. The average N_2 fixation rate based on shoot N yield was 89 kgN/ha/yr for clover and 41 kgN/ha/yr for alfalfa. The ^{15}N natural abundance results also showed evidence for BNF activity. Based on an average foliar $\delta^{15}N$ value of 2.5‰, the %Ndfa, or the reduced fraction of plant N derived from atmospheric N_2, was estimated to be 36%. The study further examined the spatial variability of the BNF potential across the six pasture fields and the variability of N content in plant material. Although alfalfa had slightly higher %N values in stem and foliage than clover, the differences in %N were not statistically significant, nor were they significantly different for $\delta^{15}N$. Knowing the contribution and efficiencies of BNF is important in the overall management of nitrogen in agricultural systems. As global populations continue to increase, it will become

increasingly important to maximize BNF as an agricultural input to minimize environmental impacts due to N losses.

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1. Introduction

Nitrogen (N) content in soils can limit and control the growth of plants. Although abundant in the atmosphere as N_2 gas, nitrogen is only usable by most plants in a fixed form of nitrogen (e.g., ammonium (NH_4^+) and nitrate (NO_3^-)). The ability to fix atmospheric N_2 comes from the symbiotic relationship between legumes and bacteria in the soil, such as *Rhizobia* (Liu et al. 2011). Through the products of photosynthesis, the legumes give the bacteria carbohydrates, while in exchange the *Rhizobia* provide legumes with N mainly in the form of NH_4^+ (Liu et al. 2011). The reduction of N_2 has a high-energy requirement, and so bacteria can only initiate the process with a large supply of carbohydrates. This process in which atmospheric N_2 is converted into forms of nitrogen that plants can take up is known as biological nitrogen fixation (BNF).

Globally, in agricultural systems, BNF accounts annually for around 50 to 70 Tg of N/yr (Herridge et al. 2008). This translates to about 14% of the annual fixation of reactive nitrogen (Nr [defined as all N compounds except N_2]) (Fowler et al. 2013). Agricultural BNF is primarily dominated by the relationship between N-fixing legumes and *Rhizobia*. With global increases in population, nitrogen input to agricultural systems is key to maintaining food security. Moreover, the potential of nitrogen fixation by N-fixing organisms to be used as a nitrogen input to agriculture is still generally underexploited.

BNF can be altered by factors on the landscape. Specifically, the rate of BNF is mainly controlled by four primary factors including the effectiveness of the symbiotic relationship between legumes and bacteria, the ability of the host legume to accumulate N, the quantity of nitrogen available in the soil, and the particular environmental constraints to BNF, such as high temperature and sensitivity to drought (Kessel et al. 2000, Bohlool et al. 1992). The fixation rate also varies by the physiological development stage of the legumes. Rates reach a maximum at the early flowering and seed-filling stages with a dramatic drop in fixation rates after that (Liu et al. 2011). Current environmental and physiological conditions must be taken into account for an accurate estimation of BNF rates. Knowing the contribution and efficiencies of BNF is important in the management of nitrogen in agricultural systems.

Locally, BNF can be an important input to an agricultural system and an alternative for mineral fertilizer use to minimize environmental impacts. The food production process requires an input of Nr, such as ammonium (Erisman 2007). In conventional agricultural systems, this input tends to come from the addition of synthetic N-fertilizer produced by the Haber-Bosch process. Since 1970, Nr creation by humans has increased by 120% (Galloway et al. 2008). Furthermore, a majority of this created Nr gets lost in the food production process, for instance from fertilizer runoff leaching into aquifers and rivers (Galloway et al. 2007). Crops in cereal-based farming systems tend to have low uptake efficiencies of nitrogen, due to its volatile and mobile nature (Crews et al. 2004). Nitrogen can therefore be lost in agricultural systems largely through nitrate leaching, ammonia volatilization and denitrification, in some cases, leading to adverse environmental conditions (Crews et al. 2004, Fowler et al. 2013). These environmental problems can vary from local to regional to global scale. Some local and regional impacts range from soil acidification, nitrate contamination of groundwater, eutrophication, anoxic dead zones and overall biodiversity loss (Crews et al. 2004, Erisman et al. 2008). Excess reactive nitrogen, on a larger scale, can also enhance smog, acid deposition, and greenhouse gas emissions from soils (Galloway et al. 2004).

In comparison, cultivation-induced BNF, or the N fixed from legumes grown in agricultural systems, reduces fertilizer N requirements, thus potentially decreasing reactive nitrogen loss. Although Nr can still be lost from legume-based cropping systems and contribute to environmental degradation, the risks are significantly less than traditional fertilizer systems. For example, Owens et al. (1994) completed an experiment on nitrate levels of groundwater after a switch in N source from traditional fertilizer to inter-seeded alfalfa in a pasture grazed by beef cattle. The study concluded that nitrate leaching decreased between 48 and 76% with the switch from ammonium fertilizers to legumes as the source of N (Owens et al. 1994). Legumes act as an alternative N source and can provide a benefit to subsequent crops in a field by enriching the N content in soils.

Alternative farming techniques aim to reduce these environmental impacts from food production. The USDA National Organic Standards Board (NOSB) defines organic principles of farming as "based on minimal use of off-farm inputs and on management practices that restore, maintain and enhance ecological harmony (Gomiero 2011)." Certified organic systems forbid chemical impacts like the use of synthetic fertilizers, pesticides, or hormones, minimizing environmental pollution. However, organic agricultural yields tend to be lower than on conventional farms (Seufert et al 2012). BNF has a high energy cost, and so fixation rates can be limited as a result of energy availability. Therefore, when Nr is added to soils through fertilizers or is already naturally abundant, nitrogen-fixing plants tend to obtain nitrogen from the available forms in the soil instead of from BNF.

Permaculture farming is an example of a non-conventional agriculture system that models the existing ecosystem processes and promotes natural nutrient cycling throughout the farm (Mollison 1988). It evaluates the farm as an entire system by strategically rotating the livestock and crops to minimize off-farm inputs and to maximize efficiency. Nitrogen use efficiency (NUE) is one measure for quantifying the amount of nitrogen added in to a system that is actually incorporated into the products. NUE is therefore equivalent to the intended N outputs (e.g., meat, eggs) divided by the intended N inputs (e.g., feed purchases, fertilizer application). Leach (2014) compared the NUE of conventional farms with those of a permaculture livestock farm by quantifying the nitrogen budgets and virtual N factors (VNFs). VNFs refer to the total Nr lost to the environment during the production of food (Leach 2014). The N surplus, or the unintended outputs to the environment, at the permaculture farm was 10-50% less on a per ha basis of that at conventional farms, as well; the beef VNF was substantially lower at the permaculture farm compared to the beef VNF at conventional farms (Leach 2014). Both results indicate that the permaculture farm has less of a negative local environmental impact than a conventional farm. Moreover, in comparison to organic systems, permaculture farming has not been as heavily researched (Leach 2014). As the approach gains traction and grows, new research is pertinent in evaluating the long-term effects and nitrogen efficiencies of permaculture farming.

