NITROGEN BENEFITS WHEN INTERSEEDING RED CLOVER INTO CORN

By

Hannah Francis

A thesis submitted in partial fulfillment of
the requirements for the degree of

Master of Science
(Agroecology and Soil Science)

at the

UNIVERSITY OF WISCONSIN-MADISON

2020

APPROVED BY:

Matthew D. Ruark

Erika Marin-Spiotta

Doug Soldat

Rodrigo Werle

ACKNOWLEDGMENTS

I owe enormous gratitude to the soil science and agroecology communities here at UW Madison. First, my advisor Dr. Matt Ruark has welcomed me into projects where I could adapt my geoscience background and grow in agricultural knowledge. He gave me flexibility to follow-through on statistical curiosity with enough scaffolding to be directive. Matt set goals for research and publication at when I began at UW-Madison, and has actively encouraged, pushed, and finessed the process to achieve those goals. I have certainly grown my knowledge base and as a researcher with Matt's guidance.

My peers in the Ruark Lab (Miranda Sikora, Alexandra Walters, Kelsey Kruger, Ashmita Rawal) provided consistent support, help in the field, encouragement, and were valuable resources through classes and trouble-shooting research. My agroecology cohort (Marnie Macgregor, Margaux Crider, Korede Olugbenle, Kelsey Kruger, Kase Wheatley, Dana Johnson, Ambar Carvallo, and honorarily Ashley Becker and Anika Rice) stretched my mind and engagement with soil science and agriculture toward a necessary inclusion of social science, with stimulating conversations and life-long friendships. All these people have encouraged me to question current systems and think imaginatively and regeneratively.

I would not have completed any field work without a great team of field techs (Chelsea Zeigler, Jessica Ross), folks at Arlington Agricultural Research Station (Andy Larson and

Dan Smith), and undergraduate employees over the summer (Zach Tracy, Conner Becka, Emma Nelson) who confidently executed field work and taught me how to correctly collect samples. I similarly could not have felt as supported in the UW-Soil Science building or Agroecology program without the support from the wonderfully kind and incredibly knowledgeable faculty and staff. I learned a lot from engaging classes, rigorous field trips, and intentional assignments.

Finally, though certainly these few paragraphs hardly name all of the people who have been instrumental in this journey, I am ever grateful to my family. Michelle, Lee, Laila and the extended Gittler/Francis contingent taught me to feel empowered to ask questions, that pursuit of social justice is expected, and that it’s important to find time to play outside. Thanks for soliciting 6 a.m. (Michelle and Lee) and 11 p.m. (Laila) phone calls that nourished a need to process the world and still find ways to laugh uncontrollably.

TABLE OF CONTENTS

Table of Contents

LIST OF FIGURES

LIST OF TABLES

Introduction

This study investigates the nitrogen benefits of interseeding red clover into corn. As of 2019 in Wisconsin, there are around 4,000,000 acres of corn planted (USDA NRCS, 2019). This presents a challenge for farmers who are interested in incorporating more cover crops into their fields; there are too few growing degree days after harvesting corn to establish a cover crop. To successfully incorporate more cover crops into fields in Wisconsin, farmers need more options to integrate cover cropping into current rotational systems. Interseeding red clover presents a solution to this problem.

Red clover builds biomass better than many over cover crops, over-winters from fall into the following year's spring, and grows well in low radiation environments (J. Stute & Shelley, 2009). As a legume cover crop, red clover has added value in the potential to reduce synthetic nitrogen fertilizer inputs through biological nitrogen fixation (Peoples et al., 2009; Sarrantonio & Gallandt, 2003; J. Stute & Shelley, 2009). Clover has a low C/N ratio (between 13.6. to 16.7) so the nitrogen in its biomass can become quickly available to a succeeding corn crop, where a study in Wisconsin showed 50% mineralization two weeks after clover termination (Bruulsema & Christie, 1987; J. K. Stute & Posner, 1995a). Other studies report yield increases following red clover cover cropping, potentially increasing the value of red clover beyond input reduction (A. Gaudin et al., 2013; White et al., 2017).

The first chapter of this study will present a statistical method for comparison of the optimum nitrogen fertilizer rates. While a common objective of agronomic research is to determine differences in management practices in relation to optimum nitrogen fertilizer rates, the comparison is not statistically standardized. Most studies compare outputs from agronomic studies with coarse nitrogen fertilizer rate trials, or compare fertilizer response curve models, but do not have a statistical method to compare the optimum fertilizer rate. This chapter presents a simple bootstrapping approach with a quadratic plateau curve model as a way to standardize this statistical comparison. To this end, I worked with a statistical consultant to create an R package (FertBoot) to process data using this method.

The second chapter addresses the viability of interseeding red clover in a continuous corn, no-till system in Wisconsin. Field studies were conducted at Arlington Agricultural Field Station in Arlington, Wisconsin from 2017-2020 on two nearby fields. The field study was a randomized complete-block, split-plot design with clover and no clover whole plot treatments, and nitrogen fertilizer rates as split plot treatments. The bootstrapping approach was employed to process the fertilizer response curve data for corn grain yield. The first site year found a statistically significant reduction in nitrogen fertilizer needed when corn followed corn interseeded with red clover, however the second site year did not find any meaningful nitrogen input from the clover. The lack of nitrogen input from clover is explained through biomass establishment, which is reflected in the soil plant available nitrogen.

Future field studies that explore interseeding cover crops, and interseeding red clover are encouraged to process data for statistical significance with the bootstrapping approach. While this current study did not recommend interseeding red clover as a way to incorporate more cover crops into the Wisconsin landscape, agronomic research should explore interseeding red clover with considerations of corn row spacing, planting density, relative maturity, and look to rely on qualitative data gathering from farmers using red clover already.

Chapter 1. Toward a standardized statistical methodology comparing optimum N rates among management practices: a bootstrapping approach

Abstract

Agronomic research lacks statistical standardization to compare optimum nitrogen fertilizer rates. There are a range of approaches to compare differences between or among optimum nitrogen fertilizer rates resulting from different management practices, but traditional statistical approaches often fail or are inappropriate when directly comparing optimum nitrogen fertilizer rates determined from fertilizer response curve models. Statistical comparisons fail when sample sizes are too small and fertilizer rates are too coarse for a response curve. In addition, traditional methods require assumptions about the underlying population distribution that may not necessarily apply to agronomic data. Previous studies use a variety of statistical methods to evaluate experimental designs with small sample sizes (3-4 replicates) and a range of nitrogen fertilizer rates (5-8 discrete rates). While this approach allows some statistical comparison of the resulting N response curves, the resulting optimum N rates produced from non-linear regression most often remain statistically incomparable. We know of no single approach used in the agronomic literature that allows for the direct comparison of optimum N rates produced with quadratic-plateau response curves. To provide the statistical rigor needed for clear recommendations for greater or less N need based on specific management practices (e.g. tillage, N source, or cover cropping), here we propose a bootstrapping approach that resamples residuals with replacement. The importance of sharing and shaping methodology motivated the creation of an R package called "FertBoot" to be used with open-source software RStudio. While bootstrapping is not new to data processing in agronomic fields, here, we provide examples of how to conduct residual-resampled bootstrapping with non-linear regression to identify differences in response curves, optimum N rates, and maximum yields using the FertBoot package in RStudio. Two example data sets assessing differences between red clover cover crop and no cover crop were used for this analysis. After the bootstrapping approach was completed, response models were compared using the log likelihood ratio (LLR) test and response variables of interest were tested using a two-sample bootstrap test. Our example data sets provide clear evidence of the value of the bootstrapping approach, as it can aid in determining significant differences between even relatively small differences in optimum N rate (e.g. 20 kg ha^{-1}). We encourage adoption of this approach as a way to accurately evaluate differences in optimum fertilizer between or among treatments to better inform future agronomic decision making.

Introduction

A common objective of fertilizer research is to determine how management practices affect the optimum nitrogen fertilizer rate. Examples of such practices include tillage, manure application, residue management, and cover cropping, with the difference in optimum N rates between the treatment and the control being considered the N fertilizer replacement value of an input or practice. A common experimental design to address this objective is a randomized complete block, split plot design with the agronomic treatment as the main plot factor and nitrogen rate as the split-plot factor. There are a range of approaches that are used to analyze the data and report treatment differences. First, studies may use ANOVA to identify treatment and interaction effects and then conduct linear or nonlinear regression when ANOVA effects were significant (e.g. Caldwell et al., 2014; Ruark et al., 2018). Then studies will use either R^2 or root mean square error (RMSE) to determine if linear, quadratic, linear-plateau, or quadratic plateau (or often other models) are the model of best fit, with the model of best fit being reported (e.g. Pantoja et al., 2015; Woli et al., 2016) or multiple models being reported (e.g. Finney et al., 2016). Along with model coefficients, the resulting optimum N rate (the N rate at which the yield plateaus) and maximum (the plateau) yield are reported. However, most studies report the calculated optimum fertilizer rates without statistical comparison (Chatterjee et al., 2018; Ruark et al., 2018; Rubin et al., 2016; Sawyer et al., 2010; Schmidt et al., 2002; Steinke et al., 2015; Woli et al., 2016), highlighting a severe limitation of our traditional statistical approach. Without the statistical ability to determine if there are significant difference between or among optimum N rates or maximum yield as determined from the nonlinear models, we cannot properly assess the validity of the calculated fertilizer replacement value associated with a new input or management practice. This highlights a need for a standardized statistical approach that allows a meaningful comparison between or among optimum N rates from different treatments.

The difference in optimal N rate is an important variable of interest, but traditional statistical approaches do not allow researchers to identify if this difference is statistically true. An approach using bootstrapping residuals with non-linear regression offers that ability. Bootstrapping is a statistical method used to address the problem of finding a sampling distribution of a variable when the probability distribution is unknown (Efron, 1979). To estimate the sampling distribution of a variable, the bootstrap approach uses independent resampling with replacement from an existing data set (resampling usually upwards of 1000 or more times) to produce new data sets. Each replicate of the bootstrapping approach develops a new response curves by estimating a new Y value for each X value by adding the original predictive value to a randomly selected residual value. In our example with nonlinear regression modeling, the new data set contains model coefficients, plus the optimum N rate and maximum yield (the plateau yield) calculated from each model. Statistics can then be applied to this data set to determine if there were differences between or among models, optimum N rates, and maximum yield.

However, bootstrapping is not new to agronomic research. There are many fields of research where they have been utilized. For example, in agronomic-focused journals we see it used with meta-analyses (Wortman, 2016; Wortman et al., 2017; Zhang et al., 2020), field-crops research (King & Blesh, 2018; Wang et al., 2013; Zheng et al., 2009), ecological modeling (e.g. Heikkinen & Mäkipää, 2010), in conjunction with artificial neural networks (Zeng et al., 2016), as cross-validation of variables (Corstanje et al., 2009), in spectroscopy (Yang et al., 2020) and hyperspectral methods (Kawamura et al., 2013; Li et al., 2014) which all employ bootstrapping to determine confidence intervals or compare regressions. While bootstrapping has also been utilized to determined confidence intervals for optimum fertilizer rates, the approaches other studies did not focus on replicability or statistical comparison of the optimum fertilizer rates (Hernandez and Mulla, 2008; Qin et al., 2019). In contrast to studies that have used bootstrapping in the past, this bootstrapping technique uses commonly used and relatively simple models that focus on the optimum nitrogen fertilizer rate as a function of model parameters. Our approach is novel because we use bootstrapping both for predictive distributions of our variable of interest and for statistical testing between treatment differences. The bootstrapping technique proposed here can be utilized in any study where researchers want to identify how differences in management (e.g. tillage, cover cropping, nitrogen fertilizer source) affect optimum N rates. This approach is specifically valuable to determine differences in management practices designed to reduce the overall need for commercial N fertilizer, such as green manure cover crop use, following a stand of perennial legumes, and manure applications, ensuring that the fertilizer replacement value of these inputs is real. Here, we provide examples of how to conduct residual-resampled bootstrapping with non-linear regression to identify differences in response curves, optimum N rates, and maximum yields from two green manure cover crop studies using the FertBoot package in R (Ma & Francis, 2020b).

Materials and Methods

Description and Experimental Design of Experimental datasets

Two unpublished nitrogen response data sets are used for this study, both comparing the nitrogen replacement value of red clover (*Trifolium incarnaturm L.)* as a green manure cover crop. The first study evaluates red clover in a wheat (*Triticum aestivum*)-corn (*Zea Mays*) (WC) rotation and the second study evaluates red clover in a corn-corn (CC) rotation. In the WC study, red clover was seeded into wheat on 23 March 2018, wheat was harvested on 24 July 2018, red clover was mowed down on 30 Aug. 2018 to prevent competition with corn growth, and then chemically terminated on 18 Oct. 2018 with 2,4-d or dicamba with glyphosate. Corn was planted in 2019 with six different rates of surface applied urea coated with a urease inhibitor (Agrotain®, Koch Agronomic Services, LLC) (0, 56, 112, 168, 224, 280 kg-N ha^{-1}). In the CC study, corn was planted on 25 May 2017 and red clover was interseeded on 26 June in 2017. In 2018, corn was planted on 24 May and red clover was terminated on 1 June with 2,4-d or dicamba with glyphosate. In 2018, corn was planted again and urea coated with a urease inhibitor (Agrotain®) was surface applied at eight different rates (0, 45, 90, 135, 180, 225, 270, and 315 kg-N ha^{-1}). Red clover was not interseeded again in 2018. In both studies, the experimental design was a randomized, complete block, split plot design, with two whole plot treatments (with and without red clover) and N rates as the split plot factor. Both studies were conducted at the University of Wisconsin Arlington Agricultural Research Station on Plano silt loam (fine-silty, mixed, superactive, mesic Typic Argiuodoll) soil. Both studies were conducted without tillage during the two crop phases. Raw yield data from each study are reported in Table 1 (WC) and Table 2 (CC).

Table 1. Nitrogen fertilizer rates and corn grain yield for each replicate (R) following no cover crop (None) or red clover (RC) in 2019 the wheat-corn (WC) rotation study. Red clover was frost-seeded into wheat in 2018 and terminated in the late fall of 2018.

		Grain yield			
Treatment	N rate	R1	R2	R3	R4
	kg ha^{-1}	------------------------Mg ha^{-1}------------------------			
None	0	3.89	9.05	9.16	6.54
	56	9.22	10.2	8.88	7.57
	112	10.1	11.9	11.4	10.9
	168	12.0	14.1	12.6	13.2
	224	12.7	14.1	13.3	12.9
	280	13.5	13.2	14.4	12.5
RC	0	9.34	11.8	12.4	10.3
	56	12.4	14.0	13.8	10.0
	112	12.3	13.5	14.5	11.8
	168	12.5	13.1	14.0	12.0
	224	11.9	13.1	15.0	13.4
	280	12.3	12.7	14.8	14.0

Table 2. Nitrogen fertilizer rates and corn grain yield for each replicate (R) following no cover crop (None) or red clover (RC) in 2019 the corn-corn (CC) rotation study. Red clover was interseeded in 2018 and terminated before corn planting in 2019.

		Grain yield			

Treatment	N rate	R1	R2	R3	R4
	kg ha^{-1}	------------------------Mg ha^{-1}------------------------			
None	0	5.58	4.99	7.17	3.69
	45	8.53	8.50	9.50	9.62
	90	10.3	11.9	9.03	9.97
	135	11.0	11.3	6.78	11.6
	180	11.2	11.2	8.27	9.61
	225	10.6	11.3	10.6	11.4
	270	11.3	11.1	9.62	11.7
	315	11.5	10.4	8.70	11.3
RC	0	4.86	4.56	6.61	4.66
	45	7.76	9.26	10.4	9.12
	90	9.11	11.1	9.62	9.89
	135	8.34	10.6	7.81	8.60
	180	6.66	11.2	9.72	10.5
	225	10.8	10.2	10.4	10.5
	270	10.8	10.2	10.7	10.2
	315	10.3	10.6	10.3	10.9

Bootstrapping Statistics

In our analysis, we compared optimum nitrogen fertilizer rates from quadratic-plateau models. Preliminary analysis comparing quadratic, linear-plateau, and quadratic-plateau curves (NLIN in SAS, and confirmed with easynls package in R) was used to identify that quadratic-plateau response curves (Eq. 1) had the lowest RMSE among models for these datasets. This was expected as quadratic-plateau models have been previously shown to be the model of best fit for corn nitrogen response analysis (Cerrato and Blackmer, 1990). All data for each cover crop treatment (6 or 8 N rates and 4 replicates) were used in the analysis. Quadratic-plateau parameter estimates and standard error and

95% confidence intervals of the quadratic-plateau model are produced with the easynls package. Results of this analysis are also used to determine the optimum N rate and the maximum yield.

Quadratic Plateau:

$$y_{ij} = \begin{cases} a_i + b_i x_{ij} + c_i x_{ij}^2 & \text{, if } x_{ij} < x_{m,i} \\ y_{m,i} & \text{, otherwise} \end{cases} \qquad [1]$$

where $x_{m,i} = \frac{-b_i}{2c_i}$ is the optimal N rate and $y_{m,i} := a_i + b_i x_{m,i} + c_i x_{m,i}^2$ is the maximum yield response to N rate for the i-th treatment.

The FertBoot package in R (Ma and Francis, 2019) was used to determine significant differences in model coefficients, optimum N, and max yield between cover crop treatments. Within FertBoot, quadratic plateau models were fit and residuals of the model were resampled (with replacement) 1000 times to produce a population estimate data set of a, b, and c coefficients, and of optimum N rates and maximum y values. Once the bootstrap was complete, the bootstrapped results that included X values outside of the range of N rates in the study were dropped. In addition, if optimum N values greater than the largest N rate used in the study was determined, that value was replaced with the largest N rate used in the study which was then used to recalculate the maximum yield. The bootstrapping approach will produce different coefficients for the plateau curves compared to the original model, but ultimately produces a more robust measurement of the variability. The ggplot2 package in R can be used to plot the bootstrapped models and their 95% confidence intervals with the original data points. An example of the code to create a graph like this can be found on the author's personal website (Ting Fung Ma

2020). Density curves were produced using the bootstrapped output of optimum nitrogen fertilizer values with ggplot (geom_density) in R to visualize differences in optimal N rates.

Confidence intervals of the mean of each response variable were determined as bootstrapped, bias-corrected and accelerated 95% confidence intervals (Davidson & Hinkley, 1997). The log-likelihood ratio (LLR) test was used to determine if bootstrapped determined response models were different between the two cover crop treatments. Models are considered different if at least one coefficient estimate was significantly different ($P<0.05$) between models. Differences between optimum N rates and maximum yields were determined using a two-sample bootstrap test (using 1000 replicates) at $P<0.05$. Bootstrapping becomes more powerful with increased replication, at the cost of computational capacity. A minimum of 1000 replicates is often suggested, while increasing replication can extend an already time-intensive computation time.

Results and Discussion

Red clover in a wheat-corn rotation

Both the original and bootstrapped quadratic-plateau models produced similar coefficients, with the exception that the optimum N value was 133 kg ha^{-1} for the original and 158 kg ha^{-1} for bootstrapped (Table 3). The 95% confidence intervals on model coefficients remain large even with bootstrapping (Table 3), although the two models (None and RC) were determined to be different using LLR test (p-value = $6.12*10^{-5}$). The red clover treatment did not affect maximum yield (Table 3). Bootstrapped quadratic-plateau curves appear visually different with respect to optimum N rate (Figure 1) and confirmed different by the two-sample bootstrap test. The two-sample bootstrapped t-test was able to determine optimum N rates were significantly different even with large 95% confidence intervals because the newly produced bootstrapped data set is sufficient in size (n) (as visually observed in Figure 2). We would not have been able to make assertions relative to if the difference of 135 kg ha^{-1} (determined as the difference between two quadratic-plateau models) was a true effect using traditional methods. Although, with a difference of 135 kg ha^{-1} between optimum N rates we would likely assume this result is real.

Red clover in a corn-corn rotation

The data set for determining the N replacement value of red clover in a corn-corn rotation differed from the previous dataset by having a greater number of N rates (eight vs six). In addition, this dataset produced a much smaller difference in optimum N rates between response curves. However, the results of this analysis produced similar quadratic-plateau curves and large 95% confidence intervals of the coefficients between the none and RC treatments. The similarities between the two treatment models are reflected in the results from the LLR test; the two models were significantly different (p=0.0048). The optimum N rates differed between the two treatments by 20.2 kg ha^{-1} using the original curves, and by 16.9 kg ha^{-1} using the bootstrapped model (Figure 3, Table 4). The two-sample bootstrap test determined that the difference of 16.9 kg ha^{-1} was significantly different than zero and the density plots revealed a small but statistically meaningful shift in distribution (Figure 4). Knowing that this difference was statistically meaningful can help with both research and outreach objectives. First, by determining statistical significance, we can discuss the results as being real, as opposed to simply speculating based on the 95% intervals of the model coefficients and relative size of the difference. We can then appropriately assess agronomic and economic relevance of this significant difference. From an outreach perspective, as we build datasets comparing the yield or nitrogen effect of red clover cover crops we can decide to include a difference (e.g. 16 kgha-1) into the average response, or if we do not determine a difference in optimum N rate, include a difference of zero. Interestingly, this approach determined a significant difference between the maximum yields of 10.5 Mg ha^{-1} (None) and 10.0 Mg ha^{-1} (RC), providing the ability to assess the impact of the cover crop on crop production (in this case a yield reduction).

Table 3. Parameter estimates from quadratic plateau response curves determined from the original analysis and bootstrapped analysis for no cover crop (None) and red clover cover cropped (RC) treatments in 2019 in the WC study. Optimum N or maximum yield values for bootstrapped results with different letters indicate differences at the alpha =0.5 significance level.

Treatment	Model	Parameter	Estimate	Std. Error	95% Confidence Interval Lower	Upper
None	Original	a	7.75	0.619	6.47	9.04
		b	0.0541	0.0107	0.0318	0.0765
		c	-0.000101	0.0000381	-0.000179	-0.0000224
		Optimum N (kg ha^{-1})	268			
		Maximum yield (Mg ha^{-1})	15.9			
	Bootstrap	a	7.55	0.578	6.22	8.62
		b	0.0627	0.0334	0.0500	0.0992
		c	-0.000137	0.000370	-0.0005063	-9.86E-05
		Optimum N (kg ha^{-1})	266 a		176	308
		Maximum yield (Mg ha^{-1})	13.3 a		12.6	14.0
RC	Original	a	12.3	0.692	10.8	13.7
		b	0.0388	0.0265	-0.0165	0.0939
		c	-0.000146	0.000189	-0.000538	0.000243
		Optimum N (kg ha^{-1})	133			
		Maximum yield (Mg ha^{-1})	14.9			
	Bootstrap	a	12.2	0.522	10.9	13.4
		b	0.0435	0.0383	0.0189	0.102
		c	-0.000200	0.000532	-0.000847	-5.78E-05

	Optimum N (kg ha^{-1})	158 b	80.5	298
	Maximum yield (Mg ha^{-1})	13.3 a	12.7	13.9

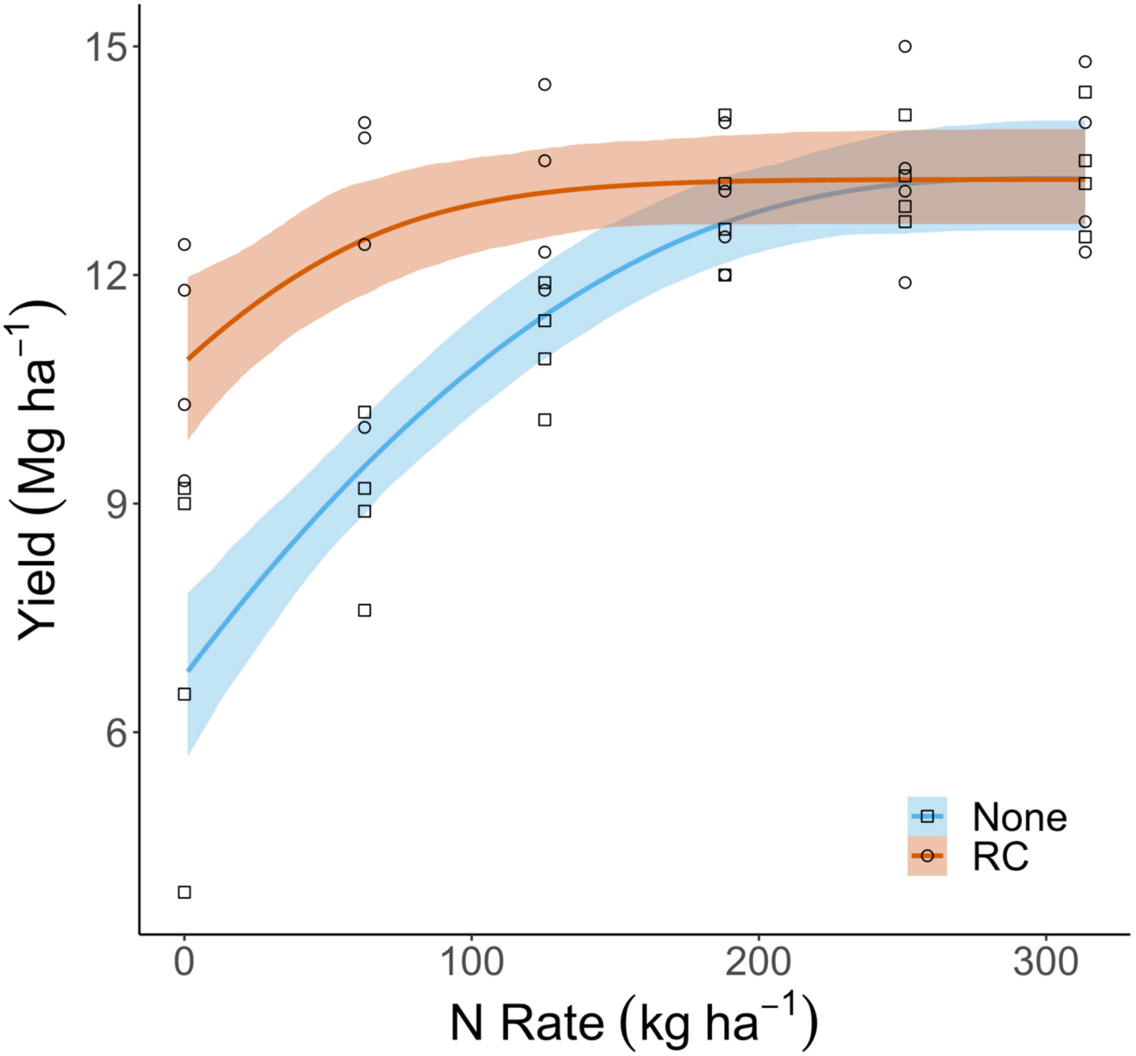


Figure 1. Fertilizer response curve of no red clover (None) and red clover as green manure (RC) in the no-till treatment. The colored bands are the bias corrected (BCa) 95% confidence intervals determined by bootstrapping residuals. Results from field experiments were fit to quadratic plateau models, which were then taken through the bootstrapping residuals procedure.

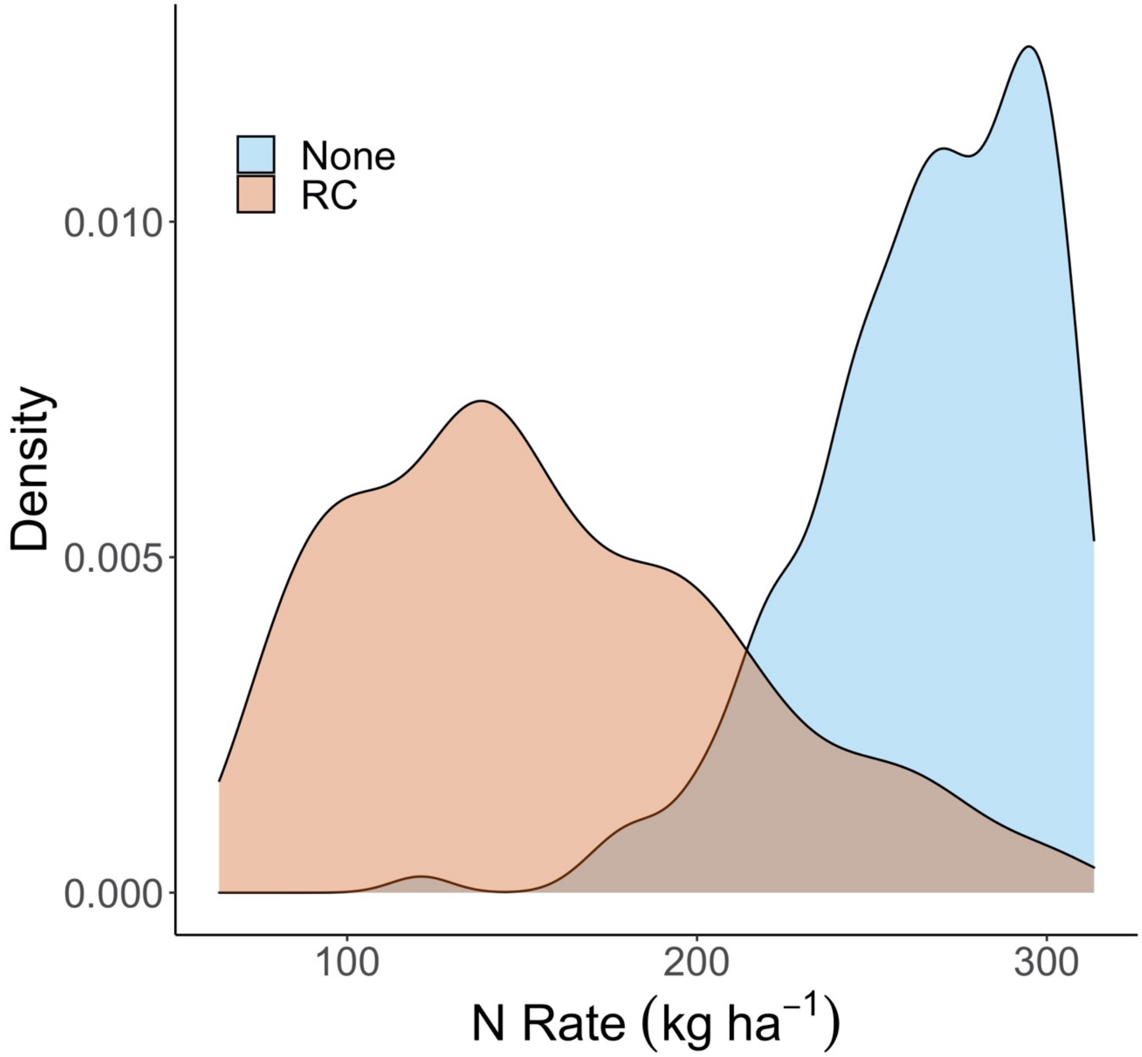


Figure 2. Density curves showing the bootstrapped outputs for optimal nitrogen fertilizer rates in the no clover (None) and clover treatment (RC).

Table 4. Parameter estimates from quadratic plateau response curves determined from the original analysis and bootstrapped analysis for no cover crop (None, R^2=0.68 from original analysis) and red clover cover cropped (RC, R^2=0.72 from original analysis) treatments in 2018 in the CC study. Optimum N or maximum yield values for bootstrapped results with different letters indicate differences at the alpha =0.05 significance level.

Treatment	Model	Parameter	Estimate	Std. Error	95% Confidence Interval Lower	95% Confidence Interval Upper
None	Original	a	5.37	0.613	4.12	6.62
		b	0.1048	0.0314	0.0405	0.169
		c	-0.000540	0.000292	-0.00113	0.000062
		Optimum N (kg ha^{-1})	97.0			
		Maximum yield (Mg ha^{-1})	10.45			
	Bootstrap	a	5.34	0.578	4.02	6.29
		b	0.123	0.0334	0.0714	0.198
		c	-0.000773	0.000370	-0.00179	-0.000305
		Optimum N (kg ha^{-1})	89.1 a		61.9	174
		Maximum yield (Mg ha^{-1})	10.5 b		9.95	10.9
RC	Original	a	5.17	0.5464	4.05	6.29
		b	0.124	0.0353	0.0523	0.1965
		c	-0.000810	0.000422	-0.00167	0.0000530
		Optimum N (kg ha^{-1})	76.8			
		Maximum yield (Mg ha^{-1})	9.95			
	Bootstrap	a	5.17	0.522	3.87	5.96
		b	0.142	0.0383	0.0743	0.226
		c	-0.00111	0.000532	-0.00253	-0.000368
		Optimum N (kg ha^{-1})	72.2 b		50.4	144
		Maximum yield (Mg ha^{-1})	9.98 a		9.57	10.4

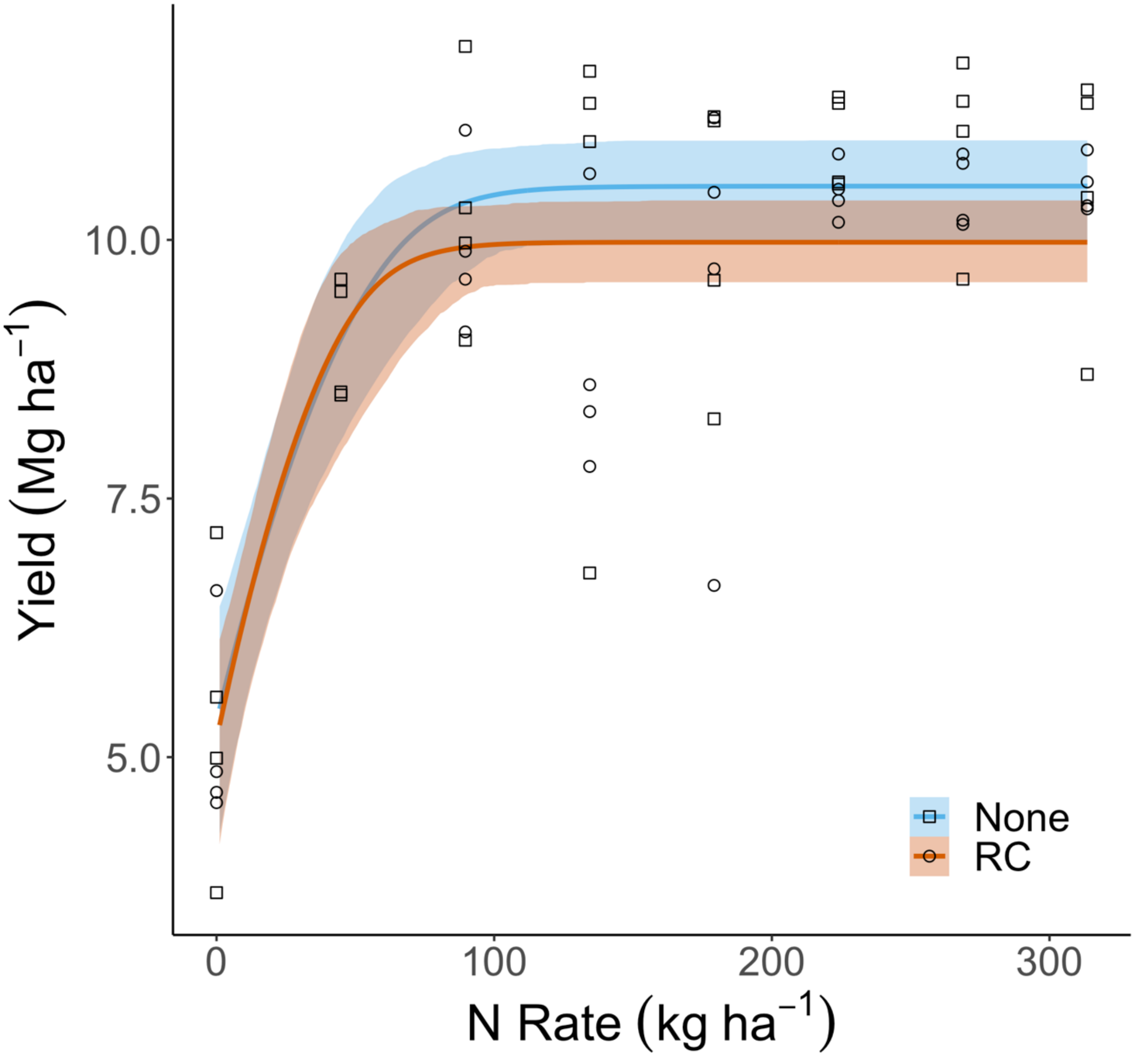


Figure 3. Fertilizer response curves with banded 95% confidence intervals from bootstrapped residuals for treatments without red clover (None) and with inter-seeded red clover (RC).

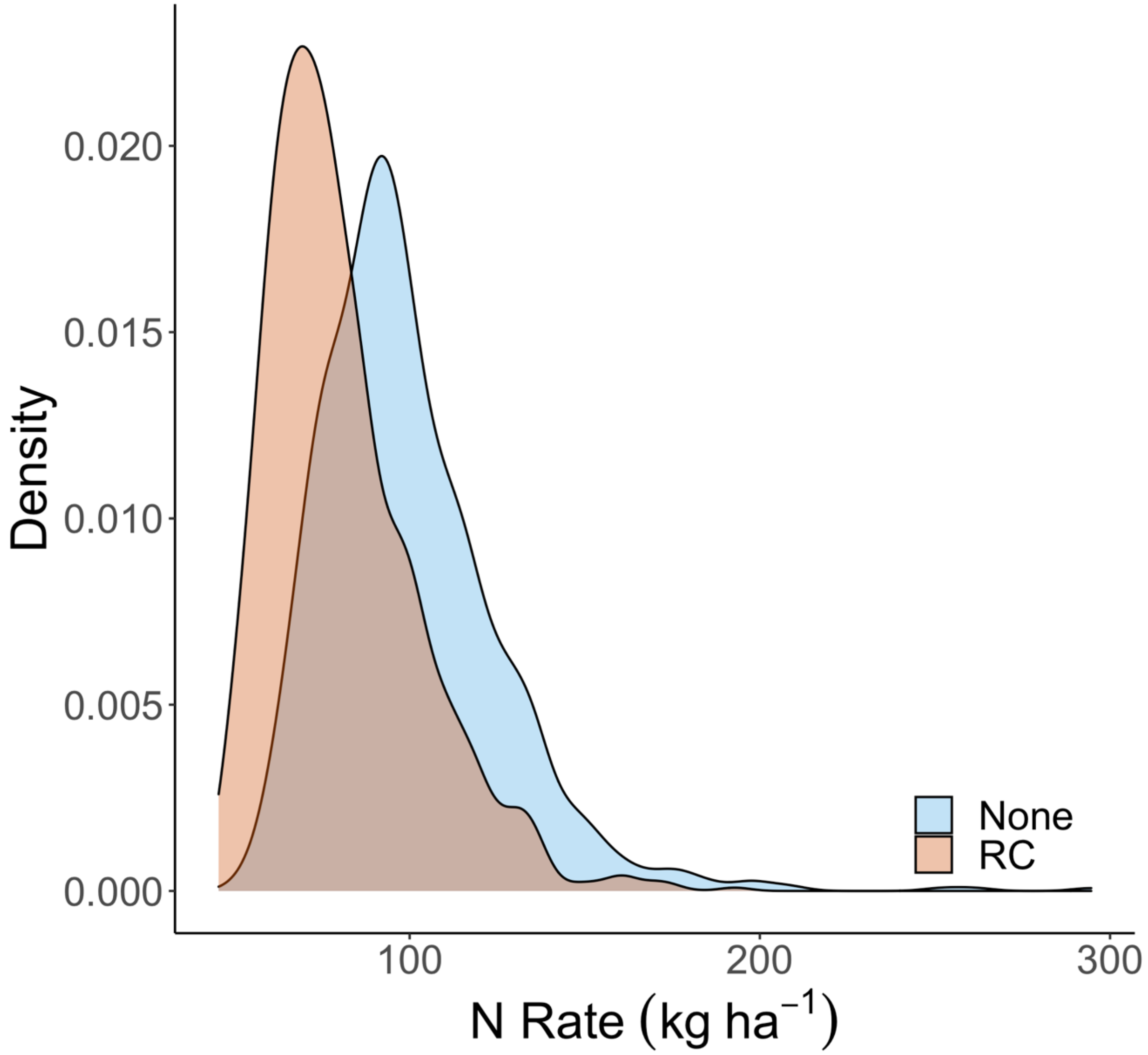


Figure 4. Density plot of the 2018 Interseed study optimum nitrogen fertilizer rate values with treatments of no red clover (None) and with inter-seeded red clover (RC).