Accurately estimating BNF contributions to agricultural systems is important in assessing their potential as an alternative to commercial fertilizer use. The most common methods for estimating N_2 fixation are through N difference, N balance, natural ^{15}N abundance, isotope dilution, and acetylene reduction (Herridge et al. 2008, Kessel et al. 2000, Anglade et al. 2015). ^{15}N natural abundance can be used to estimate BNF because of isotopic discrimination. The $^{15}N/^{14}N$ isotopic ratio in plant tissues is different for nitrogen-fixing plants, in comparison to plants that rely only on soil nitrogen (Carlsson et al. 2003). A mass spectrometer is commonly used to measure the differences in ^{15}N labeling of plant tissues and the amount of N yield (Carlsson et al. 2003).

Furthermore, Anglade et al. (2015) provide a straightforward way to estimate total BNF inputs, which includes the role of belowground contributions of N. They conducted a statistical analysis with data from existing literature that showed a strong relationship between shoot N yields and N_2 fixation rates in temperate regions globally. For alfalfa and clover, two different linear relationships to estimate N_2 fixed in plant shoots were developed, based on dry matter and N yield. Globally, there still lies a general uncertainty of the belowground contribution of fixed N (i.e. fixed N that is allocated to plant roots). Anglade et al. (2015) also include an estimate of belowground contributions by roots, nodules and rhizodeposition as a multiplicative factor of 1.7 for alfalfa and clover to estimate total N_2 fixation. Moreover, for forage legumes, belowground contributions varied from 19 to 75% of total plant derived N. The variability can largely be attributed to technical bias in the field, since rhizodeposits are not found in a well-defined structure, and legume root systems can be difficult to fully retrieve from the soil (Anglade et al. 2015). By using estimates from Anglade et al. (2015), this study is able to apply an existing relationship to different fields in a similar temperate zone.

This project examined the role and dynamics of BNF in pasture areas as an input to the overall nitrogen budget at Timbercreek Farm, a permaculture-like livestock farm, located in Albemarle County, Virginia. Leach (2014) completed a preliminary estimate of BNF in the pastures and in the forest at Timbercreek through a field survey and literature review. The total BNF was determined to be 0.7 t/N/yr with no nitrogen-fixing trees on the farm property. This current study provides a more intensive analysis of the role of BNF, and contributes to a better understanding of the nitrogen budget at Timbercreek Farm and permaculture systems in general. Permaculture farms are driven by the idea of using natural resources to deliver products efficiently. In order for a permaculture operation to be successful, it needs to enhance natural inputs of nitrogen with BNF.

Although the farm owners identify Timbercreek as approaching permaculture practices, it is worth noting that it is not a true permaculture farm. A true permaculture farm exemplifies a closed system that it does not require nutrient inputs because both crop and livestock production are used in rotation. Moreover, an estimation of BNF at Timbercreek will help the owners better understand the role of nitrogen-fixing plants and the potential to expand. The findings could also be used as a comparison for other livestock permaculture farms, but may not be suitable for true permaculture systems. Moreover, for the purpose of this study, Timbercreek Farm will be referred to as a permaculture farm.

The overarching objective of this study is to investigate the role of BNF at Timbercreek Farm. This objective is addressed through the following research questions:

1. BNF Budget at Timbercreek
    - How much does BNF contribute to nitrogen inputs in a local permaculture livestock farm?
2. Spatial Variability
    - What is the spatial variability of this potential of BNF across six pasture fields?
3. Variability of N Content in Plant Material
    - How does N content vary across different legume species across different fields?
    - How much does N-fixation contribute to N content in legumes?

2. Methods

2.1 Field Site Description

This study took place at Timbercreek Farm, a permaculture livestock farm, located in Albemarle County, Virginia (38.1° N, 78.6° W) at an elevation of 160m (Leach 2014) (Figure 1). The average annual temperature is 14.0°C, with an average seasonal range of 3.3°C to 24.2°C (NOAA 2016). This region has a total annual average precipitation of 120cm (NOAA 2016). The soils on the farm are dominated by loam (80%) and clay loam (20%) (USDA-NRCS 2010).

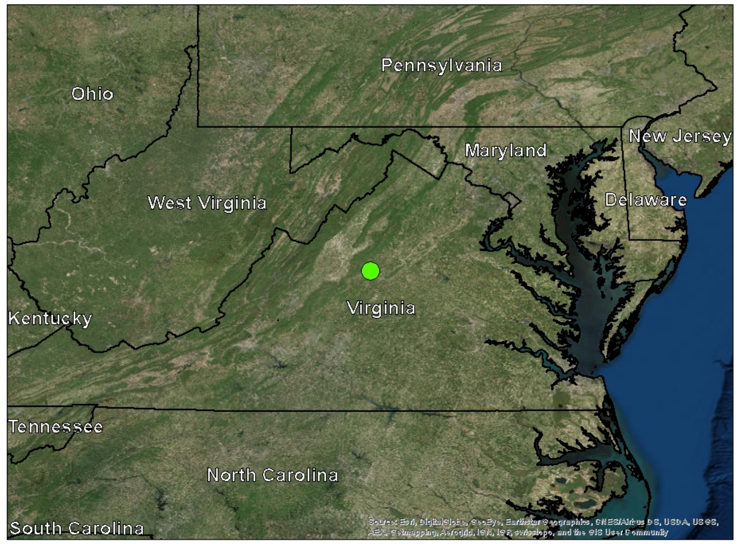

Figure 1. Timbercreek Farm is located in central Virginia in Albemarle County (38.1° N, 78.6° W).

Timbercreek Farm began its operations in 2010 and has a total area of 165 ha with 49 ha as pasture. Prior to the start of its operations, the land had been used for agriculture for centuries. The main products of the farm are poultry, beef, pork and eggs. As a permaculture farm, Timbercreek utilizes a rotational grazing method while keeping the farm pesticide, herbicide, fertilizer, antibiotic and hormone free. The farm is divided into seven distinct fields. Fields one through six are pastures, while Field seven is defined as a silvopasture, with a mixture of grasses and trees (Figure 2). The division is based on the geographic separation and land use history of the farm.

2.2 A vegetation survey to determine the legume density at Timbercreek Farm

A vegetation survey was conducted in August 2015 in each of the six fields that were identified as pastures. The point-line transect method was utilized to estimate the percent coverage of legumes (Abrahamson et al. 2011). Through GIS, three 50-meter transects were picked randomly in each field by generating three random points and a set of ten angles. When in the field, the angle from each point was chosen based on its ability to fit a 50-meter transect and on its spatial position in comparison to the other two points/transects (Figure 2), in order to maximize coverage throughout the pasture.

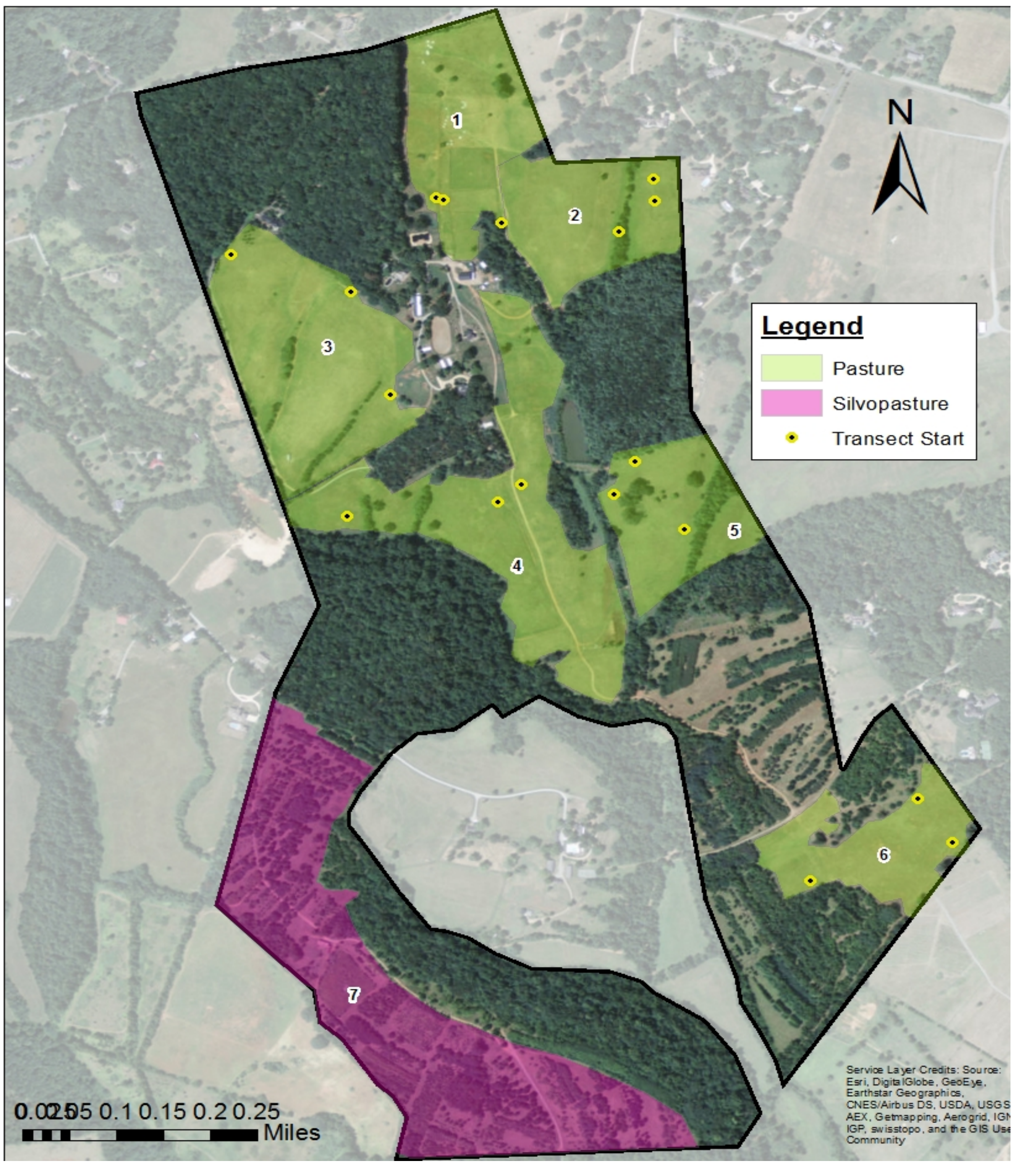

Figure 2. Timbercreek Farm property divided into seven fields. Fields 1-6 are pastures while field 7 is a silvopasture with a mix of grasses and trees. The yellow dots represent the randomly generated transect starting points.

Every meter, a pencil was dropped right above the knee. The cover type closest to the tip was identified as either legume, grass, or bare. If a legume, the plant was further identified by species. The average percent coverage among the three transects was assumed to be representative of the vegetation distribution in the entire field. The total vegetation distribution of legumes was used to scale up the N yield of individual plants to all the pastures.

2.3 Laboratory analysis to determine N and ^{15}N content in vegetation

Within each field, three samples of each found legume species and associated soils were extracted. It was important to extract the entire root system, so depending on the species and root depth the sample size ranged from 10x10x5cm to 10x10x10cm. All extracted samples were taken to the laboratory for processing and analysis. For each sample, the dry weight for each component (foliage, stems, roots, soil and litter) of the sample was recorded. The samples were then finely ground and analyzed with a mass spectrometer in Professor Stephen Macko's laboratory in the Department of Environmental Sciences at the University of Virginia in order to estimate the carbon, nitrogen, and ^{15}N contents in the material (Unkovich et al. 2008).

2.4 Estimating N_2 fixation by field and legume species

The amount of fixed N was estimated by first applying the linear relationships from the Anglade et al. (2015) study. The first estimate used dry matter (DM) data of the shoot material (kgN/ha/yr) (Equation 1 and Equation 2).

Equation 1: $y = 20.3DM + 2.49$ $r^2 = 0.83$ (Alfalfa)

Equation 2: $y = 25.6DM + 14.0$ $r^2 = 0.65$ (Clover)

The second estimate from Anglade et al. (2015) used the N yield (Ny) of shoot (kgN/ha/yr) (Equation 3 and Equation 4).

Equation 3: $y = 0.81Ny - 13.9$ $r^2 = 0.94$ (Alfalfa)

Equation 4: $y = 0.78Ny + 3.06 \quad r^2 = 0.94$ (Clover)

The natural abundance of ^{15}N in plant material was used as a proxy for rates of N- fixation.

A two-tail t-test was performed for each variable to determine the significance in the difference in N content of plant material between alfalfa and clover. The %N of stem, foliage and roots were compared between alfalfa and clover and the $\delta^{15}N$ of stem, foliage and roots were also compared between alfalfa and clover.