Conclusion

The large variation in fertilizer response data in field studies can make some methods for comparisons between treatments of interest statistically inappropriate or cumbersome using traditional methods of evaluation. We propose bootstrapping residuals of fertilizer response curves as an approach to facilitate statistical comparisons between treatments given the constraints of the amount of data collected in field research. The original non-linear models (here, quadratic-plateau models) yield similar coefficients to the bootstrapped quadratic-plateau model coefficients, though both methods maintain large 95% confidence intervals. While alternative methods rely on the 95% confidence interval or standard error terms to determine if differences between optimum nitrogen fertilizer rates are significantly different, the two-sample bootstrap test following bootstrapping residuals of the fertilizer response curve models directly compares optimum N rates between treatments. Determination of statistically significant differences in optimum nitrogen fertilizer rates can inform changes in fertilizer application rates as agronomic research continues to explore management practices that reduce inputs in a movement toward sustainable intensification. We provided examples of how to conduct residual-resampled bootstrapping with non-linear regression to identify differences in response curves, optimum N rates, and maximum yields from two green manure cover crop studies using the FertBoot package in R (T. F. Ma & Francis, 2020), and encourage this method for future studies evaluating input differences between agronomic treatments.

Acknowledgements

Thank you to Lorna Yen for their "An introduction to the bootstrapping method" article (https://towardsdatascience.com/an-introduction-to-the-bootstrap-method-58bcb51b4d60) that served as a source of inspiration for this paper. Funding for this research was provided by the Wisconsin Fertilizer Research Council, Project 280-15 and 282-16.

References

Caldwell, B., Mohler, C. L., Ketterings, Q. M., & DiTommaso, A. (2014). Yields and profitability during and after transition in organic grain cropping systems. *Agronomy Journal*, *106*(3), 871–880. https://doi.org/10.2134/agronj13.0286

Chatterjee, A., Subedi, K., Franzen, D. W., Mickelson, H., & Cattanach, N. (2018). Nitrogen fertilizer optimization for sugarbeet in the Red River valley of north Dakota and Minnesota. *Agronomy Journal*, *110*(4), 1554–1560. https://doi.org/10.2134/agronj2017.12.0694

Corstanje, R., Portier, K. M., & Reddy, K. R. (2009). Discriminant analysis of biogeochemical indicators of nutrient enrichment in a Florida wetland. *European Journal of Soil Science*, *60*(6), 974–981. https://doi.org/10.1111/j.1365-2389.2009.01186.x

Davison, A. C. & Hinkley, D. V. (1997). Bootstrap Methods and their Application. Cambridge University Press.

Efron, B. (1979). Bootstrap methods: another look at the jackknife. *Annals of Statistics*, *7*(1), 1–26.

Finney, D. M., White, C. M., & Kaye, J. P. (2016). Biomass production and carbon/nitrogen ratio influence ecosystem services from cover crop mixtures. *Agronomy Journal*, *108*(1), 39–52. https://doi.org/10.2134/agronj15.0182

Heikkinen, J., & Mäkipää, R. (2010). Testing hypotheses on shape and distribution of ecological response curves. *Ecological Modelling*, *221*(3), 388–399. https://doi.org/10.1016/j.ecolmodel.2009.10.030

Kawamura, K., Watanabe, N., Sakanoue, S., Lee, H. J., Lim, J., & Yoshitoshi, R. (2013). Genetic algorithm-based partial least squares regression for estimating legume content in a grass-legume mixture using field hyperspectral measurements. *Grassland Science*, *59*(3), 166–172. https://doi.org/10.1111/grs.12026

King, A. E., & Blesh, J. (2018). Crop rotations for increased soil carbon: Perenniality as a guiding principle: Perenniality. *Ecological Applications*, *28*(1), 249–261. https://doi.org/10.1002/eap.1648

Li, X., Zhang, Y., Bao, Y., Luo, J., Jin, X., Xu, X., Song, X., & Yang, G. (2014). Exploring the best hyperspectral features for LAI estimation using partial least squares regression. *Remote Sensing*, *6*(7), 6221–6241. https://doi.org/10.3390/rs6076221

Ma, T. F., & Francis, H. (2020). *FertBoot: Fertilizer Response Curve Analysis by Bootstrapping Residuals*. R package version 0.4.0.

Pantoja, J. L., Woli, K. P., Sawyer, J. E., & Barker, D. W. (2015). Corn Nitrogen Fertilization Requirement and Corn–Soybean Productivity with a Rye Cover Crop. *Soil Science Society of America Journal*, *79*(5), 1482. https://doi.org/10.2136/sssaj2015.02.0084

Ruark, M. D., Chawner, M. M., Ballweg, M. J., Proost, R. T., Arriaga, F. J., & Stute, J. K. (2018). Does cover crop radish supply nitrogen to corn? *Agronomy Journal*, *110*(4), 1513–1522. https://doi.org/10.2134/agronj2017.06.0352

Rubin, J. C., Struffert, A. M., Fernández, F. G., & Lamb, J. A. (2016). Maize yield and nitrogen use efficiency in upper midwest irrigated sandy soils. *Agronomy Journal*, *108*(4), 1681–1691. https://doi.org/10.2134/agronj2015.0552

Sawyer, J. E., Pedersen, P., Barker, D. W., Ruiz Diaz, D. A., & Albrecht, K. (2010). Intercropping corn and kura clover: Response to nitrogen fertilization. *Agronomy Journal*, *102*(2), 568–574. https://doi.org/10.2134/agronj2009.0392

Schmidt, J. P., DeJoia, A. J., Ferguson, R. B., Taylor, R. K., Young, R. K., & Havlin, J. L. (2002). Corn yield response to nitrogen at multiple in-field locations. *Agronomy Journal*, *94*(4), 798–806. https://doi.org/10.2134/agronj2002.7980

Steinke, K., Rutan, J., & Thurgood, L. (2015). Corn response to nitrogen at multiple sulfur rates. *Agronomy Journal*, *107*(4), 1347–1354. https://doi.org/10.2134/agronj14.0424

Wang, L., Feng, Z., & Schjoerring, J. K. (2013). Effects of elevated atmospheric CO2 on physiology and yield of wheat (Triticum aestivum L.): A meta-analytic test of current hypotheses. *Agriculture, Ecosystems and Environment*, *178*(2013), 57–63. https://doi.org/10.1016/j.agee.2013.06.013

Woli, K. P., Boyer, M. J., Elmore, R. W., Sawyer, J. E., Abendroth, L. J., & Barker, D. W. (2016). Corn era hybrid response to nitrogen fertilization. *Agronomy Journal*, *108*(2), 473–486. https://doi.org/10.2134/agronj2015.0314

Wortman, S. E. (2016). Weedy fallow as an alternative strategy for reducing nitrogen loss from annual cropping systems. *Agronomy for Sustainable Development*, *36*(4). https://doi.org/10.1007/s13593-016-0397-3

Wortman, S. E., Holmes, A. A., Miernicki, E., Knoche, K., & Pittelkow, C. M. (2017). First-season crop yield response to organic soil amendments: A meta-analysis. *Agronomy Journal*, *109*(4), 1210–1217. https://doi.org/10.2134/agronj2016.10.0627

Yang, M., Mouazen, A., Zhao, X., & Guo, X. (2020). Assessment of a soil fertility index using visible and near-infrared spectroscopy in the rice paddy region of southern China. *European Journal of Soil Science*, *71*(4), 615–626. https://doi.org/10.1111/ejss.12907

Yen, L. (2019). *An Introduction to the Bootstrap Method*. Medium.

Zeng, W., Xu, C., Wu, J., & Huang, J. (2016). Sunflower seed yield estimation under the interaction of soil salinity and nitrogen application. *Field Crops Research*, *198*, 1–15. https://doi.org/10.1016/j.fcr.2016.08.007

Zhang, Y., Xie, D., Ni, J., & Zeng, X. (2020). Conservation tillage practices reduce nitrogen losses in the sloping upland of the Three Gorges Reservoir area: No-till is better than mulch-till. *Agriculture, Ecosystems and Environment*, *300*(May 2019), 107003. https://doi.org/10.1016/j.agee.2020.107003

Zheng, B. S., Le Gouis, J., Daniel, D., & Brancourt-Hulmel, M. (2009). Optimal numbers of environments to assess slopes of joint regression for grain yield, grain protein yield and grain protein concentration under nitrogen constraint in winter wheat. *Field Crops Research*, *113*(3), 187–196. https://doi.org/10.1016/j.fcr.2009.05.001

Chapter 2: Field studies with interseeded red clover in a continuous corn, no-till system

Abstract

Interseeding red clover provides an alternative method to incorporate cover crops into continuous corn rotations. Red clover is a leguminous cover crop that can grow in low radiation environments and is winter hardy for much of the northern USA. In addition to the well-known benefits of cover crops to soil health and fertility, systems with red clover have previously demonstrated improved corn yield and a nitrogen credit. However, the potential nitrogen credit and the effect of interseeding red clover on subsequent corn yields has yet to be rigorously evaluated. The objectives of this project were to determine the effect of interseeding red clover on (1) corn yield in the interseeded year and subsequent year, (2) response to N fertilizer in the interseeded year and the subsequent year and, (3) residual (post-harvest) and early season soil N content in a continuous corn, no-till management system. The plot design was a randomized, complete block-split with four replications, treated with or without red clover, at eight rates of N-fertilizer (0 to 315 kg-N ha^{-1} in 45 kg-N ha^{-1} intervals). Corn yields were evaluated when red clover was continuously interseeded, or not interseeded following the first interseeding year. Red clover accumulated up to 300 kg ha^{-1} biomass pre-termination (10 kg N ha^{-1}) when interseeded with corn at the V4-V5 growth stage without detriment to yield. In 2018, corn following the interseeded year out-yielded corn on plots with no history of interseeded red clover with a small, but significant nitrogen credit. In 2019, there was not a significant difference in corn yield data between treatments. A statistical approach with

bootstrapping was used to determine statistically significant nitrogen credits. Overall, we observed that interseeding red clover into continuous corn did not provide an agronomically meaningful nitrogen benefit to the cropping system.

Introduction

Cover crop use in Wisconsin has increased in recent years and is projected to continue increasing (CTIC & SARE, 2020). The benefits of cover crops in agroecosystems are well established, however, each cover crop species and system come with its challenges. This study focuses on red clover (*Trifolum pratense L.*), a cover crop whose use has resulted in no detriment to, or even increased, corn yields (Coombs et al., 2017a; Hesterman et al., 1992; Miguez & Bollero, 2005; White et al., 2016). Red clover has been in use since the European agricultural revolution to add nitrogen to agronomic systems (Taylor, 1985), yet despite historic relevance, the advent of synthetic nitrogen fertilizer correlated with a decline in cover crop use. Now, interest in cover crop use is increasing, and farmers cite reasons for adopting cover crops ranging from input reduction to increased yields and improved soil structure (CTIC & SARE, 2020). As a legume cover crop, yield benefits from red clover are thought to come from improvements to soil structure and from a nitrogen credit that red clover, when used as green manure, provides nitrogen to a subsequent crop (Raimbault & Vyn, 1991; Vyn et al., 1999). Despite estimates of the nitrogen credit red clover delivers to continuous corn or rotational corn systems based on height or coarse fertilizer rate studies, statistical significance between nitrogen credits has yet to be established.

Cover crops have been widely studied and regarded for their synergistic effects in promoting and sustaining soil health. Cover crops reduce soil erosion (Snapp et al., 2005), reduce nitrate leaching by reducing residual soil nitrate concentrations (De Notaris et al., 2018; Gabriel et al., 2012; Randall et al., 2008; Thapa et al., 2018; Vyn et al., 2000), suppress weeds (den Hollander et al., 2007; Donovan et al., 2001; Sarrantonio & Gallandt, 2003), build soil organic matter (Snapp et al., 2005), improve soil structure (Vyn 1991), and stabilize or improve yields (Baributsa et al., 2008; Curran et al., 2018; Grabber et al., 2014; Jones et al., 1998; Ketterings et al., 2015).

The value of legume cover crops encompass the ecological benefits of cover crops detailed above, with the additional benefit of working with nitrogen fixing bacteria to symbiotically derive plant available nitrogen from atmospheric nitrogen, then providing this nitrogen to a subsequent crop upon termination. Historically, symbiotic biological nitrogen fixation by leguminous crops provided the bulk of the plant available nitrogen for other crops (Smil, 2001). With increased nitrogen availability, legume crop residues have been shown to increase N uptake, C accumulation, and yields of a subsequent crop (Lupwayi & Soon, 2016). The residues of legumes are often called “green manures” and contribute to the quantity of soil N, reducing the need for reliance on synthetic nitrogen fertilizers. The availability of plant-available N is dependent on microbial activity, which is mediated by a variety of soil abiotic factors such as moisture, temperature, pH, soil type and texture, as well as soil management practices, including tillage (Sarrantonio & Gallandt, 2003).

Red clover is a legume cover crop that provides the ecological benefits of other cover crops while also providing nitrogen to a subsequent crop when used as a green manure.

Red clover is of particular interest because it fills a niche in its capability of surviving in a lower radiation environment, and can therefore be included in cropping systems like continuous corn that with a higher plant density and leave only a short growing period following harvest (Baributsa et al., 2008; Gaudin et al., 2013; Kendall and Stringer, 1985). Compared to other cover crops, and even other clover species, red clover has a greater establishment rate and produces more biomass (Wyngaarden et al., 2015). Red clover has the positive attribute of decaying rapidly; it releases approximately 50% of biomass nitrogen in the four weeks following termination, and this release slows considerably at the corn silking stage, approximately 10 weeks after termination (J. K. Stute & Posner, 1995b). Inter-seeding leguminous cover crops into corn has been shown to provide a nitrogen credit to a subsequent corn crop (Gentry et al., 2013; Henry et al., 2010). Corn has been the most widely fertilized crop in the U.S. since the 1950s and therefore has the largest share of the total nitrogen consumption (Smil, 2001). Crop harvest and removal necessitates nitrogen input since the nitrogen supply is not recycled back into the system. Leguminous cover crops provide a management buffer where there is a guaranteed nitrogen input to the soil while simultaneously preventing nitrogen fertilizer loss.

When red clover is interseeded into corn, the cover crop is treated like an annual to release the nitrogen stored in its biomass to the next corn corn. When interseeded, red clover is terminated when it is at 50% bloom stage, which is near peak N-accumulation, and therefore a high protein content (>14-15% protein). Interseeding red clover into corn not only provides nitrogen credit to the subsequent crop without sacrificing yield, but also works to improve soil fertility (Baributsa et al., 2008).The timing is a balance for red clover—while the cover crop should be allowed to grow as long as possible to fix nitrogen and suppress weeds, it can be detrimental in dry conditions as it will compete for soil moisture with the cash crop. A robust stand of red clover requires approximately 6.7 kg clover seed ha^{-1}, where there are an estimated 125,000 seeds per kilogram. Average yields of red clover biomass are between 390-560 kg ha^{-1}, with low yields a typical reflection of a lack of pollinating insects, which for clovers are typically bees (John & Ogle, 2008). The genetic diversity that accompanies planting red clover (a dicotelydon) with corn (a monocotelydon) interrupts pest and disease cycles (Wyngaarden et al., 2015).

There are several perceived and agronomic, field-level limitations to legume cover crop use in commodity-crop systems. Despite the well-known measurable influences on soil and environmental health, there are still barriers to cover crop adoption (Blanco-Canqui et al., 2015). Prior to the development of synthetically produced nitrogen fertilizer at an industrial scale, cover crops were a widely used strategy to maintain soil quality (Smil, 2001). This changed in the post-Green Revolution tendency toward “industrial, commodity-oriented monoculture systems” was a historic factor that would influence cover crop adoption (Roesch-Mcnally et al., 2018). Cover crops never left the agronomic stage entirely, and farmers began using cover crops in these commodity-crop systems. One of the biggest barriers to adoption is the timing of cover crop establishment, where in northern climates it is challenging to establish a cover crop in the fall and terminate it in the spring (Roesch-Mcnally et al., 2018). Additionally, economic returns on cover crop use may be low with high production costs, despite a study that showed cover crop use was correlated with yield increase in corn and soybeans for 43% of farmers surveyed, and a quarter of all yield increase occurred with red clover as a cover crop (Singer, 2008). Red clover in this survey occupied the largest portion of cover crops that were associated with yield increases. A survey evaluated by Singer (2008) found that 64% of farmers who use cover crops prefer cover crops that fix nitrogen. As a leguminous cover crop, red clover also fulfills the preference for a nitrogen-fixing cover crop, where other legume cover crops have a limitation of synchroneity of nitrogen release (J. K. Stute & Posner, 1995a). The limitations of cover crop use existed as perceptions in commodity-crop systems, and agronomic studies are attempting to address those gaps in cover crop knowledge in current agroecosystems. A survey conducted by the Sustainable Agriculture Research and Education (SARE) program and the Conservation Technology Information Center (CTIC) from 2019-2020 reported that of the people that started the survey, 94% of farmers surveyed across the U.S. had some experience with cover crops, with no detriment to corn yields. However, the net economic benefit of using red clover as an interseeded, leguminous cover crop remains to be verified, and is an additional obstacle to overcome grower reluctance to incorporate cover crops (Sarrantonio & Gallandt, 2003). More farmers are increasingly likely to remain with cover crop use once adopted even through economic volatility (CTIC & SARE, 2020), reiterating the understanding of the long-term benefits to soil health. The majority of non-users, while not currently using cover crops, still demonstrate a desire for more knowledge.

To address many of the limitations that planting legume cover crops in northern climates presents, interseeding has become an increasingly common practice to maintain healthier soils and ensure better establishment. Still, the majority of farmers who are already using cover crops have not tried interseeding as a practice (CTIC & SARE, 2020). This practice still presents a challenge of stand establishment, and the perception of poor establishment has been reported by farmers as a main reason to refrain from growing cover crops (Youngerman et al., 2018). In a survey conducted in 2016, clover represented 14% of inter-seeded cover crop species (Conservation Technology Information Center (CTIC), 2017), suggesting room for increased adoption. The agronomic trials in this study seek to assess the biomass establishment of red clover in the context of nitrogen fertilizer replacement values.

The goal of this research is to evaluate the potential fertilizer replacement value of red clover and the cover crop viability in a continuous corn, no-till system in Wisconsin. The objectives of the study were to determine differences in (1) the response of plant available nitrogen in residual (post-harvest) and early season soil N to nitrogen fertilizer in clover and no clover treatments, (2) red clover biological nitrogen fixation in an interseeded system with varying nitrogen fertilizer rates, and (3) corn yields with interseeded red clover in the interseeded year and the subsequent year,. The hypotheses are: (1) to see an increase of in-season nitrogen in the upper 30 cm of soil that would suggest the terminated clover is supplying nitrogen to the system, (2) the biological nitrogen fixation will decrease as nitrogen fertilizer rates increase in the clover plots, since supplying nitrogen to a nodulating legume can shut down biological nitrogen fixation, and (3) red clover will supply nitrogen to corn, where maximum corn yields occur at a lower nitrogen fertilizer rate in the interseeded treatment with clover than the treatment without clover.. Ultimately, this information will help growers better utilize cover-crop systems.

Materials and Methods

Field description and sampling

The study was conducted at the University of Wisconsin Arlington Agricultural Research Station from 2017-2020. The research station is in south central Wisconsin, 30 kilometers north of Madison (43°18’9.47″N, 89°20’43.32″W), on a Plano silt loam (fine-silty, mixed, superactive, Mesic Typic Argiuodoll). The study site has a mean annual temperature of 6.9°C and a mean annual precipitation of 898 mm (National Climate Data Center). Two fields were used in the study only a kilometer apart; the first field (field 1) was sampled from 2017-2019 and the second field (field 2) was used from 2019-2020. Routine soil analyses at 0-15 cm depth were collected from each sampling location (field 1 and field 2) prior to corn planting each year, and averaged across site-years. Soil pH (1:1 water) was 6.3 and 6.5, soil P (mg kg^{-1}) was 37 and 34 (Bray 1), soil K (mg kg^{-1}) was 100 and 64 (Bray 1), and soil OM (%) was 3.0 and 4.0 (LOI) in fields 1 and 2, respectively.

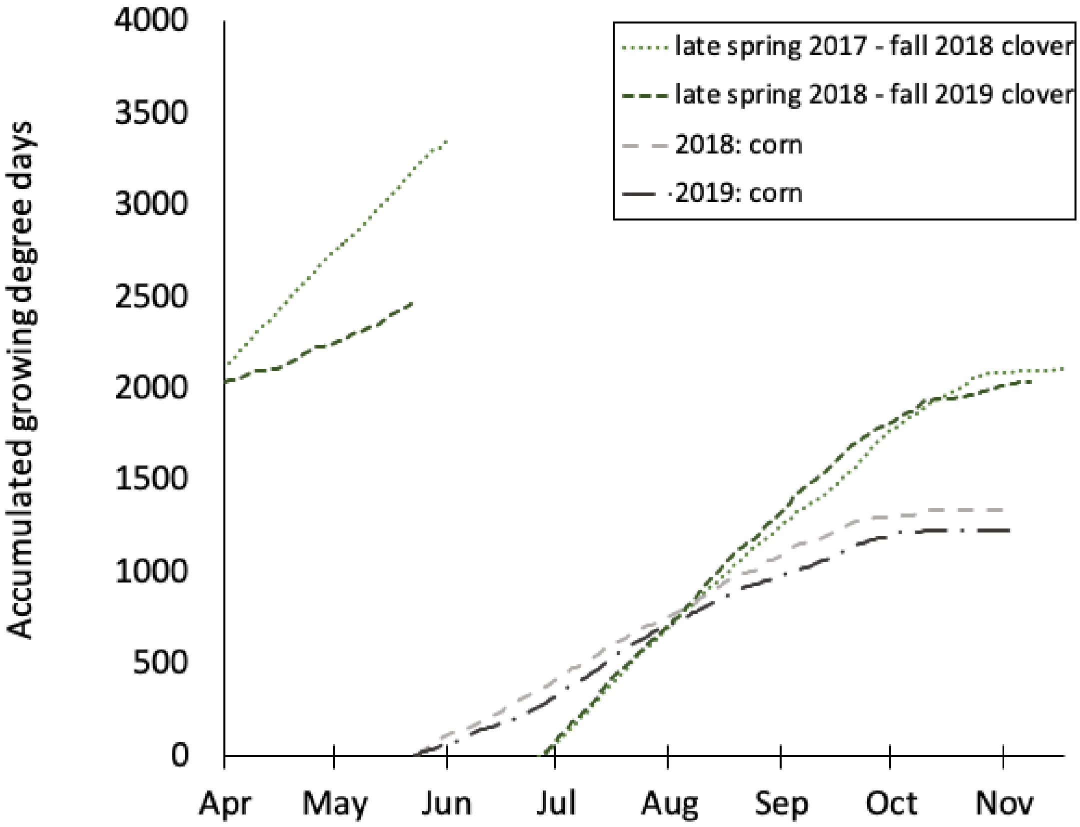


Figure 5. Accumulated growing degree days (GDDs) for one site-year showing the termination of the cover crop in May and corn planting at the end of May. GDDs were calculated with temperature data from the NOAA NOWData using Growing Degree Days (°C) = [(T(°C)max+ T(°C)min)/2]- T_{base} where for corn Tbase(°C)=10°C and clover was calculated with a T_{base}°C=0.62°C (Baxter et al., 2019).

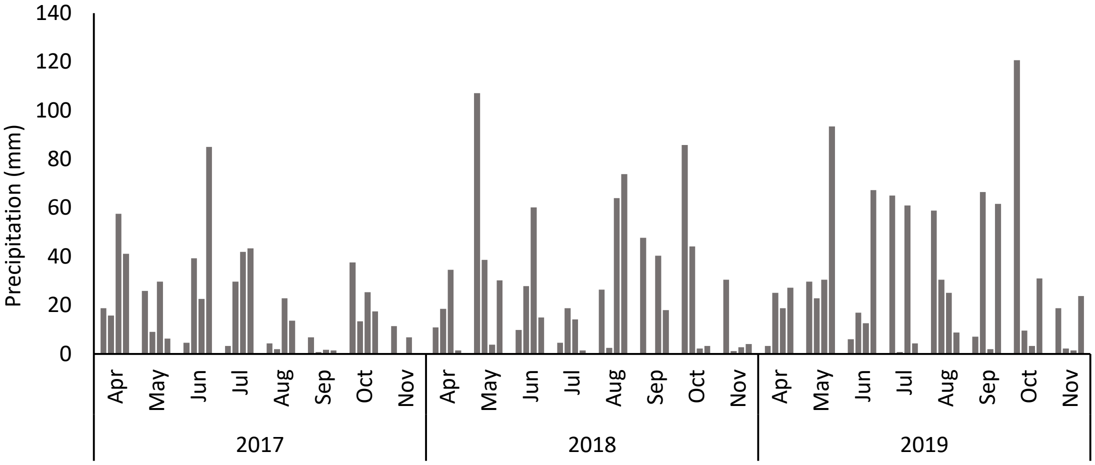


Figure 6. Weekly accumulated precipitation for the corn growing season at Arlington, WI from 2017-2019. Corn was planted at the end of May each year and harvested late October or early November. Cover crop planting occurred in June of each year and termination was in May.

The experimental design was a randomized, complete-block split-plot design with four replicates. The whole plot factor was red clover cover crop and the split plot factor was N rate. There were three whole plot treatments, one without red clover (No Clover) and two that had interseeded red clover. The split-plot treatments were eight N-fertilizer rates (0-313.6 kg ha^{-1} in 44.8 kg ha^{-1} intervals) or one uniform nitrogen fertilizer application rate during the first interseeding site-year (210 kg ha^{-1} with a 0 kg ha^{-1} reference). The effect of interseeded red clover on corn yield N response was evaluated when clover was interseeded the previous year or interseeded both the previous year and the year of the N rate study. Field 1 was established in 2017 and corn was planted and clover was interseeded. The next year in 2018, the whole plot treatments were no red clover (No Clover), red clover planted in 2017 to be terminated in spring of 2018 (Clover 2017), and clover planted in 2017, terminated in 2018, and interseeded again in 2018 (Clover Always). The difference between Clover 2017 and Clover Always is to determine differences in N response following clover termination if clover is interseeded again or not. In 2018, red clover was interseeded into corn for the N response year in 2019 (Clover 2018). In 2019, the N response was evaluated in field 1 with N rates in repeated on the same plots in No Clover and Clover Always, while Clover 2018 had the eight N rates on plots that had previously received just one N application of 210 kg ha^{-1}. A second field, field 2, was established in 2019 to have one more year of a N response following clover. Site-years were analyzed separately to account for differences in cover crop establishment between years.

In the establishment year of the second field there was one treatment without clover interseeded with N rates, one treatment where clover was interseeded with N rates, and one treatment where clover was interseeded without N rates (one application of 210 kg ha^{-1}). In 2020, the final year of the study for the second field, all three treatments were repeated, and this time both clover treatments received N rates. An error in herbicide application meant that the Clover Always treatment was not interseeded in 2020, effectively rendering both clover treatments to Clover 2019 where one received N rates and one had just one N rate applied the previous year. Reference rye plots adjacent to the experimental clover plot were planted at a seeding rate 134.4 kg ha^{-1} and had no nitrogen fertilizer or 210 N kg ha^{-1} fertilizer applications. Individual plot sizes were 4.5 m wide (6 corn rows) x 9 m long with 1.5 m alleys. The effect of interseeding red corn yield was evaluated in the interseeding year in 2017, 2018, and 2019 (Table 5). The following figures (Figures 7-11) are all examples of one replicate of each treatment system.

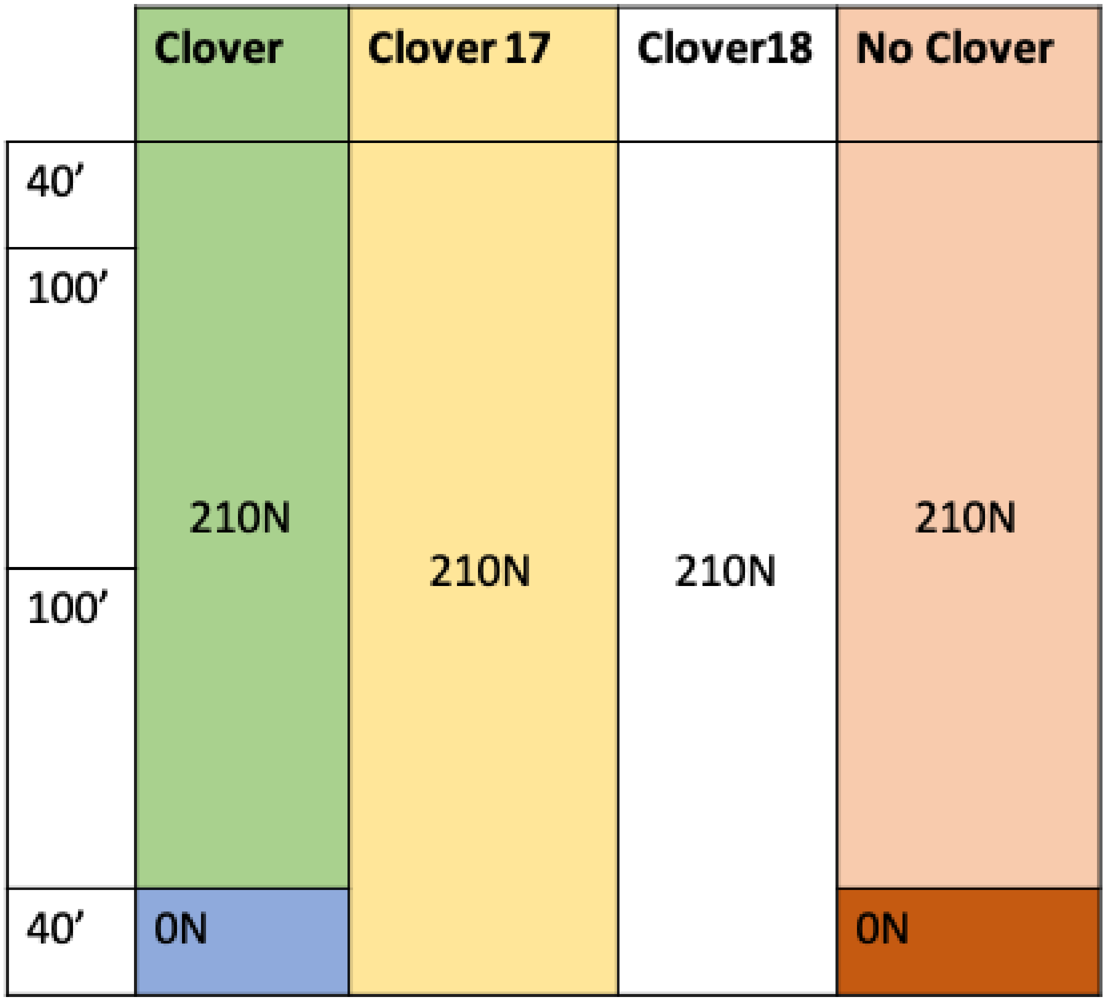
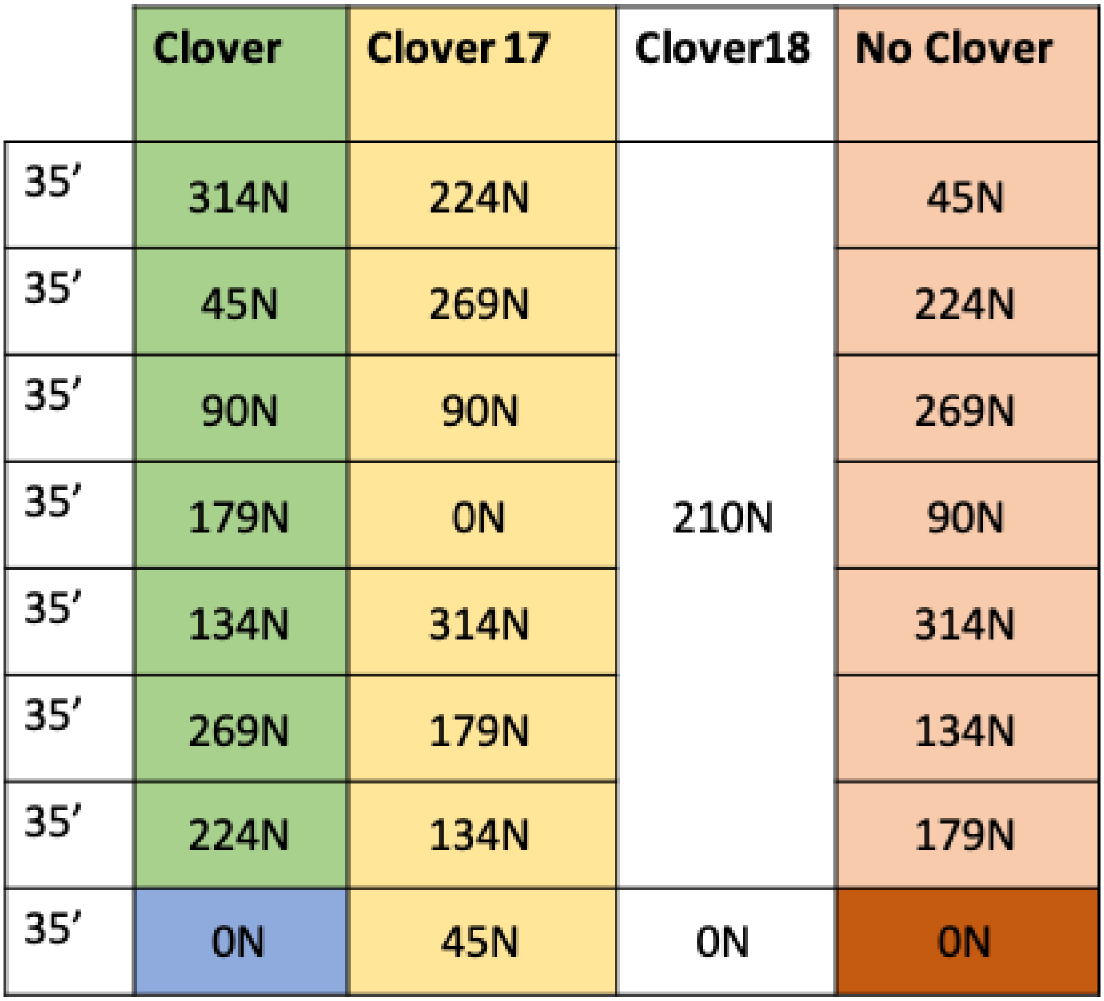
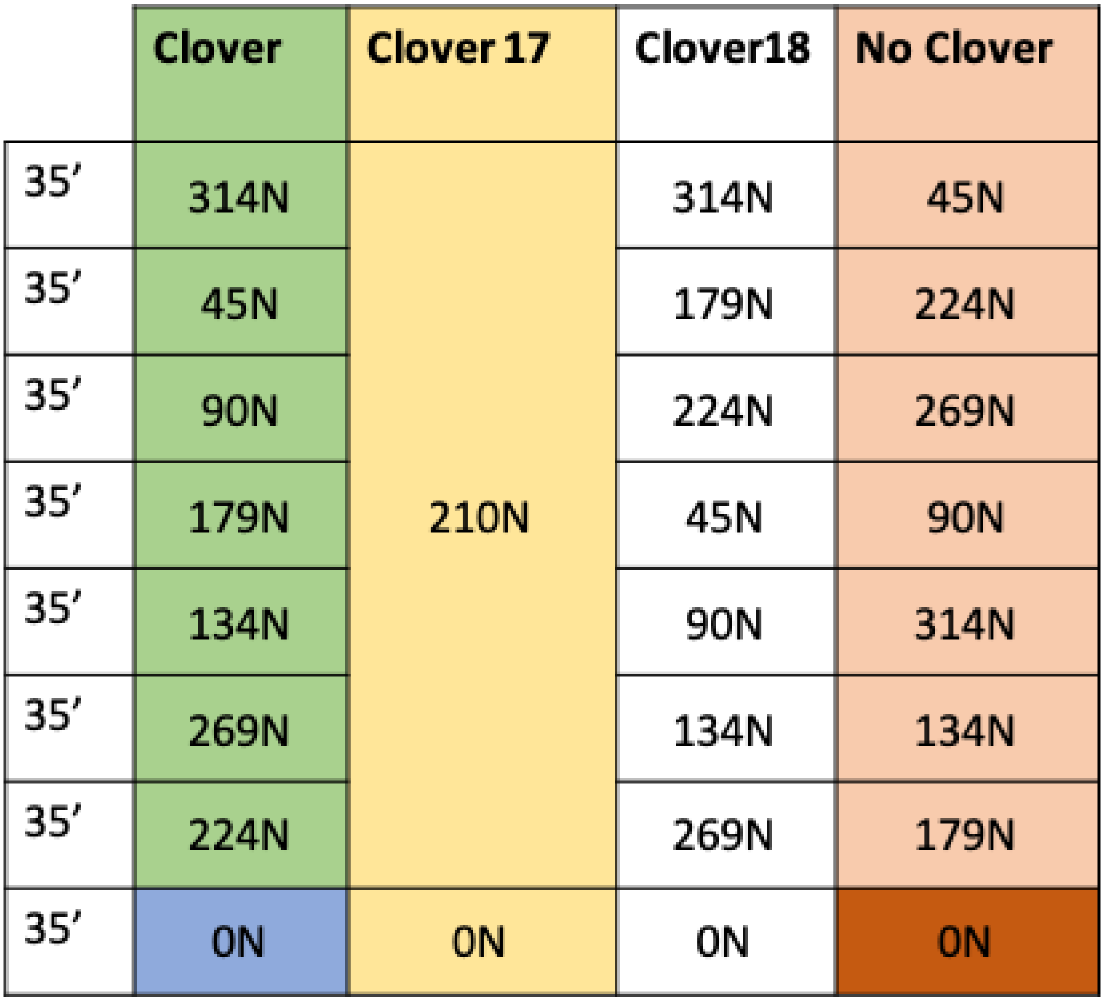


Figure 7. Field 1 2017 plot plan. N rates are in kg-N ha^{-1}. Plot width measurements are in feet.