3. Results

3.1. BNF Contribution and Spatial Variability

3.1.1 Vegetation Survey

The vegetation survey determined the percent coverage and distribution of legumes (Figure 3). Of the six fields surveyed, three had low legume coverage of 10-17% and the remainder had moderate to high coverage of 26-37% (Table 1). In total, three legume species were identified in the pastures: *Medicago sativa* (alfalfa), *Trifolium repens* (white clover), and *Trifolium campestre* (hop clover). However, hop clover was only identified on two transects in Field 1. For the purpose of this study, hop clover and white clover were grouped as clover. All of the pasture fields observed both clover and alfalfa on at least one transect except for Field 4, where we only observed clover.

Table. 1. The percent coverage of legumes, grass and bare ground for all six-pasture fields and the average coverage at Timbercreek Farm.

	Field 1	Field 2	Field 3	Field 4	Field 5	Field 6	Average
Total area (ha)	7.6	6.2	10.3	13.7	5.1	5.6	
Legume (%)	17	26	37	10	15	29	22
Alfalfa (%)	*6*	*3*	*14*	*0*	*12*	*26*	*10*
Clover (%)	*11*	*23*	*23*	*10*	*3*	*3*	*12*
Grass (%)	44	65	54	47	84	67	60
Bare (%)	39	9	9	43	1	4	18

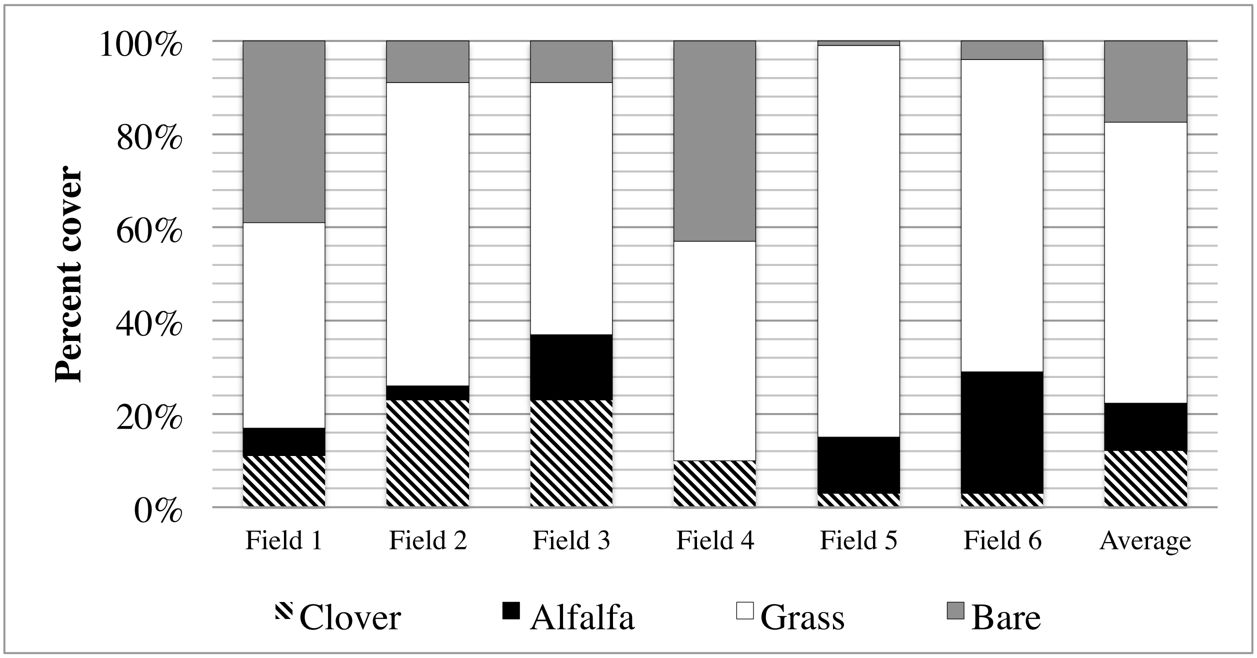

Figure 3. The percent coverage determined from a vegetation survey of six pasture fields at Timbercreek Farm in August 2015. The diagonal line bars indicate clover, black bars are alfalfa, white bars represent grass, and gray bars indicate bare spots.

3.1.2 Estimation of N_2 fixation based on dry matter (DM) yield

By applying the first set of linear relationships (Equation 1 and Equation 2) developed from Anglade et al. (2015), the amount of fixed N (kgN/ha) was estimated using shoot DM yield (t/ha). The method provided an estimate of total legume BNF in the pastures at Timbercreek to be 621 $kgNyr^{-1}$ (Figure 4). Anglade et al. (2015) included confidence intervals on the slope coefficients and intercept so the range of total BNF was calculated to be 484 to 758 kgN/yr. On a per unit area, Field 6 was contributing the most BNF at 27 kgN/ha/yr, and Field 5 had the lowest contribution at 5 kgN/ha/yr (Table 2). The calculated average rate of N_2 fixation was 49 kgN/ha/yr for alfalfa and 57 kgN/ha/yr for clover (Table 3).