	Clover	Clover 17	Clover18	No Clover
35’	314N	224N	210N	45N
35’	45N	269N		224N
35’	90N	90N		269N
35’	179N	0N		90N
35’	134N	314N		314N
35’	269N	179N		134N
35’	224N	134N		179N
35’	0N	45N	0N	0N

Figure 8. Field 1 2018 plot plan. N rates are in kg-N ha^{-1}. Plot width measurements are in feet.

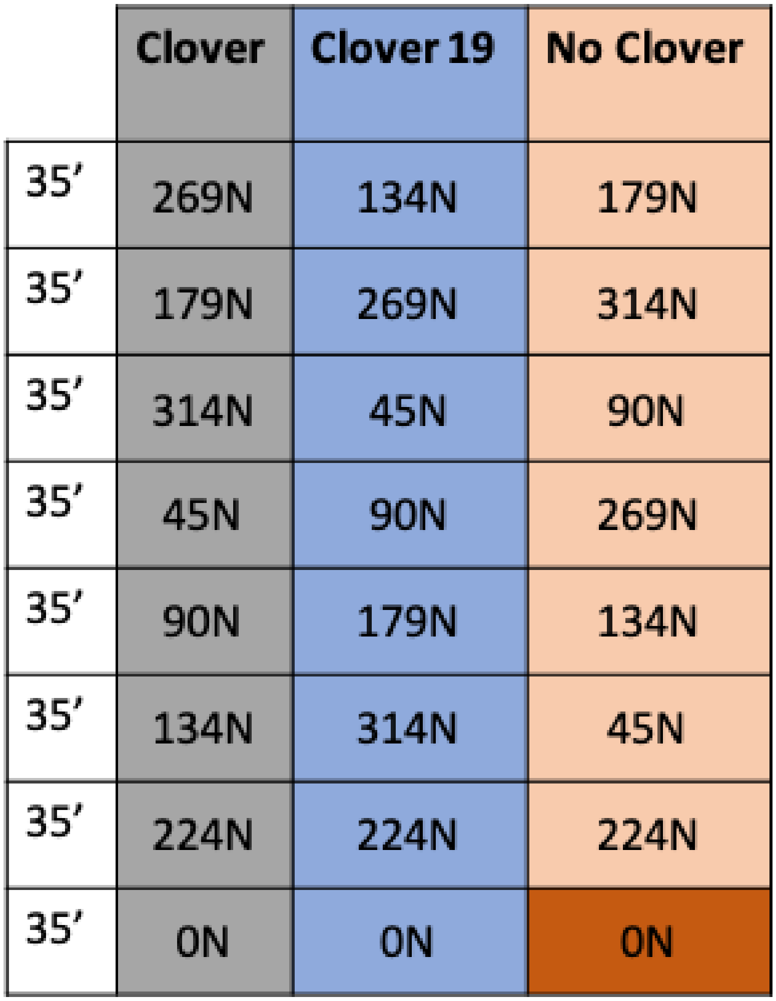


	Clover	**Clover 17**	**Clover18**	**No Clover**
35′	314N	210N	314N	45N
35′	45N		179N	224N
35′	90N		224N	269N
35′	179N		45N	90N
35′	134N		90N	314N
35′	269N		134N	134N
35′	224N		269N	179N
35′	0N	0N	0N	0N

Figure 9. Field 1 2019 plot plan. N rates are in kg-N ha^{-1}. Plot width measurements are in feet.

	Clover	Clover 19	No Clover
35′	269N	210N	179N
35′	179N		314N
35′	314N		90N
35′	45N		269N
35′	90N		134N
35′	134N		45N
35′	224N		224N
35′	0N	0N	0N

Figure 10. Establishment year of Field 2, 2019 plot plan. N rates are in kg-N ha^{-1}. Plot width measurements are in feet.



	Clover	Clover 19	No Clover
35’	269N	134N	179N
35’	179N	269N	314N
35’	314N	45N	90N
35’	45N	90N	269N
35’	90N	179N	134N
35’	134N	314N	45N
35’	224N	224N	224N
35’	0N	0N	0N

Figure 11. Field 2 2020 plot plan. N rates are in kg-N ha^{-1}. Plot width measurements are in feet.

Table 5. Cover crop treatments for site years

Treatment	2017	2018	2019	2020
Clover Always	Clover + N*	Clover + N rates	Clover + N rates	Clover + N rates
Clover 2017	Clover + N	No Clover + N rates	No Clover + N	No Clover + N
Clover 2018	No Clover + N	Clover + N	No Clover + N rates	No Clover + N
No Clover	No Clover + N	No Clover + N rates	No Clover + N rates	No Clover + N rates

*N represents one nitrogen fertilizer application rate of 210 N kg ha^{-1} with a 0 N kg ha^{-1} plot in the same row
N rates are the eight split plot rates of 0-313.6 kg ha^{-1} in 44.8 kg ha^{-1} intervals

Red clover was drill seeded (13.45 kg ha^{-1}) into corn at 1 cm depth at the V4-V5 growth stage with a modified grain drill. Prior to drill seeding, whole blocks were treated with the herbicide Round-Up PowerMax (1.54 kg ha^{-1} in AMS). Nitrogen fertilizer was applied at sidedress near the time of interseeding as urea coated with Agrotain®. Non-replicated plots of rye and rye interseeded into corn were established as reference plants for ^{15}N analysis. Rye was chosen as the reference cover crop because it is a non-nodulating (non-nitrogen fixing) cover crop and is similarly winter-hardy. Red clover was chemically terminated each spring with 2, 4-d or dicamba with glyphosate.

Corn was planted May each site-year and red clover was interseeded a few weeks later in June. Red clover was terminated at or just after corn planting with 2,4-d or dicamba with glyphosate. Urea coated with a urease inhibitor (Agrotain®) was surface applied at eight different rates (0, 45, 90, 135, 180, 225, 270, and 315 kg-N ha^{-1}) in late June each site-year. This study was conducted without tillage during the two crop phases (Table 6).

Table 6. Field activities for all site-years

	Year			
Field activity	**2017**	**2018**	**2019**	**2020**

Pre-termination clover sample		24-May	22-May	22-May
Early spring and routine soil sampling	25-May	24-May	22-May	22-May
Corn planting	25-May	24-May	23-May	22-May
Clover termination		1-Jun	22-May	22-May
Sidedress fertilizer	26-Jun	28-Jun	25-Jun	18-Jun
Late spring soil sampling	26-Jun	28-Jun	25-Jun	18-Jun
Clover drill-seeded into corn	26-Jun	28-Jun	25-Jun	18-Jun
Reference rye planted	26-Jun	28-Jun		
Corn harvested	3-Nov	3-Nov	5-Nov	
Clover fall biomass sample	16-Nov	1-Nov	30-Oct	
Fall soil sampling	16-Nov	1-Nov	30-Oct	

Soil and biomass analysis

Red clover and rye were sampled post-harvest in the fall, and in the spring pre-termination for total N and ^{15}N analysis by the UC Davis Stable Isotope Laboratory. The results were used with the natural abundance method to calculate the nitrogen content in the cover crop biomass derived from the atmosphere (Unkovich et al., 2008). Interseeded rye with corn was used as the reference plant, and a B-value of -0.94 was used as an average of four studies that independently evaluated the B-value of red clover (Appendix 4, Unkovich et al., 2008). Red clover biomass was harvested from a representative 0.6 x 0.6-m (0.36-m^2) quadrat per plot. Living red clover was clipped at ground level, bagged and dried at 65°C for at least 5 days and weighed. The width between corn rows where clover is interseeded is 0.9 m, so the dry biomass weight was scaled to a kg ha^{-1} using a 0.9 x 0.6 m sample area (0.54 m^2) to account for the area between corn rows without red clover. Dried clover samples were ground and milled to a fine powder, and tin-rolled. UC

Davis Stable Isotope Laboratory uses a continuous flow isotope ratio mass spectrometer to analyze ^{15}N.

Soil was sampled at three times; before corn planting, after corn was planted pre-fertilizer, and at harvest. Soil samples were collected as composites of 5 sub-samples per plot. Prior to corn planting soil was sampled (0-15 cm) for routine analysis (organic matter, phosphorus, potassium, and pH). Soil samples were collected at the time of red clover sampling (0-30 cm, and 30-60 cm) and analyzed for nitrate and ammonium- N with a potassium chloride extraction at the Soil and Forage Analysis Laboratory in Marshfield, WI. Soil was additionally sampled in-season, prior to corn harvest (0-30cm) for preplant soil nitrogen content analyzed by potassium chloride extraction and after planting for an in-season analysis of soil nitrogen also with potassium chloride extraction content on a per block basis for treatments within zero N and optimum N rates (determined by 2017 and 2018 yields).

Statistical analysis

To compare the overall significance of clover crop treatment and nitrogen fertilizer rate, analyses of variance and statistical data analysis were performed using SAS software (SAS Institute, Cary, NC) and RStudio (R Core Team, 2020). Analysis of variance (ANOVA) was conducted with the lme4 and agricolae package in R (Bates et al., 2015; Mendiburu, 2020) was used to determine (1) the effect of cover crop on fall and spring nitrate and (2) the effect of cover crop, block, nitrogen fertilizer rate, and their interaction on in-season nitrate levels. In the mixed model, block, block*cover crop treatment, block*cover crop treatment*nitrogen fertilizer rate were all random effects in a split block design. Assumptions for normality were tested for NO_3-N and PAN using QQ-plots and plotting residuals from un-transformed data. The NO_3-N and PAN data did not meet assumptions for normality, and a Box-Cox transformation procedure was conducted to determine the optimal transformation for both data sets with the car package in RStudio, resulting in log-transforming all NO_3-N and PAN data for ANOVA (Fox & Weisberg, 2019). Data from in-season sampling were analyzed by sampling date. Soil sampling for nitrate and ammonium (summed for plant available N) before planting and at harvest were analyzed by year.

A bootstrapping technique with the FertBoot package (Ma & Francis, 2020a) was used to determine statistical differences in treatment fertilizer response curve models, maximum yield, and optimum nitrogen fertilizer rates using non-linear models. A detailed explanation of the bootstrapping approach can be found in Chapter 1.

Results

Nitrogen biomass and total N uptake

Red clover biomass varied within each treatment and between site-years (Tables 7, 8 and 9). See Appendix I. for photos of clover biomass establishment between treatments and site-years. The most clover biomass in any sampling period occurred in fall of the establishment year (2017) in the first field (Table 7). Following the establishment year, where stand establishment was not as robust, the clover biomass (kg ha^{-1}) was as low as 50 kg ha^{-1} and at most 397.4 kg ha^{-1} (Table 9).

Table 7. Interseeded red clover and reference rye biomass in Fall 2017- Spring 2018. Standard errors reported. Clover with N treatments are averaged between different N rates (180 kg ha^{-1} and 210 kg ha^{-1})

Sampling Time	Treatment	Biomass	Total N	Biomass SE	Total N SE
		-----kg ha^{-1}-----			
Fall 2017	Clover No N	699.1	7.54	62.5	0.60
	Clover With N	699.1	9.43	144.3	0.17
	Rye/Corn With N	609.5	11.64	-	-
	Rye/Corn No N	627.4	9.63	-	-
Spring 2018	Clover No N	158.6	5.6	69.6	2.38
	Clover With N	197.6	6.09	68.0	2.85
	Rye/Corn With N	166.7	4.13	-	-
	Rye/Corn No N	611.3	15.12	-	-

Table 8. Interseeded red clover and reference rye biomass in Fall 2018- Spring 2019. Standard errors reported. Clover with N treatments are averaged between different N rates (180 kg ha^{-1} and 210 kg ha^{-1})

Sampling Time	Treatment	Biomass	Total N	Biomass SE	Total N SE
		-----kg ha^{-1}-----			
Fall 2018	Clover No N	247.5	7.26	67.8	2.11
	Clover With N	263.9	8.81	77.6	77.60
	Rye/Corn With N	259.9	8.08	-	-
	Rye/Corn No N	188.2	6.45	-	-
Spring 2019	Clover No N	397.4	11.76	199.0	6.78
	Clover With N	99	3.38	131.9	2.88
	Rye/Corn With N	663.3	15.61	-	-
	Rye/Corn No N	1857	48.67	-	-

Table 9. Interseeded red clover and reference rye biomass in Fall 2019- Spring 2020. Clover with N treatments are averaged between different N rates (180 kg ha^{-1} and 210 kg ha^{-1})

Sampling Time	Treatment	Biomass	Total N	Biomass SE	Total N SE
		-----kg ha^{-1}-----			
Fall 2019	Clover No N	68.8	1.35	19.0	0.51
	Clover With N	54.7	0.97	10.8	0.21
	Rye/Corn With N	30.5	1.16	-	-
	Rye/Corn No N	37.6	1.14	-	-
Spring 2020	Clover No N	176.1	5.36	29.0	1.06
	Clover With N	162.7	4.98	32.8	2.46

Soil Nitrogen

Soil nitrogen was sampled by site-year in fall (at corn harvest) and early spring (just prior to corn planting) for soil nitrate and ammonium at two sampling depths (Table 10). Soil nitrate was high (>29 mg kg^{-1} in the upper 30 cm or >8 mg kg^{-1} in the 30-60 cm sampling depth) in the clover plots with a nitrogen application rate (210 kg ha^{-1}) in the fall of 2017, then decreased in successive years to much lower nitrate values (2-6 mg kg^{-1} in the upper 30 cm or 0-7 mg kg^{-1} in the 30-60 cm sampling depth). Each year, the early spring sampling period is during clover establishment when corn is not yet planted. Plant available N (PAN) followed a similar trend to the nitrate, where PAN was higher (>35 mg kg^{-1}) at both depths in the plots with nitrogen. The second interseeded field was established at Arlington Agricultural Research Station in 2019 with the same treatment structure and nitrogen fertilizer rates. The 2019-2020 soil fall nitrate levels were between 1-3 mg kg^{-1} in the upper 30 cm and between 1-4 mg kg^{-1} in the lower 30-60 cm depth. Fall soil PAN values were between 6-8 mg kg^{-1} at both depths and higher in the treatments with nitrogen fertilizer applied. Means were reported for the four replicates for each nitrate and PAN value (Tables 10 and 11).

The early spring soil NO_3-N and PAN values were highest in the first year of the study in 2018, with maximum values in the upper 30 cm in the no clover treatment with nitrogen in 2018 at 5.9 mg kg^{-1} NO_3-N and 12.6 mg kg^{-1} PAN. The NO_3-N values are higher at the 30-60 cm depth in 2018 and 2019 than the upper 0-30cm measurements, while PAN values are higher in the upper 0-30 cm in 2018, then are higher in the 30-60 cm depth in 2019 and 2020. The late spring sampling time served as a snapshot of in season nitrate in the upper 30 cm after corn planting once clover had been terminated (Table 10). The 2018 and 2019 in-season soil nitrate levels were between 2-9 mg kg^{-1} and 10-16 mg kg^{-1} PAN. The plots with nitrogen fertilizer generally had higher values than the plots without nitrogen fertilizer added. Soil nitrogen measurements were variable between years and there was not a consistent trend between clover or no clover treatments (Table 10).

Table 10. Soil nitrate and plant available N (PAN as NO_3-N+NH_4-N) in fall, early spring, and in-season late spring sampling periods in the first field. The first field encompasses the 2017-2019 years.

Clover Treatment		Nitrogen	NO_3-N		PAN (NO_3-N+NH_4-N)	
			2017	2018	2017	2018
Fall			------------------------mg kg^{-1}------------------------			
0-30 cm	No Clover	With	29.4	4.93	35.1	12.6
		None	3.63	4.08	8.48	11.9
	Clover	With	36.3	5.35	41.0	13.5
		None	3.70	3.95	8.43	11.2
30-60 cm	No Clover	With	11.8	4.55	16.2	12.2
		None	1.68	3.58	5.95	12.4
	Clover	With	8.98	5.13	13.2	12.1
		None	1.50	3.40	5.53	10.2
Early Spring			2018	2019	2018	2019

0-30 cm	No Clover	With	5.90	4.53	12.6	9.18
		None	4.00	4.48	8.40	9.08
	Clover	With	4.75	4.13	9.88	9.15
		None	3.55	3.30	8.23	8.88
30-60 cm	No Clover	With	7.75	6.08	12.2	10.4
		None	4.20	5.30	8.15	9.55
	Clover	With	6.30	5.95	10.4	10.7
		None	3.48	3.85	7.43	9.38
Late Spring			2018	2019	2018	2019
0-30 cm	No Clover	With	5.30	8.78	11.32	15.43
		None	4.70	9.25	10.83	16.14
	Clover	With	5.93	6.00	11.28	13.30
		None	5.53	5.45	10.90	11.48

Table 11. Soil nitrate and plant available N (PAN as NO_3-N+NH_4-N) in Fall, early spring, and in-season late spring sampling periods in the second field, which was established in 2019 through 2020

Clover Treatment		Nitrogen	NO_3-N	PAN
			2019	
Fall			-----mg kg^{-1}----	
0-30 cm	No Clover	With	2.68	8.28
		None	2.00	7.03
	Clover	With	2.93	7.68
		None	2.15	7.00
30-60 cm	No Clover	With	2.75	8.83
		None	1.48	6.50
	Clover	With	3.84	9.09
		None	1.56	6.99
Early Spring			2020	
0-30 cm	No Clover	With	2.90	7.88
		None	2.80	7.53
	Clover	With	2.20	7.23
		None	1.98	6.93
30-60 cm	No Clover	With	2.25	8.90
		None	1.28	6.43
	Clover	With	1.71	8.33

		None	1.54	8.68
Late Spring			2020	
0-30 cm	No Clover	With	4.45	13.80
		None	3.18	12.33
	Clover	With	3.61	13.06
		None	2.94	12.01

Soil nitrate and PAN were compared across experimental cover crop treatments, nitrogen fertilizer rate, and sampling depth using a mixed model ANOVA with the split-plot function in the lmer and agricolae package in R (Bates et al., 2015; Mendiburu, 2020). In the mixed model, cover crop, nitrogen rate (without or with N; 0 or 210 kg ha^{-1}), and depth were variables analyzed and block*cover crop*nitrogen rate were random effects.

Fall

In the fall sampling periods for each site-year, soil nitrate and PAN (as a sum of NO$_3$-N+NH$_4$-N) were not significantly different between cover crop treatment, however, they were significantly different between treatments with or without applied N fertilizer (Table 12). The treatments with nitrogen fertilizer applied had significantly more nitrate and PAN in the soil than treatments without nitrogen applied in the fall sampling period. There was significantly more nitrate in the upper 30cm in Fall of 2017 and 2018, and higher PAN in the upper 30cm in 2017 only (Table 12). There was one interaction effect of applied nitrogen fertilizer and depth in 2017, where samples with nitrogen fertilizer in the upper 30cm had more PAN (24.9mg kg^{-1}) than samples without nitrogen fertilizer at sampling depths of 30-60cm (8.65mg kg^{-1} PAN).