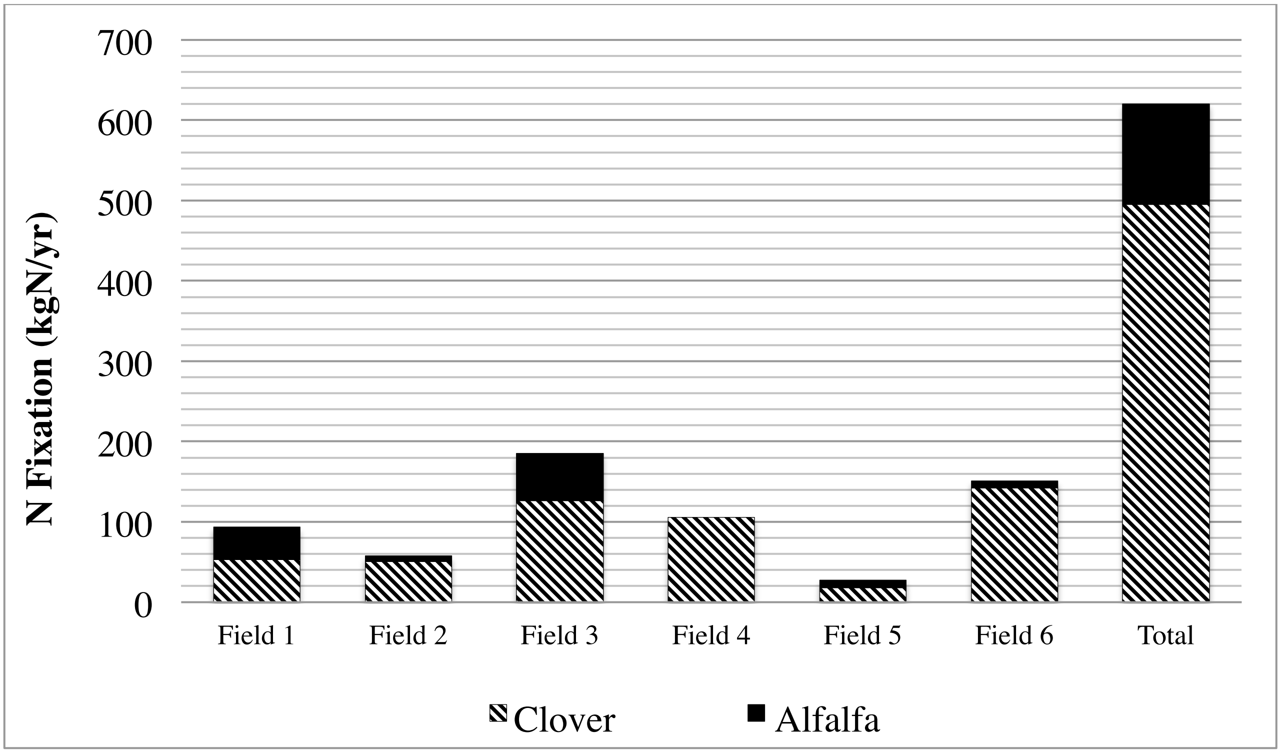

Figure 4. Estimates of total biological nitrogen fixation at Timbercreek Farm in 2015 based on the shoot DM yield and Anglade et al. (2015) linear relationships. The stripes represent clover and the black was alfalfa.

Table 2. Field estimations of total biological nitrogen fixation based on shoot DM yield (tN/yr) and Anglade et al. (2015) linear relationships.

	Field 1	Field 2	Field 3	Field 4	Field 5	Field 6	Total
Total area (ha)	7.6	6.2	10.3	13.7	5.1	5.6	48.5
Clover BNF (kgN/yr)	53	50	126	106	18	143	496
Alfalfa BNF (kgN/yr)	40	7.0	59	0.0	10	8.5	125
Legume BNF (kgN/yr)	93	57	185	106	28	151	621
Min. Legume BNF (kg/N/yr)	75	42	141	88	19	119	484
Max. Legume BNF (kg/N/yr)	112	73	230	124	36	181	758

Table 3. Field estimations of shoot N_2 fixed (kgN/ha/yr) based on shoot DM yield (t/ha) and Anglade et al. (2015) linear relationships.

	Field 1	Field 2	Field 3	Field 4	Field 5	Field 6	Average	Min.[1]	Max.[1]
Clover BNF Rate (kgN/ha/yr)	63	35	53	77	65	51	57	47	69
Alfalfa BNF Rate (kgN/ha/yr)	88	38	41	0	29	98	49	43	74

[1]Range of average N_2 fixation rates based on confidence intervals on the slope coefficients and intercepts from Anglade et al. (2015).

3.1.3 Estimation of N_2 fixation based on N yield

By applying the second set of linear relationships (Equation 3 and Equation 4) from Anglade et al. (2015), the amount of fixed N (kgN/yr), including the belowground contribution, was calculated using shoot N yield. This method provided an estimate of total legume BNF on the six pastures at Timbercreek to be 633 kg/N/yr and a range of 534-772 kg/N/yr (Figure 5). Field 5 had the lowest fixed N at 62 kg/N/yr, and Field 3 had the largest estimate at 213 kgN/yr (Table 4). The average rate of N_2 fixation was 89 kgN/ha/yr for clover and 40 kgN/ha/yr for alfalfa (Table 5). N_2 fixation rates ranged from 48 to 170 kgN/ha/yr for clover and from 4 to 91 kgN/ha/yr for alfalfa.

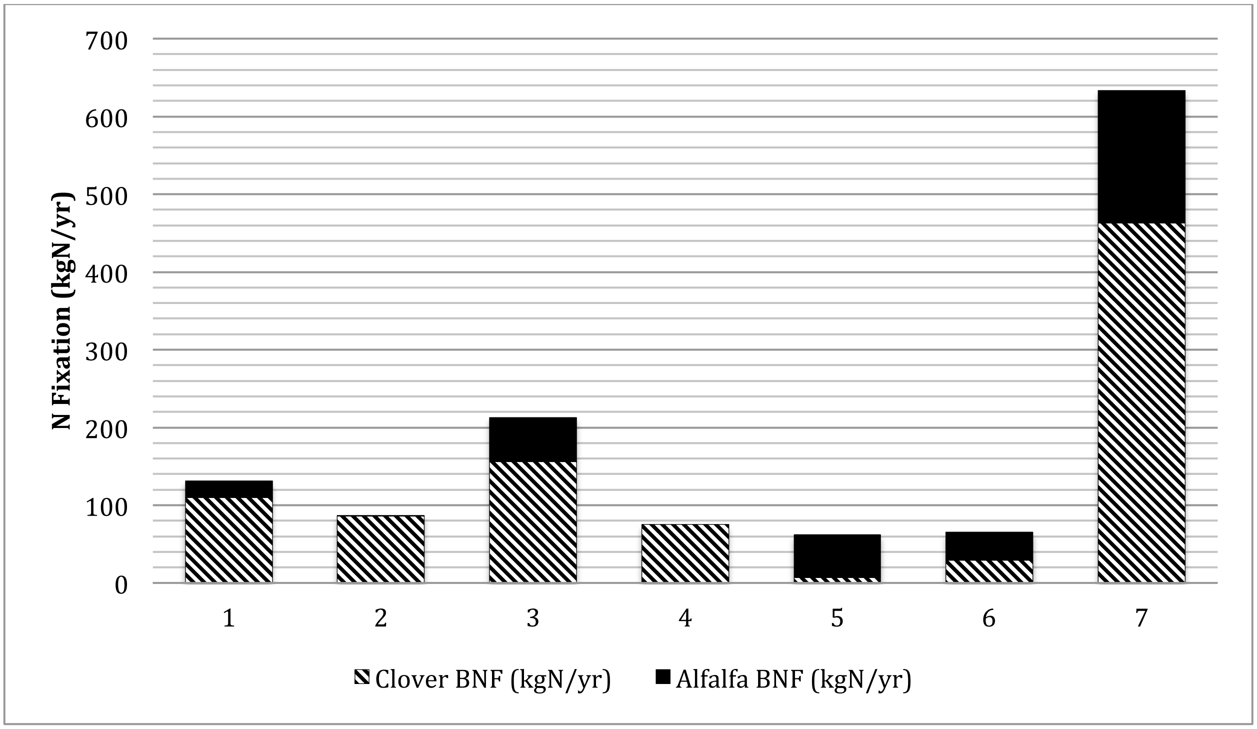

Figure 5. Estimates of total biological nitrogen fixation at Timbercreek Farm in 2015 based on the shoot N yield and Anglade et al. (2015) linear relationships. The stripes represent clover and the black was alfalfa.