Early Spring

There was one significant difference in cover crop treatments in the first field study site with in early spring 2018 where the no clover treatments had more soil nitrate (5.5mg kg-1) and PAN (10.3) than the clover treatment nitrate (4.5mg kg^{-1}) and PAN (8.99) (Table 12). The nitrate levels were not significantly different between different nitrogen fertilizer rates in the early spring sampling period except in 2018 where treatments with nitrogen fertilizer applied had more soil nitrate than those without. In 2018 and 2020, PAN was also higher in the treatments with nitrogen fertilizer applied (Table 13). There were significant differences between sampling depth in most sampling periods. In early spring of 2018 and 2019, there a was greater concentration of nitrate in the 30-60c m depth than the 0-30 cm depth, while in 2020 that changed to more nitrate in the upper 30 cm (Table 13). The PAN was significantly different between depths in 2020, where there was significantly higher PAN at 30-60 cm (8.09) than at 0-30 cm (7.39 mg kg^{-1}). There was one instance of statistically significant interaction with nitrogen rates and depth where treatments with nitrogen at the 30-60 cm sampling depth had significantly more nitrate (5.80 mg kg^{-1}) than any other interaction in 2018, though the lowest nitrate level of the interactions was not even 2mg kg-1 lower in the treatment with no nitrogen fertilizer applied at the 0-30 cm depth (4.18 mg kg^{-1}) (Table 13).

In-season sampling late spring

The in-season sampling periods in late spring of 2018 and 2020 show no significant effects on soil nitrate concentration with cover crop treatment (clover or no clover), nitrogen fertilizer application, and the interaction between the two (Table 14). In 2019, there is a statistically significant difference in cover crop treatment, where the no clover treatment had more PAN (15.8 mg kg^{-1}) than the clover treatment (12.4 mg kg^{-1}).

Table 12. Fall average nitrate (NO_3-N) and plant available nitrogen (PAN) values with ANOVA results as affected by cover crop treatment (clover or no clover), nitrogen fertilizer application (with or none), and at different sampling depths (0-30 cm and 30-60 cm). Within each column for significant treatment factors, means followed by the same letter are not significantly different ($\alpha = 0.05$).

	NO_3-N			PAN (NO_3-N+NH_4-N)		
Treatment	2017	2018	2019	2017	2018	2019
	-------------------	-------------------	mg kg^{-1}	-------------------	-------------------	-------------------
Cover crop (CC)						
Clover	12.6a	4.46a	2.62a	17.0a	11.8a	7.69a
No Clover	11.6a	4.29a	2.23a	16.4a	12.3a	7.66a
Nitrogen (N)						
With	21.6a	4.99a	3.05a	26.4a	12.6a	8.47a
None	2.63b	3.75b	1.80b	7.10b	11.4b	6.88b
Depth (D)						
0-30 cm	18.3a	4.58a	2.44a	23.3a	12.3a	7.50a
30-60 cm	5.99b	4.17b	2.41a	10.2b	11.7a	7.85a
Source of variation			*P*-Value			
CC	0.571	0.268	0.964	0.601	0.226	0.927
N	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
D	<0.001	0.038	0.731	<0.001	0.881	0.613
CC × N	0.866	0.060	0.839	0.910	0.279	0.563
CC × D	0.216	0.669	0.561	0.285	0.193	0.433
N × Depth	0.183	0.086	0.161	<0.001	0.880	0.230
CC × N × D	0.432	0.792	0.582	0.468	0.746	0.886

Table 13. Early spring average nitrate (NO_3-N) and plant available nitrogen (PAN) values with ANOVA results as affected by cover crop treatment (clover or no clover), nitrogen fertilizer application (with or none), and at different sampling depths (0-30 cm and 30-60 cm). Within each column for significant treatment factors, means followed by the same letter are not significantly different ($\alpha = 0.05$).

	NO_3-N			PAN (NO_3-N+NH_4-N)		
Treatment	2018	2019	2020	2018	2019	2020
	-------------------	-------------------	mg kg^{-1}	-------------------	-------------------	-------------------
Cover crop (CC)						
Clover	4.5b	4.31a	1.86a	8.99b	9.53a	7.79a
No Clover	5.5a	5.10a	2.31a	10.3a	9.55a	7.69a

Nitrogen (N)						
With	6.18a	5.17a	2.27a	11.3a	9.86a	8.09a
None	3.81b	4.23a	1.90a	8.05b	9.22a	7.39b
Depth (D)						
0-30 cm	4.55b	4.11b	2.47a	9.78a	9.07a	7.39b
30-60 cm	5.43a	5.30a	1.70b	9.55a	10.0a	8.09a
Source of variation			*P*-Value			
CC	0.00188	0.156	0.171	0.00675	0.619	0.649
N	<0.001	0.0877	0.209	<0.001	0.0607	0.0161
D	0.00288	0.00153	0.00113	0.766	0.433	0.0154
CC × N	0.727	0.78	0.316	0.129	0.953	0.0216
CC × D	0.552	0.476	0.216	0.792	0.332	0.00469
N × Depth	0.00466	0.539	0.268	0.16	0.671	0.16
CC × N × D	0.942	0.466	0.179	0.518	0.728	0.00951

Table 14. Late spring in-season average nitrate (NO_3-N) and plant available nitrogen (PAN) values with ANOVA results as affected by cover crop treatment (clover or no clover), nitrogen fertilizer application (with or none), and at different sampling depths (0-30 cm and 30-60 cm). Within each column for significant treatment factors, means followed by the same letter are not significantly different ($\alpha = 0.05$).

	NO_3-N			PAN (NO_3-N+NH-4)		
Treatment	2018	2019	2020	2018	2019	2020
	------------------------------------mg kg^{-1}------------------------------------					
Cover crop (CC)						
Clover	5.73a	5.73a	3.28a	11.1a	12.4b	12.5a
No Clover	5.00a	9.02a	3.82a	11.1a	15.8a	13.1a
Nitrogen (N)						
With	5.62a	7.39a	4.03a	11.3a	14.4a	13.4a
None	5.12a	7.35a	3.06a	10.9a	13.8b	12.2a
Source of variation			*P*-Value			
CC	0.234	0.0567	0.353	0.708	0.0178	0.513
N	0.0839	0.0709	0.202	0.245	0.0123	0.151
D	0.557	0.866	0.632	0.748	0.615	0.632

^{15}N analysis

The nitrogen derived from the atmosphere (Ndfa (%)) values varied within clover treatments between replicates. Clover treatments with nitrogen had more instances of zero biological nitrogen fixation than clover without nitrogen fertilizer application (Table 15).

Table 15. Nitrogen derived from the atmosphere in clover and no clover treatments within site-years in fields 1 and 2

	Fall								Spring							
	Clover No N				Clover With N				Clover No N				Clover With N			
Site year 1: Fall 2017 - Spring 2018																
Rep	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Ndfa (%)	44.83	38.33	46.43	58.10	0.00	0.00	0.00	22.77	77.33	83.65	77.00	102.09	77.33	61.00	70.33	79.08
Mean				46.92				5.69				85.02				71.94
SD				8.23				11.39				11.79				8.21
CV (%)				17.54				200.00				13.86				11.42
Site year 2: Fall 2018 - Spring 2019																
Ndfa (%)	60.96	0.00	65.99	73.44	0.00	0.00	31.39	21.62	76.33	47.01	82.92	95.14	42.02	53.17	43.27	73.87
Mean				50.10				13.25				75.35				53.08
SD				33.79				15.81				20.44				14.73
CV (%)				67.45				119.33				27.12				27.74
Site year 3: Fall 2019																
Ndfa (%)	60.96	0.00	65.99	73.44	0.00	0.00	31.39	21.62			N/A					
Mean				50.10				13.25								
SD				33.79				15.81								
CV (%)				67.45				119.33								
Field 2: Fall 2019- Spring 2020																
Ndfa (%)	41.05	60.26	5.09	53.34	38.13	10.43	14.38	4.14	44.90	0.00	0.00	76.74	19.40	0.00	0.00	93.60
Mean				39.93				16.77				30.41				28.25
SD				24.55				14.85				37.44				44.52
CV (%)				61.48				88.55				123.13				157.6

An accompanying reference rye to the red clover was used each sampling period with the natural abundance method. While rye is commonly used as a reference plant for other cover crops, the $\delta^{15}N$ varied widely between sampling periods (Table 16). The $\delta^{15}N$ is higher for rye and clover samples with a nitrogen fertilizer application (210 kg ha^{-1}) in every year except Fall of 2017 for the rye cover crop reference (Table 16).

Table 16. Reference plant $\delta^{15}N$ variability

Sampling Date	**Plot**	**$\delta^{15}N$ (permil)**
11/16/17	Rye in Corn no N	1.97
11/16/17	Rye in Corn with N	1.22
5/24/18	Rye/Corn with N	4.23
5/24/18	Rye/Corn no N	2.04
11/1/18	Rye/Corn no N	1.13
11/1/18	Rye/Corn with N	3.29
5/22/19	Rye/Corn no N	1.78
5/22/19	Rye/Corn with N	2.52
10/30/19	Rye/Corn no N	1.75
10/30/19	Rye/Corn with N	3.62

Table 17. Red clover biomass $\delta^{15}N$ variability

Sampling Date	**Plot**	**$\delta^{15}N$ (permil)**
11/16/17	Clover no N	0.60
11/16/17	Clover with N	1.58
5/24/18	Clover no N	-0.21
5/24/18	Clover with N	0.42
11/1/18	Clover no N	0.11
11/1/18	Clover with N	3.53
5/22/19	Clover no N	-0.27
5/22/19	Clover with N	0.68
10/30/19	Clover no N	-0.26
10/30/19	Clover with N	1.60
6/18/20	Clover no N	1.17
6/18/20	Clover with N	3.13

Yield and Optimum Nitrogen Fertilizer Rates

Bootstrapped residuals were used to calculate 95% confidence intervals of the optimum nitrogen fertilizer rates and maximum yields for each cover crop treatment (Figure 12). While Chapter 1 discussed this and the following results more thoroughly, they are presented again here. The log likelihood ratio test for differences in models (prior to bootstrapping the data) suggest models are significantly different (p=0.006). In 2018, there was a small (15-17 kg ha^{-1}), but statistically significant fertilizer replacement value in the clover treatments (Table 17). The corn that was not interseeded with red clover required significantly more nitrogen to reach a maximum yield than the treatments that had red clover interseeded (Figures 12 & 13, Table 17). Maximum yields are significantly different between treatments, where Clover 2017 treatment had a slightly higher yield (10.7 Mg ha^{-1}) than the No Clover treatment (10.5 Mg ha^{-1}), which were both higher than the Clover Always treatment (10.5 Mg ha^{-1}). While the bootstrapping technique allowed for determination of statistically significant differences between yields, the differences here may not be agronomically significant in this agricultural system.

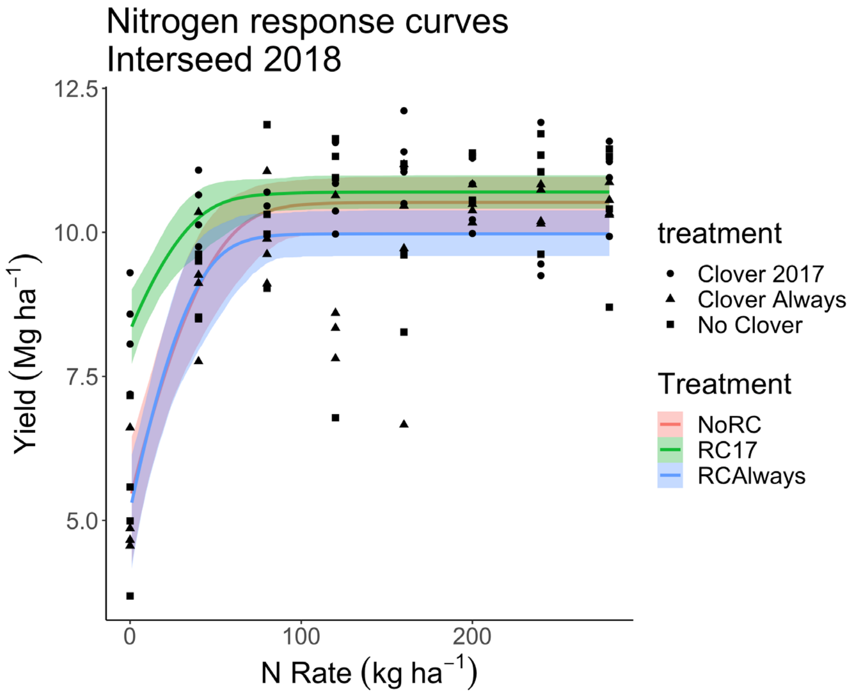


Figure 12. Fertilizer response curve quadratic plateau models determined by bootstrapping residuals for clover treatments No Clover (NoRC), Clover 2017 (RC17), and Clover Always (RC Always). Data points are the actual sample. Banding represents the bootstrapped 95% confidence intervals.

Table 18. Optimum N rate and yield for the 2017-2018 site-year determined by bootstrapping residuals. Different letters suggest significant differences ($p<0.05$).

				95% Confidence Interval	
Treatment	Parameter	Estimate	Std. Error	Lower	Upper
None	a	5.34	0.578	4.02	6.29
	b	0.123	0.0334	0.0714	0.198
	c	-0.000773	0.000370	-0.00179	-0.000305
	Optimum N (kg ha^{-1})	89.1 a	23.1	61.9	174
	Maximum yield (Mg ha^{-1})	10.5 b	0.245	9.95	10.9
Clover Always	a	5.17	0.522	3.87	5.96
	b	0.142	0.0383	0.0743	0.226
	c	-0.00111	0.000532	-0.00253	-0.000368
	Optimum N (kg ha^{-1})	72.2 b	0.207	50.4	144
	Maximum yield (Mg ha^{-1})	9.98 a	19.1	9.57	10.4
Clover 2017	a	8.29	0.359	7.63	8.96
	b	0.0719	0.0232	0.0310	0.119
	c	-0.000574	0.000308	-0.00136	-0.000133
	Optimum N (kg ha^{-1})	73.6b	26.0	49.7	209.2
	Maximum yield (Mg ha^{-1})	10.7c	0.148	10.4	11.0

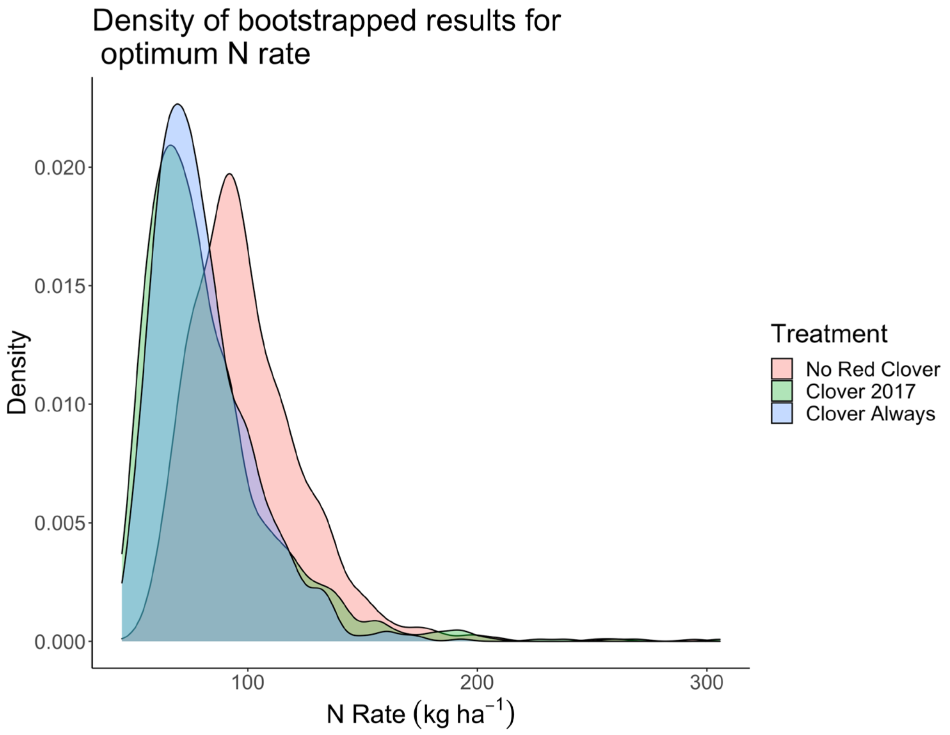


Figure 13. Density plot of the bootstrapped results (where the data was resampled 1000 times) for the optimum nitrogen fertilizer rate for each treatment. The density plots are constructed with the results from bootstrapping residuals.

In 2019, there was not a significant fertilizer replacement value in the clover treatments (Table 18). Models in 2019 were not significantly different determined by the log likelihood ratio test (p=0.580). The corn that was not interseeded with red clover required as much nitrogen to reach a maximum yield as the treatment where clover was only inteseeded in 2018 (Figure 14 &15, Table 18). The treatment with clover inter-seeded every year had an optimum nitrogen fertilizer rate that was significantly higher than the treatments without clover or with clover interseeded only in 2018 (Table 18 and Figure 15).

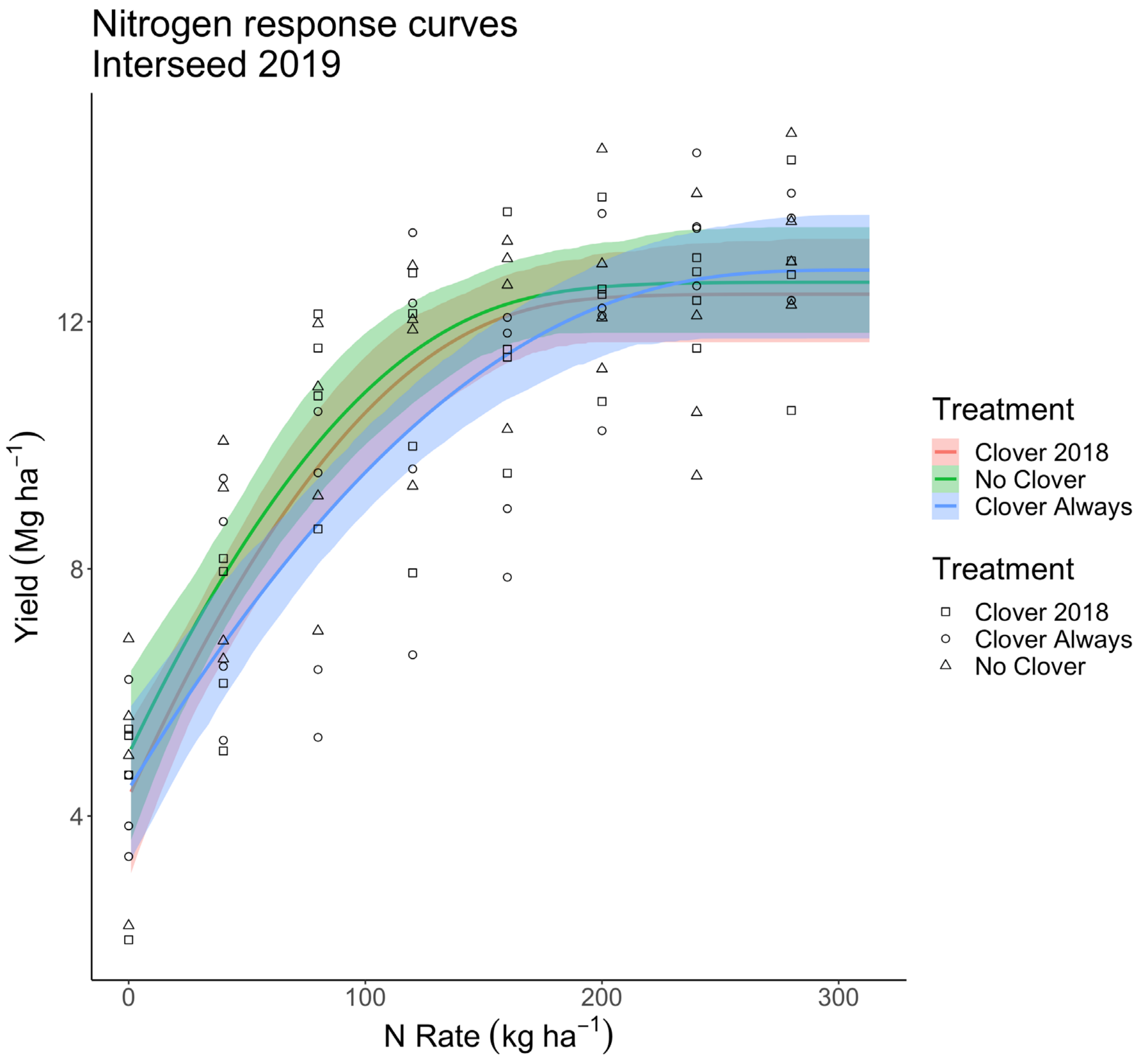


Figure 14. Fertilizer response quadratic plateau models determined by bootstrapping residuals for clover treatments No Clover, Clover 2018, and Clover Always. Data points are the actual sample. Banding represents the bootstrapped 95% confidence intervals.