Table 4. Field estimations of total biological nitrogen fixation based on shoot N yield (kgN/yr) and Anglade et al. (2015) linear relationships.

	Field 1	Field 2	Field 3	Field 4	Field 5	Field 6	Total
Total area (ha)	7.6	6.2	10.3	13.7	5.1	5.6	48.5
Clover BNF (kgN/yr)	110	86	156	75	7.3	29	463
Alfalfa BNF (kgN/yr)	21	0.83	57	0	55	36	149
Legume BNF (kgN/yr)	131	87	213	75	62	65	633
Min. Legume BNF (kgN/yr)	113	73	172	98	36	42	534
Max. Legume BNF (kgN/yr)	146	103	256	126	57	84	772

Table 5. Field estimations of shoot N_2 fixed (kgN/ha/yr) based on shoot N yield (kgN/yr) and Anglade et al. (2015) linear relationships.

	Field 1	Field 2	Field 3	Field 4	Field 5	Field 6	Average	Min. [1]	Max. [1]
Clover BNF Rate (kgN/ha/yr)	132	60	66	55	48	170	89	83	104
Alfalfa BNF Rate (kgN/ha/yr)	47	4	40	0	91	25	41	22	49

[1]Range of average N_2 fixation rates based on confidence intervals on the slope coefficients and intercepts from Anglade et al. (2015)

3.2 Variability of N content in plant material

By performing a t-test, the differences in N content between alfalfa and clover were evaluated. Although alfalfa had slightly higher mean %N values in stem and foliage than clover, the differences were not statistically significant. The mean %N value in stems for alfalfa was 2.5% and for foliage was 2.6%, whereas, %N of stems for clover was 2.4% and for foliage was 2.5%. The %N value for roots was also lower. For alfalfa the average value was 2.4% and for clover was 2.3%. The mean $\delta^{15}N$ value for foliage and stem was 2.5‰ for clover. The mean $\delta^{15}N$ value in foliage for alfalfa was 2.6‰ and in stem was 2.4‰. The mean $\delta^{15}N$ value for roots was lower for both alfalfa and clover. The average across the six pastures was 2.2‰ for alfalfa and 2.3‰ for clover (Figure 6).

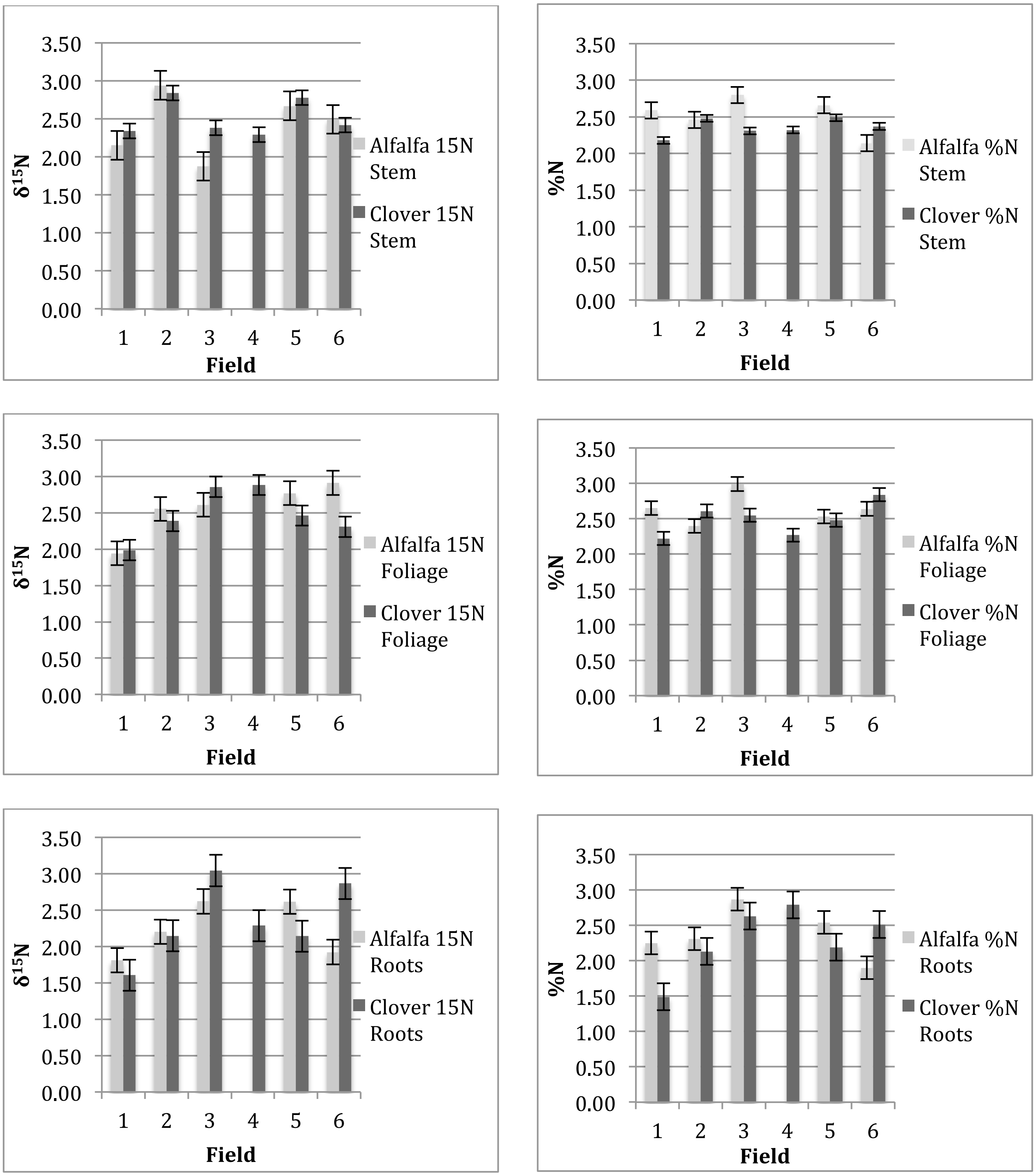

Figure 6. Mean δ^{15}N and %N content for alfalfa and clover samples from each of the six pasture fields at Timbercreek Farm with standard error.

4. Discussion

4.1 BNF Contribution at Timbercreek and Variability of N Content

4.1.1 Estimation of BNF based on Anglade et al. (2015) relationships

The rates of N_2 fixation, calculated with the linear relationships from Anglade et al. (2015), were generally consistent with the range of fixation rates from the literature. The average rates of N_2 fixation (based on shoot N yield) were 89 kgN/ha/yr for clover and 41 kgN/ha/yr for alfalfa (Table 7). Herridge et al. (2008) conducted a study on the estimates of global inputs of BNF in several agricultural systems. For legume-*Rhizobia* relationships in pasture and fodder agricultural systems, the rate of BNF was estimated to be between 110 to 227 kgN/ha/yr. Ledgard (2001) studied nitrogen cycling in temperate legume/grass pastures similar to those of Timbercreek Farm. For these low input, legume-based agricultural systems, N_2 fixation rates were estimated for clover in grazed pastures to be on average 59 kgN/ha/yr.

As mentioned earlier, Leach (2014) completed a preliminary study on BNF at Timbercreek Farm. BNF was estimated by observing root nodule effectiveness and average N_2 fixation rates from the literature. The literature review determined the range of N_2 fixation rates to be 88-234 kgN/ha/yr for alfalfa and 67-240 kgN/ha/yr for white clover (Table 7). The average rate was 169 kgN/ha/yr for alfalfa and 155 kgN/ha/yr for white clover. Leach (2014) estimated total legume BNF in all six pastures and in Field 7, a silvopasture, to be 700 kgN/yr or 4 kgN/ha/yr with a 20% effective nodule rate and the average N_2 fixation rates from the literature (Table 7). When accounting for just the six pastures, total BNF was estimated to be 438 kgN/yr. These results are consistent with my rates of total BNF calculated from Anglade et al. (2015). BNF, in the six pasture fields at Timbercreek Farm, was estimated in this study to be 621 kgN/yr based on the DM approach and 633 kgN/yr based on the N yield approach.

The two estimates of total BNF calculated in this study using Anglade et al. (2015) assumed all legume plants were fixing N. In comparison, the estimate from Leach (2014) assumed only a 20% effective nodule rate based on observations of nodules from legume samples. The Leach (2014) study also occurred in June 2014 while my study took place in August 2015. The difference in total BNF could be attributed to the two different sets of methods being used, the difference in sampling time, or perhaps even due to a change in the BNF rate at the farm.

Table 6. A summary of the N_2 fixation rates (kgN/ha/yr) from the DM and N yield estimates, from the literature review (Leach 2014), and from Leach (2014) estimates at Timbercreek Farm based on a 20% effective nodule rate.

	Methods for estimating pasture N fixation rates			
	Anglade et al. (2015) DM	Anglade et al. (2015) N yield	Leach (2014) Literature Review	Leach (2014) 20% Effective Nodule Rate
Clover Avg. N fixation rate (kgN/ha/yr)	57	89	155	
Alfalfa Avg. N fixation Rate (kgN/ha/yr)	49	41	169	
Legume Avg. N fixation rate (kgN/ha/yr)	53	67	140	4

4.1.2 Estimation of BNF based on the natural abundance of ^{15}N

The ^{15}N natural abundance results show evidence for BNF activity. Typically, the $\delta^{15}N$ of N_2 fixing legumes can be compared to the $\delta^{15}N$ of a non-fixing reference plant to generate estimates for %Ndfa, or the reduced fraction of plant N derived from atmospheric N_2 (Equation 5). In the case of this study, $\delta^{15}N$ estimates were only obtained for the plant material of legumes. However, if legume species were solely dependent on symbiotic N_2 fixation for growth, the isotopic composition would be similar to atmospheric N_2 ($\delta^{15}N$ = 0 ‰) (Unkovich et al. 2008). The $\delta^{15}N$ of a non-fixing plant, on the other hand, would be very similar to the $\delta^{15}N$ values of the soil mineral N (Unkovich et al. 2008). Therefore, legumes such as clover and alfalfa will have $\delta^{15}N$ values indicative of the relative inputs of the two N sources, atmosphere and soil. In the case of this study, the $\delta^{15}N$ of foliage for alfalfa and clover was on average 2.5‰. From Wang et al. (2007) and Aranibar et al. (2004), a likely range of $\delta^{15}N$ soil values for this region is 2.6 to 5.6‰ based on the age of the field and the precipitation. By taking the average of those values and the average foliage $\delta^{15}N$ value, the %Ndfa was calculated for the farm.

$$\%Ndfa = \frac{\delta^{15}\text{N of soil N} - \delta^{15}\text{N of N}_2\text{ fixing legume}}{\delta^{15}\text{N of soil N} - \delta^{15}\text{N of N}_2} * \frac{100}{1}$$

With an average soil $\delta^{15}N$ value of 3.9‰ and a foliage $\delta^{15}N$ of 2.5‰, the %Ndfa at Timbercreek was estimated to be 36%. This means that an estimated 36% of N in the plant could have derived from N_2 fixation or atmospheric N_2.

4.1.3 Comparison of methodologies for determining total BNF

The abundance of ^{15}N is just one way to measure N_2 fixation. It requires technical skills and sophisticated, well-maintained equipment to accurately assess the isotopic composition of plant and soil material. However, it provides a high level of confidence and a powerful means for directly evaluating N_2 fixation within the field. Besides growing plants in a soil N-free environment, abundance of ^{15}N is the only methodology to unambiguously verify active N_2 fixation (Unkovich et al. 2008). In comparison, the application of Anglade et al. (2015) provides an easy and cost effective method for determining BNF through measurable variables. It does not require the same level of technicality, however, the results have a much lower certainty as Anglade et al. (2015) assumes that N_2 fixation is occurring as long as there are legumes present.

The level of certainty also varies between the two Anglade et al. (2015) relationships. The linear relationships based on DM, or strictly biomass, have a much lower certainty and lower r^2 values of 0.65 for clover and 0.89 for alfalfa. It assumes the more biomass there is in the legume sample, the more N_2 fixation. The N yield linear relationships have a higher r^2 value of 0.94 for both legume species and take into account %N instead of simply biomass.

Another important aspect of this study was the inclusion of belowground N in the linear relationships of N yield from Anglade et al. (2015). In the past, it was assumed that shoot N was a reasonable estimate for total plant N. However, evidence shows 30-50% of the total plant N of legumes can be allocated to (nodulated) roots (Unkovich et al. 2008). The estimates of N_2 fixation derived from Anglade et al. (2015) based on N yield included a 1.7 multiplicative factor for belowground contribution under the assumption that the harvested aboveground yield was equivalent to the total aboveground production, which it likely was.