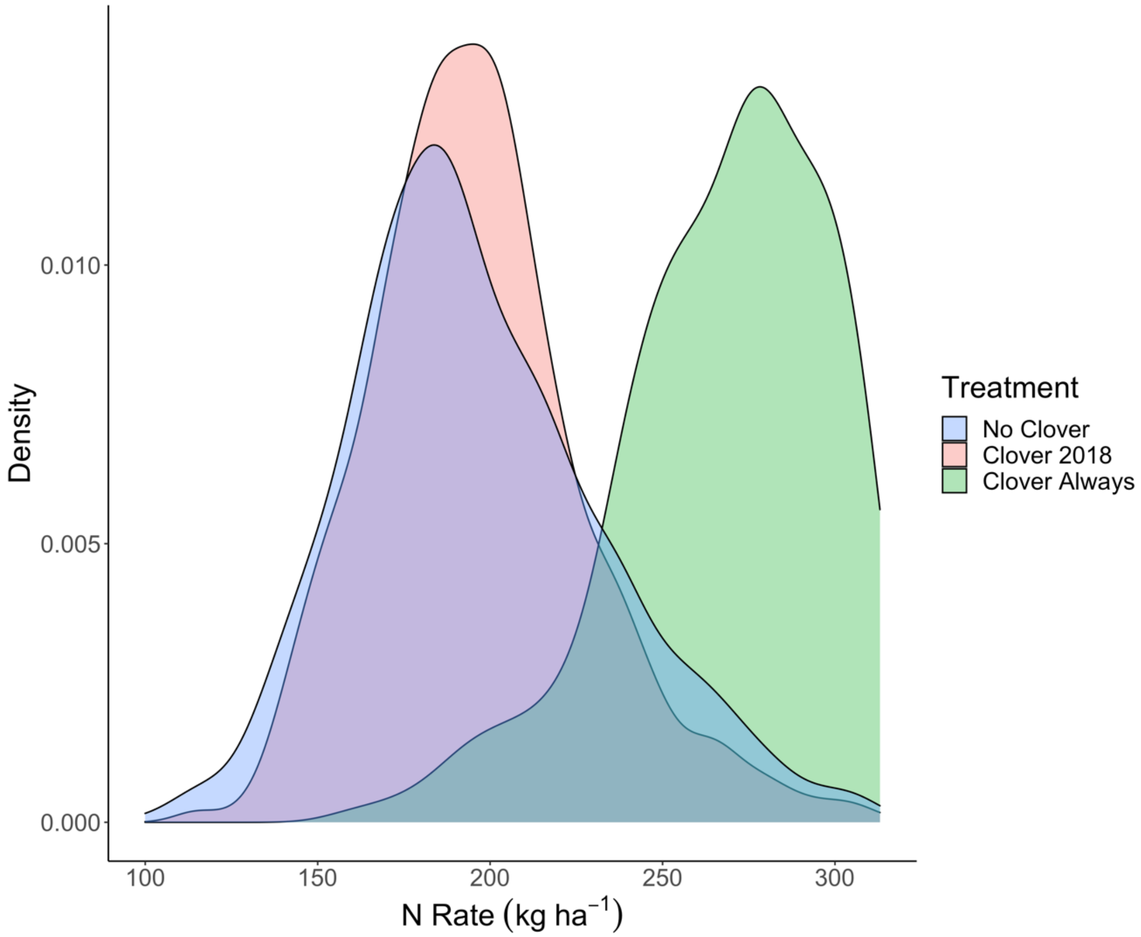


Figure 15. Density plot of the bootstrapped results for 2019 interseed yield data (where the data was resampled 1000 times) for the optimum nitrogen fertilizer rate for each treatment. The density plots are constructed with the results from bootstrapping residuals.

Table 19. Optimum N rate and yield for the 2018-2019 site-year determined by bootstrapping residuals. Different letters suggest significant differences ($p<0.05$).

Model	Model Parameter	Estimate	Std. Error	95% Confidence Interval Lower	Upper
No Clover	a	5.00	0.736	3.40	6.27
	b	0.0807	0.0180	0.0526	0.126
	c	-0.000221	9.20e-05	-0.000506	-0.000106
	Optimum N (kg ha^{-1})	196.3b	36.9	141.8	293.9
	Maximum yield (Mg ha^{-1})	12.6	0.434	11.87	13.59
Clover Always	a	4.43	0.672	3.22	5.80
	b	0.142	0.00937	0.0485	0.0865
	c	-0.0011	3.31e-05	-2.45e-04	-8.78e-05
	Optimum N (kg ha^{-1})	267.9b	30.1	184.8	309.0
	Maximum yield (Mg ha^{-1})	12.8a	0.527	11.71	13.70
Clover 2018	a	4.31	0.648	2.94	5.45
	b	0.0849	0.0156	0.0596	0.123
	c	-0.000227	7.633e-05	-0.000452	-0.000125
	Optimum N (kg ha^{-1})	196.5b	31.3	146.3	278.5
	Maximum yield (Mg ha^{-1})	12.4c	0.41	11.7	13.4

DISCUSSION

Biomass

Red clover above-ground biomass establishment and production were inconsistent when interseeded into corn. Previous studies have reported more red clover biomass when red clover was interseeded into winter wheat in a corn-soybean-wheat system. In one study, biomass accumulated to just over 2000 kg ha^{-1} prior to termination in plots without nitrogen and a minimum of 1090.2 kg ha^{-1} with nitrogen (A. C. M. Gaudin et al., 2014). Red clover biomass increased by a substantial amount (912.5 kg ha^{-1} with roots, 222 kg ha^{-1} shoots) after wheat was harvested, suggesting that interseeding created competition for red clover biomass accumulation. As nitrogen fertilizer rates increase, canopy cover also increased, decreasing solar radiation accessible by the clover, and yielding a decrease in clover biomass. Another study reported a range of 688-1184 kg ha^{-1} clover biomass accumulation in a relatively dry year, and in a wetter year, yielded upwards of 2300 kg ha^{-1} clover biomass (Queen et al., 2009).

Clover biomass accumulation declined after the establishment year due environmental controls in a continuous corn system. The most productive year for biomass occurred in the establishment year of the first field in November 2017 (699 kg ha^{-1}), at the lower end, or much further below biomass values observed in other studies. Beyond the establishment year, clover biomass production values decreased and the establishment of the clover stand between replicates and within plots was variable. To inform future agronomic practices, it is important to note that stand establishment was a primary concern for farmers that were interested in cover crop adoption (Conservation Technology Information Center (CTIC), 2017; CTIC & SARE, 2020; Roesch-Mcnally et al., 2018). The variable stand establishment in this study suggests that this no-till, interseed system might not be the best option for a hearty red clover stand. The small amount of biomass accumulation prior to termination justifies the very small nitrogen fertilizer replacement value in 2018, and the lack of any nitrogen fertilizer replacement value in 2019. As a no-till system, corn stover remains on top of the soil after harvest. In addition to light interception by corn, the cover is thick enough that it might inhibit clover emergence after successive years of stover surface build-up. While the amount of corn stover may explain much of the variation in clover biomass growth, red clover is known to have problems with stand evenness due to soil moisture and topography (Wyngaarden et al., 2015). The understory environment when clover is interseeded into corn simply did not allow for clover to build enough biomass to be useful to the next season of corn.

Yield effect

Interseeded red clover may not provide enough nitrogen to corn to be a promoted as a productive system for farmers in Wisconsin. The treatments with interseeded red clover required significantly less nitrogen (15-17 kg ha^{-1}) at the optimum nitrogen rate than the treatment without clover interseeded. This was a small difference, and while statistically significant, might not be agronomically significant to a farmer. There was a small yield increase from the clover treatment "clover always", which was consistent with other cover crop studies and meta-analyses that showed increased plant available nitrogen and increased or maintained corn yield compared to a control without a cover crop (Coombs et al., 2017b; Marcillo & Miguez, 2017). However, the other clover treatment called "clover 2017" did not show a yield increase, and in fact, showed a decrease even though it also required less nitrogen than the treatment without clover interseeded.

The 2017-2018 site-year was the establishment year and did not have years of continuous corn cultivation on a no-till field to build up corn stover. Since the establishment year in 2017 did not have abundant corn stover, the clover was able to grow a more robust stand without impediment. In the subsequent years, corn residue accumulated, leading to substantial corn stover accumulation, limiting clover emergence. The treatments with clover interseeded in 2018-2019 required more nitrogen than the treatments without clover. Once again, the treatments with clover interseeded in 2018 had a slight yield drag compared to the no clover treatments, while the treatment where clover was interseeded in both 2017 and 2018 had a slight yield increase compared to the plot without clover. Since other research correlates clover with a slight increase in corn yield, and clover competition with corn is minimal, it is unlikely that clover is causing the yield depression. Instead, the yield decrease might be a result of a system where corn is grown after corn instead of rotated with other crops (Brooker et al., 2020).

N response effect

The soil nitrogen data does not suggest a reliable release of nitrogen from red clover biomass in the upper 30 cm following red clover cover crop termination. The cover crop treatments with a nitrogen fertilizer application (210 kg ha^{-1} for treatments without the split N rates, and otherwise 190 kg ha^{-1} for the "with" nitrogen sampling in split plots) have a statistically significantly higher soil nitrate and PAN. Part of the lack of a nitrogen flush is explained by the variability in the amount of cover crop biomass. The higher level of soil nitrate and PAN in fertilized plots at corn harvest in the fall suggests more nitrogen from synthetic fertilizer input rather than from biological nitrogen fixation in the clover biomass. In early spring when the clover was planted, there was either significantly less or not a significant difference in NO_3-N and PAN in the plots with clover compared to the no clover plots. This sampling period was at the time of clover termination prior to planting corn. We expected a slight amount of nitrogen drawdown by the cover crop during this period if it does not receive enough nitrogen from biological nitrogen fixation. Since there was less nitrogen available in the clover cover crops without nitrogen fertilizer application, growing clover appeared to be taking up slightly more nitrogen from the soil than the clover that has access to nitrogen fertilizer. The in-season PAN was not significantly different across treatments except for the 2019 late spring test in the second study field before the trial was established. This suggests that there was not a release of nitrogen from the clover as it decomposed post-termination after corn planting, a time when farmers might sample to adjust for N fertilizer applications.

Biological nitrogen fixation

In addition to the variability in biomass accumulation, there was also a variable range of biological nitrogen fixation in the clover between years, treatments, and replicates. The variability in biological nitrogen fixation makes it unclear how much new nitrogen (derived from the atmosphere, available when clover biomass decomposes) enters the system. Other studies have observed that environmental factors rather than specific legume species are significant controls (site-year, special variation) on biological nitrogen fixation in legumes (Parr et al., 2011). Unlike other studies that conduct greenhouse experiments to determine their own baseline $\delta^{15}N$ values for nodulating legumes (Parr et al., 2011), we used a B-value of -0.94 as an average of values (-0.78 to -1.30) reported by four separate studies for red clover in the appendix of a review by Unkovich et al., (2008) that explored different means of measuring plant-associated nitrogen fixation. This estimation of a B-value can be a source of potential error; the reference plant variation is large between years and treatments. Rye interseeded into corn was chosen as the non-legume reference plant for this study in line with other studies that use rye as a reference plant to compare with legume cover crops (Parr et al., 2011). There is a clear pattern of lower $\delta^{15}N$ values for rye interseeded into corn without nitrogen fertilizer applied and higher $\delta^{15}N$ values when nitrogen fertilizer was applied. While we wouldn't expect a variation in $\delta^{15}N$ values in reference rye if the fertilizer applied was made using the Haber-Bosch process, our data suggest that fertilizer application led rye to be enriched in ^{15}N. The variation in nitrogen derived from the atmosphere in clover between years and between the plots that received synthetic nitrogen fertilizer is dependent on the reference rye plant, which varied with fertilizer application. The range in values of nitrogen derived from the atmosphere in red clover between replicates that we report suggests the reduction in biological fixation is spatially variable. Since average Ndfa(%) values were only above 50% in Fall and Spring 2017-2018 in both clover treatments and Spring 2019 in clover treatments without N, there is little evidence that we were consistently adding new nitrogen to the system.

CONCLUSION

Interseeded red clover into corn did not lead to differences in plant available nitrogen, slightly decreased or did not change the optimum nitrogen fertilizer rate to achieve maximum yield, and did not grow new nitrogen to the system through biological nitrogen fixation to point to a significant nitrogen contribution. Given the limitations of clover biomass establishment in this system, we do not recommend successively interseeding red clover into a continuous corn system in Wisconsin until additional studies can confirm reliable red clover biomass establishment. More multi-year research trials that interseed red clover are necessary to inform the economic and agroecologic benefits of interseeding red clover. We suggest future studies with red clover in no-till corn systems consider corn row spacing, corn planting density, and relative maturity with different corn species to increase light penetration to the clover in the understory and management of corn stover on the soil surface. We also suggest comparing interseeded red clover into corn at experimental plots further south in the corn belt to assess clover biomass establishment with increased growing degree days and gathering qualitative data on farmers' experiences with red clover under different growing conditions. As agriculture moves toward sustainable intensification, and demand for information on incorporating cover crops into existing systems increases, red clover is certainly worth further exploration.

REFERENCES

Baributsa, D. N., Foster, E. F., Thelen, K. D., Kravchenko, A. N., Mutch, D. R., & Ngouajio, M. (2008). Corn and cover crop response to corn density in an interseeding system. *Agronomy Journal*, *100*(4), 981–987. https://doi.org/10.2134/agronj2007.0110

Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, *67*(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Baxter, L. L., Grey, T. L., Tucker, J. J., & Hancock, D. W. (2019). Optimizing Temperature Requirements for Clover Seed Germination. *Agrosystems, Geosciences & Environment*, *2*(1), 1–7. https://doi.org/10.2134/age2018.11.0059

Blanco-Canqui, H., Shaver, T. M., Lindquist, J. L., Shapiro, C. A., Elmore, R. W., Francis, C. A., & Hergert, G. W. (2015). Cover crops and ecosystem services: Insights from studies in temperate soils. *Agronomy Journal*, *107*(6), 2449–2474. https://doi.org/10.2134/agronj15.0086

Brooker, A. P., Renner, K. A., & Basso, B. (2020). Interseeding cover crops in corn: Establishment, biomass, and competitiveness in on-farm trials. *Agronomy Journal*, *June*, 3733–3743. https://doi.org/10.1002/agj2.20355

Bruulsema, T. W., & Christie, B. R. (1987). Nitrogen Contribution to Succeeding Corn from Alfalfa and Red Clover 1 . *Agronomy Journal*, *79*(1), 96–100. https://doi.org/10.2134/agronj1987.00021962007900010020x

Caldwell, B., Mohler, C. L., Ketterings, Q. M., & DiTommaso, A. (2014). Yields and profitability during and after transition in organic grain cropping systems. *Agronomy Journal*, *106*(3), 871–880. https://doi.org/10.2134/agronj13.0286

Chatterjee, A., Subedi, K., Franzen, D. W., Mickelson, H., & Cattanach, N. (2018). Nitrogen fertilizer optimization for sugarbeet in the Red River valley of north Dakota and Minnesota. *Agronomy Journal*, *110*(4), 1554–1560. https://doi.org/10.2134/agronj2017.12.0694

Conservation Technology Information Center (CTIC). (2017). *2016-2017 cover crop survey report*. *September*. https://www.sare.org/Learning-Center/From-the-Field/North-Central-SARE-From-the-Field/2017-Cover-Crop-Survey-Analysis

Coombs, C., Lauzon, J. D., Deen, B., & Van Eerd, L. L. (2017a). Legume cover crop management on nitrogen dynamics and yield in grain corn systems. *Field Crops Research*. https://doi.org/10.1016/j.fcr.2016.11.001

Coombs, C., Lauzon, J. D., Deen, B., & Van Eerd, L. L. (2017b). Legume cover crop management on nitrogen dynamics and yield in grain corn systems. *Field Crops Research*, *201*, 75–85. https://doi.org/10.1016/j.fcr.2016.11.001

Corstanje, R., Portier, K. M., & Reddy, K. R. (2009). Discriminant analysis of biogeochemical indicators of nutrient enrichment in a Florida wetland. *European Journal of Soil Science*, *60*(6), 974–981. https://doi.org/10.1111/j.1365-2389.2009.01186.x

CTIC, & SARE. (2020). *Annual report 2019-2020 national cover crop survey* (Issue August).

Curran, W. S., Hoover, R. J., Mirsky, S. B., Roth, G. W., Ryan, M. R., Ackroyd, V. J., Wallace, J. M., Dempsey, M. A., & Pelzer, C. J. (2018). Evaluation of cover crops drill interseeded into corn across the mid-Atlantic region. *Agronomy Journal*, *110*(2), 435–443. https://doi.org/10.2134/agronj2017.07.0395

Davidson, A. C., & Hinkley, D. V. (1997). *Bootstrap Methods and their Application*. Cambridge University Press.

De Notaris, C., Rasmussen, J., Sørensen, P., & Olesen, J. E. (2018). Nitrogen leaching: A crop rotation perspective on the effect of N surplus, field management and use of catch crops. *Agriculture, Ecosystems and Environment*, *255*. https://doi.org/10.1016/j.agee.2017.12.009

den Hollander, N. G., Bastiaans, L., & Kropff, M. J. (2007). Clover as a cover crop for weed suppression in an intercropping design. II. Competitive ability of several clover species. *European Journal of Agronomy*, *26*(2), 104–112. https://doi.org/10.1016/j.eja.2006.08.005

Donovan, J. T. O., Ross, S. M., King, J. R., & Ce, R. (2001). Weed Suppression by Seven Clover Species. *Agronomy Journal*, *93*(1991), 820–827.

Efron, B. (1979). Bootstrap methods: another look at the jackknife. *Annals of Statistics*, *7*(1), 1–26.

Finney, D. M., White, C. M., & Kaye, J. P. (2016). Biomass production and carbon/nitrogen ratio influence ecosystem services from cover crop mixtures. *Agronomy Journal*, *108*(1), 39–52. https://doi.org/10.2134/agronj15.0182

Fox, J., & Weisberg, S. (2019). *An R Companion to Applied Regression* (third). Sage.