4.1.4 The context of BNF to total nitrogen inputs at Timbercreek Farm

Another important part of this study was determining the significance of BNF within the context of all nitrogen inputs to livestock production at Timbercreek. As an approaching permaculture livestock farm, besides legume BNF, the main inputs of N for the grazing livestock are from hay and N already within the imported livestock. There is also N from atmospheric deposition and the N mineralized from soil organic matter. At Timbercreek, the cattle primarily feed on forage and are only supplemented with hay during the winter months. To evaluate the current management of BNF at the farm, a rough estimate of total forage intake for the cattle was determined. Estimates of total forage intake (kg) were found by first calculating the weight gain (kg) of cattle, by subtracting live weight imported to the farm (kg) from total live weight sold (kg). The weight gain was then multiplied by an estimated feed conversion ratio, or the amount of kilograms of feed needed to produce 1 kilogram of weight gain. To determine the total forage intake (kg), the hay intake (kg) was subtracted from the total feed intake (kg) (Laura Cattell Noll, personal communication).

N in forage (kgN) was then estimated using the percent coverage of legumes per field and the dry matter %N content of the respective legume species. The %N in forage that could potentially come from BNF is represented by the ratio of total BNF to the amount of N in forage. Due to the assumptions taken into account in estimating total forage intake and the uncertainties associated with the BNF estimates of this study, the amount of BNF attributed to N content in forage is likely to have a large uncertainty. However, it still helps to understand the potential relative importance of total BNF in terms of management implications for grazing pastures. By taking the average over three years of N budget data, an estimated 30% of the N in forage could potentially come from BNF (Table 7). This estimate is similar to the earlier estimate of %Ndfa of 36%, which indicates how much of the N content in the plant is coming from BNF relative to soil N.

Table 7. Estimation of %N in forage that could potentially come from BNF based on total forage intake (kg) and the total BNF estimate at Timbercreek Farm of 633 kgN/yr.

Year	Total Forage Intake (kg)	Forage N (kgN)	%N in Forage that could come from BNF (%)
2013	138,800	3,400	19
2014	76,800	1,900	33
2015	69,900	1,700	37

4.2 Spatial Variability

4.2.1 Management implications of BNF estimations

Given that Timbercreek is a permaculture farm, the results of this study could suggest planting and inoculating legumes in more pastures. The vegetation survey showed variability in legume coverage over the six pastures. For example, Field 4 only had 10% legume coverage and Field 5 had 15% coverage. Leach (2014) found a similar variability in legume coverage in the summer of 2014. The average percent coverage of legumes was 29%, and Field 5 and Field 4 had the lowest percentages of 0% and 16% respectively. The farmer, Zach Miller, has developed a management strategy to revitalize the pasture fields and started with Field 4 in the summer of 2015 by planting millet with a mix of inoculate, or *Rhizobium* bacteria, that is specific to a particular legume species and in this case clover (Zach Miller, personal communication). Field 5 is the next field to begin this revitalization process. By increasing *Rhizobium* in the soil, rates of nitrogen fixation could be maximized.

4.3. Future Work

BNF was estimated using samples collected at Timbercreek Farm during late August. Future work could incorporate temporal variability of BNF by conducting vegetation surveys and sample collections during different seasons. By estimating rates of fixation throughout the year, total BNF can be better understood, as the livestock consume forage all year round. As farmer Zach Miller conducts the revitalization in the pastures, it would be interesting to resurvey the percent coverage of legumes and N content to verify change overtime.

This study focused on foliage N content, however, a dynamic relationship exists between plant N and soil N. Legume fixation in pastures is primarily affected by legume persistence and production, soil N status and competition with associated grasses (Ledgard and Steele 1992). To better understand BNF efficiencies and short and long term variability, it is important to further study soil N. Moreover, BNF in pasture systems can be transferred belowground through decomposition of dead plant material, mineralization, and through the return of animal excreta.

By further studying the soil N dynamics and waste dynamics at Timbercreek Farm, the overall role of BNF and its contribution to forage could be more accurately determined.

5. Conclusion

Legumes act as an alternative N source and can minimize fertilizer N requirements. As global populations continue to increase, it will become increasingly important to maximize BNF as a nutrient input to minimize environmental impacts. This study focused on Timbercreek Farm, a permaculture livestock farm, in Albemarle County, VA, which relies on BNF as an N input to its pastures. Alternative agricultural systems, like permaculture farming, need to enhance natural inputs of nitrogen with BNF as these systems discourage the use of traditional nitrogen fertilizers. The total estimation of BNF was 633 kgN/yr in 2015, which was an increase from a previous estimate by Leach (2014) of 438 kgN/yr in 2014. The average rate of BNF was 89 kgN/ha/yr for clover and 41 kgN/ha/yr for alfalfa. The $\delta^{15}N$ values showed evidence for BNF activity and a %Ndfa of 36% was estimated from the foliage $\delta^{15}N$ values. By applying DM and N yield linear relationships from Anglade et al. (2015), N_2 fixation was estimated easily and cost-effectively.

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7. Appendix

Table 8. Coordinates for the start and finish points of each of the transects generated randomly across the six pasture fields.

Field	Start/Finish	Y	X
1	Start	38.1006710	-78.5629633053851
1	Start	38.1006800	-78.5629288207214
1	Start	38.1002130	-78.5617128176528
1	Finish	38.1006140	-78.5619389412585
2	Start	38.1009160	-78.5587018374233
2	Finish	38.1005310	-78.5583953658984
2	Start	38.1005290	-78.5586919376663
2	Finish	38.1005360	-78.5586118723446
2	Start	38.1000030	-78.5594050188539
2	Finish	38.0996300	-78.5596910841035
3	Start	38.0991820	-78.5645749798781
3	Finish	38.0991320	-78.5647590926839
3	Start	38.0972120	-78.5638878241651
3	Finish	38.0971590	-78.5643684910483
3	Start	38.0987190	-78.5648640061097
3	Finish	38.0996840	-78.5674888425921
4	Start	38.0951340	-78.5648918431112
4	Finish	38.0955180	-78.5651755399208
4	Start	38.0953250	-78.5619323450018
4	Finish	38.0954860	-78.5623146634518
4	Start	38.0956120	-78.5614552993417
4	Finish	38.0952720	-78.5611245479825
5	Start	38.0959710	-78.5592313158565
5	Finish	38.0958040	-78.5585755379981
5	Start	38.0954000	-78.5599230565026
5	Finish	38.0956700	-78.5594807851505
5	Start	38.0943180	-78.5581461409205
5	Finish	38.0942890	-78.5589338330850
6	Start	38.0944070	-78.5585308430975
6	Finish	38.0886230	-78.5559984646958
6	Start	38.0885610	-78.5560233509372
6	Finish	38.0879970	-78.5545029800852
6	Start	38.0892250	-78.5537437804319
6	Finish	38.0891540	-78.5537689690614