Gabriel, J. L., Muñoz-Carpena, R., & Quemada, M. (2012). The role of cover crops in irrigated systems: Water balance, nitrate leaching and soil mineral nitrogen accumulation. *Agriculture, Ecosystems and Environment*, *155*(3), 50–61. https://doi.org/10.1016/j.agee.2012.03.021

Gaudin, A. C. M., Janovicek, K., Martin, R. C., & Deen, W. (2014). Approaches to optimizing nitrogen fertilization in a winter wheat-red clover (Trifolium pratense L.) relay cropping system. *Field Crops Research*, *155*, 192–201. https://doi.org/10.1016/j.fcr.2013.09.005

Gaudin, A., Westra, S., Loucks, C. E. S., Janovicek, K., Martin, R. C., & Deen, W. (2013). Improving Resilience of Northern Field Crop Systems Using Inter-Seeded Red Clover: A Review. *Agronomy*, *3*(1), 148–180. https://doi.org/10.3390/agronomy3010148

Gentry, L. E., Snapp, S. S., Price, R. F., & Gentry, L. F. (2013). Apparent red clover Nitrogen credit to corn: Evaluating cover crop introduction. *Agronomy Journal*, *105*(6), 1658–1664. https://doi.org/10.2134/agronj2013.0089

Grabber, J. H., Jokela, W. E., & Lauer, J. G. (2014). Soil nitrogen and forage yields of corn grown with clover or grass companion crops and manure. *Agronomy Journal*, *106*(3), 952–961. https://doi.org/10.2134/agronj13.0466

Heikkinen, J., & Mäkipää, R. (2010). Testing hypotheses on shape and distribution of ecological response curves. *Ecological Modelling*, *221*(3), 388–399.

https://doi.org/10.1016/j.ecolmodel.2009.10.030

Henry, D. C., Mullen, R. W., Dygert, C. E., Diedrick, K. A., & Sundermeier, A. (2010). Nitrogen contribution from red clover for corn following wheat in western Ohio. *Agronomy Journal*, *102*(1), 210–215. https://doi.org/10.2134/agronj2009.0187

Hesterman, O. B., Griffin, T. S., Williams, P. T., Harris, G. H., & Christenson, D. R. (1992). Forage Legume-Small Grain Intercrops: Nitrogen Production and Response of Subsequent Corn. *Journal of Production Agriculture*, *5*(3), 340–348. https://doi.org/10.2134/jpa1992.0340

John, L. St., & Ogle, D. (2008). Plant Guide Red Clover (Trifolium pratense L.). In *USDA NRCS*. https://doi.org/10.1039/c3cc48937j

Jones, M. E., Harwood, R. R., Dehne, N. C., Smeenk, J., & Parker, E. (1998). Enhancing soil nitrogen mineralization and corn yield with overseeded cover crops. *Soil and Water Conservation Society*, *53*(3), 245–249.

Kawamura, K., Watanabe, N., Sakanoue, S., Lee, H. J., Lim, J., & Yoshitoshi, R. (2013). Genetic algorithm-based partial least squares regression for estimating legume content in a grass-legume mixture using field hyperspectral measurements. *Grassland Science*, *59*(3), 166–172. https://doi.org/10.1111/grs.12026

Kendall, W. A., & Stringer, W. C. (1985). Physiological Aspects of Clover Trifolium. *Agronomy*, *25*, 111–160.

Ketterings, Q. M., Swink, S. N., Duiker, S. W., Czymmek, K. J., Beegle, D. B., & Cox, W. J. (2015). Integrating cover crops for nitrogen management in corn systems on Northeastern U.S. dairies. *Agronomy Journal*, *107*(4), 1365–1376. https://doi.org/10.2134/agronj14.0385

King, A. E., & Blesh, J. (2018). Crop rotations for increased soil carbon: Perenniality as a guiding principle: Perenniality. *Ecological Applications*, *28*(1), 249–261. https://doi.org/10.1002/eap.1648

Li, X., Zhang, Y., Bao, Y., Luo, J., Jin, X., Xu, X., Song, X., & Yang, G. (2014). Exploring the best hyperspectral features for LAI estimation using partial least squares regression. *Remote Sensing*, *6*(7), 6221–6241. https://doi.org/10.3390/rs6076221

Lupwayi, N. Z., & Soon, Y. K. (2016). Nitrogen-Related Rotational Effects of Legume Crops on Three Consecutive Subsequent Crops. *Soil Science Society of America Journal*, *80*, 306–316. https://doi.org/10.2136/sssaj2015.08.0299

Ma, T. F., & Francis, H. (2020a). *FertBoot: Fertilizer Response Curve Analysis by Bootstrapping Residuals.* (0.4.0.). R package.

Ma, T. F., & Francis, H. (2020b). *FertBoot: Fertilizer Response Curve Analysis by Bootstrapping Residuals*. R package version 0.4.0.

Marcillo, G. S., & Miguez, F. E. (2017). Corn yield response to winter cover crops: An updated meta-analysis. *Journal of Soil and Water Conservation*, *72*(3), 226–239. https://doi.org/10.2489/jswc.72.3.226

Mendiburu, F. de. (2020). *agricolae: Statistical Procedures for Agricultural Research*. R package version 1.3-3.

Miguez, F. E., & Bollero, G. A. (2005). Review of corn yield response under winter cover cropping systems using meta-analytic methods. *Crop Science*, *45*(6), 2318–

2329. https://doi.org/10.2135/cropsci2005.0014

Pantoja, J. L., Woli, K. P., Sawyer, J. E., & Barker, D. W. (2015). Corn Nitrogen Fertilization Requirement and Corn–Soybean Productivity with a Rye Cover Crop. *Soil Science Society of America Journal*, *79*(5), 1482. https://doi.org/10.2136/sssaj2015.02.0084

Parr, M., Grossman, J. M., Reberg-Horton, S. C., Brinton, C., & Crozier, C. (2011). Nitrogen delivery from legume cover crops in no-till organic corn production. *Agronomy Journal*, *103*(6), 1578–1590. https://doi.org/10.2134/agronj2011.0007

Peoples, M. B., Hauggaard-Nielsen, H., & Jensen, E. S. (2009). *The Potential Environmental Benefits and Risks Derived from Legumes in Rotations*. https://doi.org/10.2134/agronmonogr52.c13

Queen, A., Earl, H., & Deen, W. (2009). Light and moisture competition effects on biomass of red clover underseeded to winter wheat. *Agronomy Journal*, *101*(6), 1511–1521. https://doi.org/10.2134/agronj2008.0163

R Core Team. (2020). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing.

Raimbault, B. A., & Vyn, T. J. (1991). Crop Rotation and Tillage Effects on Corn Growth and Soil Structural Stability. *Agronomy Journal*, *83*(6), 979. https://doi.org/10.2134/agronj1991.00021962008300060011x

Randall, G. W., Delgado, J. A., & Schepers, J. S. (2008). Nitrogen Management to Protect Water Resources. In *Nitrogen in Agricultural Systems* (p. p.911-945). http://www.moen.gov.np/pdf_files/water_resources_strategy.pdf

Roesch-Mcnally, G. E., Basche, A. D., Arbuckle, J. G., Tyndall, J. C., Miguez, F. E., Bowman, T., & Clay, R. (2018). The trouble with cover crops: Farmers' experiences with overcoming barriers to adoption. *Renewable Agriculture and Food Systems*, *33*(4), 322–333. https://doi.org/10.1017/S1742170517000096

Ruark, M. D., Chawner, M. M., Ballweg, M. J., Proost, R. T., Arriaga, F. J., & Stute, J. K. (2018). Does cover crop radish supply nitrogen to corn? *Agronomy Journal*, *110*(4), 1513–1522. https://doi.org/10.2134/agronj2017.06.0352

Rubin, J. C., Struffert, A. M., Fernández, F. G., & Lamb, J. A. (2016). Maize yield and nitrogen use efficiency in upper midwest irrigated sandy soils. *Agronomy Journal*, *108*(4), 1681–1691. https://doi.org/10.2134/agronj2015.0552

Sarrantonio, M., & Gallandt, E. (2003). The Role of Cover Crops in North American Cropping Systems. *Journal of Crop Production*, *8*(1/2), 187. https://doi.org/10.1300/J144v08n01

Sawyer, J. E., Pedersen, P., Barker, D. W., Ruiz Diaz, D. A., & Albrecht, K. (2010). Intercropping corn and kura clover: Response to nitrogen fertilization. *Agronomy Journal*, *102*(2), 568–574. https://doi.org/10.2134/agronj2009.0392

Schmidt, J. P., DeJoia, A. J., Ferguson, R. B., Taylor, R. K., Young, R. K., & Havlin, J. L. (2002). Corn yield response to nitrogen at multiple in-field locations. *Agronomy Journal*, *94*(4), 798–806. https://doi.org/10.2134/agronj2002.7980

Singer, J. W. (2008). Corn belt assessment of cover crop management and preferences. *Agronomy Journal*, *100*(6), 1670–1672. https://doi.org/10.2134/agronj2008.0151

Smil, V. (2001). *Enriching the Earth*. MIT Press.

Snapp, S. S., Swinton, S. M., Labarta, R., Mutch, D., Black, J. R., Leep, R., & Nyiraneza, J. (2005). Evaluating Cover Crops for Benefits, Costs and Performance within Cropping System Niches. *Agronomy Journal*, *97*(i), 322–332. https://doi.org/10.2134/agronj2005.0322

Steinke, K., Rutan, J., & Thurgood, L. (2015). Corn response to nitrogen at multiple sulfur rates. *Agronomy Journal*, *107*(4), 1347–1354. https://doi.org/10.2134/agronj14.0424

Stute, J. K., & Posner, J. L. (1995a). Legume Cover Crops as a Nitrogen Source for Corn in an Oat-Corn Rotation. *Journal of Production Agriculture*, *8*(3), 311–390. https://doi.org/10.2134/jpa1995.0385

Stute, J. K., & Posner, J. L. (1995b). Synchrony between legume nitrogen release and corn demand in the Upper Midwest. *Agronomy Journal*, *87*(6), 1063–1069. https://doi.org/10.2134/agronj1995.00021962008700060006x

Stute, J., & Shelley, K. (2009). Frost Seeding Red Clover in Winter Wheat. In *University of Wisconsin Nutrient and Pest Management Program publication 1-0209-3C*. http://ipcm.wisc.edu/download/pubsNM/RedClover_0109.pdf

Taylor, N. L. (1985). *Clovers Around the World*.

Thapa, R., Mirsky, S. B., & Tully, K. L. (2018). Cover Crops Reduce Nitrate Leaching in Agroecosystems:A Global Meta-Analysis. *Journal of Environment Quality*, *47*(6), 1400. https://doi.org/10.2134/jeq2018.03.0107

Unkovich, M., Herridge, D., Peoples, M., Cadisch, G., Boddey, B., Giller, K., Alves, B., & Chalk, P. (2008). *Measuring plant-associated nitrogen fixation in agricultural systems*. www.aciar.gov.au

USDA NRCS. (2019). *Plant Guide*. The PLANTS Database National Plant Data Team, Greensboro, NC 27401-4901 USA. https://doi.org/10.1039/c3cc48937j

Vyn, T. J., Faber, J. G., Janovicek, K. J., & Beauchamp, E. G. (2000). Cover Crop Effects on Nitrogen Availability to Corn following Wheat. *Agronomy Journal*, *92*, 915–924.

Vyn, T. J., Janovicek, K. J., & Miller, M. H. (1999). Soil Nitrate Accumulation and Corn Response to Preceding Small-Grain Fertilization and Cover Crops. *Agronomy Journal*, *91*, 17–24.

Wang, L., Feng, Z., & Schjoerring, J. K. (2013). Effects of elevated atmospheric CO2 on physiology and yield of wheat (Triticum aestivum L.): A meta-analytic test of current hypotheses. *Agriculture, Ecosystems and Environment*, *178*(2013), 57–63. https://doi.org/10.1016/j.agee.2013.06.013

White, C. M., DuPont, S. T., Hautau, M., Hartman, D., Finney, D. M., Bradley, B., LaChance, J. C., & Kaye, J. P. (2017). Managing the trade off between nitrogen supply and retention with cover crop mixtures. *Agriculture, Ecosystems and Environment*, *237*, 121–133. https://doi.org/10.1016/j.agee.2016.12.016

White, C. M., Finney, D. M., Kemanian, A. R., & Kaye, J. P. (2016). A model–data fusion approach for predicting cover crop nitrogen supply to corn. *Agronomy Journal*, *108*(6), 2527–2540. https://doi.org/10.2134/agronj2016.05.0288

Woli, K. P., Boyer, M. J., Elmore, R. W., Sawyer, J. E., Abendroth, L. J., & Barker, D. W. (2016). Corn era hybrid response to nitrogen fertilization. *Agronomy Journal*, *108*(2), 473–486. https://doi.org/10.2134/agronj2015.0314

Wortman, S. E. (2016). Weedy fallow as an alternative strategy for reducing nitrogen loss from annual cropping systems. *Agronomy for Sustainable Development*, *36*(4). https://doi.org/10.1007/s13593-016-0397-3

Wortman, S. E., Holmes, A. A., Miernicki, E., Knoche, K., & Pittelkow, C. M. (2017). First-season crop yield response to organic soil amendments: A meta-analysis. *Agronomy Journal*, *109*(4), 1210–1217. https://doi.org/10.2134/agronj2016.10.0627

Wyngaarden, S. L., Gaudin, A. C. M., Deen, W., & Martin, R. C. (2015). Expanding red clover (trifolium pratense) usage in the corn-soy-wheat rotation. *Sustainability (Switzerland)*, *7*(11), 15487–15509. https://doi.org/10.3390/su71115487

Yang, M., Mouazen, A., Zhao, X., & Guo, X. (2020). Assessment of a soil fertility index using visible and near-infrared spectroscopy in the rice paddy region of southern China. *European Journal of Soil Science*, *71*(4), 615–626. https://doi.org/10.1111/ejss.12907

Youngerman, C. Z., Ditommaso, A., Curran, W. S., Mirsky, S. B., & Ryan, M. R. (2018). Corn density effect on interseeded cover crops, weeds, and grain yield. *Agronomy Journal*, *110*(6), 2478–2487. https://doi.org/10.2134/agronj2018.01.0010

Zeng, W., Xu, C., Wu, J., & Huang, J. (2016). Sunflower seed yield estimation under the interaction of soil salinity and nitrogen application. *Field Crops Research*, *198*, 1–15. https://doi.org/10.1016/j.fcr.2016.08.007

Zhang, Y., Xie, D., Ni, J., & Zeng, X. (2020). Conservation tillage practices reduce nitrogen losses in the sloping upland of the Three Gorges Reservoir area: No-till is better than mulch-till. *Agriculture, Ecosystems and Environment*, *300*(May 2019), 107003. https://doi.org/10.1016/j.agee.2020.107003

Zheng, B. S., Le Gouis, J., Daniel, D., & Brancourt-Hulmel, M. (2009). Optimal numbers of environments to assess slopes of joint regression for grain yield, grain protein yield and grain protein concentration under nitrogen constraint in winter wheat. *Field Crops Research*, *113*(3), 187–196. https://doi.org/10.1016/j.fcr.2009.05.001

Conclusions and Future Work

The results of these studies do not show large nitrogen fertilizer replacement values that would support wide adoption of interseeding red clover in continuous corn, no-till systems in Wisconsin. However, the field studies were responsive, and the fertilizer response curves generated from the studies suggest the limitations to the system lie in clover biomass establishment. We suggest future studies investigate corn row spacing and corn planting density, both of which could allow for more light penetration beneath the corn canopy, benefitting the clover. A later corn relative maturation date could give clover an advantage in establishment before the canopy closes in. An exploration of this system at latitudes further South could make more growing degree days available to the clover. Corn stover management under no-till could be explored in corn silage systems that remove the majority of the corn plant, rather than the corn grain system explored in this study that left behind crop residue.

Another output of this work was the FertBoot R package that I built with a statistical consultant, Ting Fung Ma. This package is intended to be used, critically assessed, and adapted by researchers evaluating differences between optimum nitrogen fertilizer rates. We intended this to be a user-friendly code with limited modifications needed by researchers. We hope this encourages future decision making and prompts discussion about agronomic meaning in statistical analyses.

Appendix I. Red clover biomass photos

Figure 16. May 2018 biomass samples. Plot on the left received 135 kg N ha^{-1}. The clover on the right received 224 kg N ha^{-1}. Corn stover covers the soil between red clover emergence.

Figure 17. May 2019 biomass establishment. The healthy green stand is in-between plots in the alleys. Note the lack of clover between the harvested corn rows.

Figure 18. Clover biomass in October 2019. Early frost and a lot of fall precipitation did not create ideal growing conditions.

Figure 19. May 2020 biomass establishment. Lots of corn stover remained from last harvest, with uneven clover stands

Appendix II. A brief guide to FertBoot

1. Download the open-source program RStudio at rstudio.com
2. Install the package FertBoot to the RStudio library. Load FertBoot

```
library(FertBoot)
```

3. Set a working directory (where the data is saved)

```
setwd("~/folder_with_data")
```

4. Read data file
    a. Format data with treatment, fertilizer rate, and yield in separate columns
    b. Create data frames of fertilizer rate and yield for each treatment

```
df_1 = subset(data.frame, treatment = 1)

df_1 = df_1(x=df_1$nrate,y=df_1$yield)
```

5. Start the bootstrapping procedure by creating the model that will later be used to bootstrap the residuals. Fit each treatment data frame to a model (quadratic plateau, linear plateau, or quadratic) using multiple initial values

```
m.df_1 = f.quad.plateau(df_1,
start=list(a = 1, b = 1, c = 1),  plus_minus=1e2, n.start=10000,
msg=FALSE)
```

6. Delineate the range of fertilizer application rates with (here, maximum nitrogen fertilizer rates applied were 314 kg ha^{-1})

```
x.range = df_1(x=1:314)
```

7. Bootstrap the residuals of the quadratic plateau. This can take several hours.

```
results.df_1 =  boot.resid.quad.plateau(m.df_1, df_1, x.range=x.range,
B=1e3-1, plus_minus = 1e2, n.start=10000, print.progress=TRUE)
```

8. Save results to the working directory

```
saveRDS(results.df_1, file = "results.df_1.RDS")
```

9. Trim any outliers if bootstrapped x-values exceed maximum fertilizer rate applied

```
results.df_1 = results.df_1[results.df_1$max_x <= 314,]
```

10. Compare optimum fertilizer rates (bootstrapped estimate for optimum fertilizer rate and 95% confidence interval) for reaching yield plateau, and yield plateaus

```
c(mean(results.df_1$max_x), FertBoot::boot.CI(results.df_1$max_x,
alpha=0.05)$CI.percent)
```

    a. change max_x to max_y to compare maximum yield

11. Test for statistical significance between optimum fertilizer rates of different treatments

```
compare.two.sample(results.df_1$max_y, result. results.df_2$max_y,
R=1e4)$p.value
```

    a. change max_x to max_y to compare maximum yield

12. Use the log-likelihood ratio test to determine general differences between treatments.
    a. First, fit the models to quadratic plateau curves

```
df_1_log = f.quad.plateau(d=df_1)
df_2_log = f.quad.plateau(d=df_2)
```

The null model is the two data sets are the same

```
null_log = f.quad.plateau(d=rbind(df_1, df_2))
```

    b. Calculate the test statistic

```
ts =  as.numeric(logLik df_1_log $nls.model) + logLik(df_2_log $nls.model) -
logLik(null_log$nls.model))
```

1 - pchisq(ts, df = 6) # degrees of freedom (df) = 3 for comparing 2 groups as there are 3 more parameters in the alternate model (3*2 - 3)

13. Reference the FertBoot code in the CRAN repository at

    https://github.com/cran/FertBoot

    a. The authors (Francis and Ma) provided example code for figures here:

    https://github.com/rtfma/FertBoot/blob/main/ExampleGraphs_Interseed2018.pdf