

# Feedbacks between nitrogen fixation and soil organic matter increase ecosystem functions in diversified agroecosystems

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*Abstract.* Nitrogen (N) losses from intensified agriculture are a major cause of global change, due to nitrate ( $\text{NO}_3^-$ ) export and the eutrophication of aquatic systems as well as emissions of nitrous oxide ( $\text{N}_2\text{O}$ ) into the atmosphere. Diversified agroecosystems with legume cover crops couple N and carbon (C) inputs to soil and reduce N pollution, but there is a need to identify controls on legume  $\text{N}_2$  fixation across ecosystems with variable soil conditions. Here, I tested the hypothesis that N mineralization from turnover of soil organic matter (SOM) regulates legume  $\text{N}_2$  fixation across 10 farms that spanned a gradient of SOM levels. I separated soil samples into two SOM fractions, based on size and density, which are indicators of soil nutrient cycling and N availability (free particulate organic matter and intra-aggregate particulate organic matter [POM]). This study indicates downregulation of legume  $\text{N}_2$  fixation in diversified agroecosystems with increasing N availability in intra-aggregate POM and increasing N mineralization. Intercropping the legume with a grass weakened the relationship between N in POM and  $\text{N}_2$  fixation due to N assimilation by the grass. Further, mean rates of N and C mineralization across sites increased with two seasons of a legume-grass cover crop mixture, which could enhance this stabilizing feedback between soil N availability and  $\text{N}_2$  fixation over time. These results suggest a potential mechanism for the diversity–ecosystem-function relationships measured in long-term studies of agroecosystems, in which regular use of legume cover crops increases total soil organic C and N and reduces negative environmental impacts of crop production.

*Key words:* agroecosystem; biological nitrogen fixation; cover crop; legume; mineralization; particulate organic matter; soil carbon; soil organic matter.

## INTRODUCTION

Nitrogen (N) fertilizer use in agricultural landscapes is a major driver of global change. Increases in reactive N production, dominated by agricultural intensification (Galloway et al. 2008), and N applications that exceed plant need, contribute to eutrophication of surface waters and to greenhouse gas emissions (Diaz and Rosenberg 2008, IPCC 2013). Despite decades of research and technological development to improve N fertilizer use efficiency, N pollution is a persistent environmental problem (Diaz and Rosenberg 2008). Applying ecological principles to manage plant diversity in agroecosystems for functions such as N retention may offer greater potential to mitigate pollution. For example, agroecosystems with legume  $\text{N}_2$  fixation as a primary N source can reduce N losses (Drinkwater et al. 1998, Syswerda et al. 2012) and sequester carbon (C) in

soil (McDaniel et al. 2014, King and Blesh 2018). Given the potential for legume N sources to alleviate environmental impacts, there is a critical need to understand patterns of, and ecological controls on, legume  $\text{N}_2$  fixation across variable agroecosystem conditions.

An ecological perspective offers insights into the mechanisms driving N losses from intensified agriculture. Inorganic N fertilizers are vulnerable to leaching and gaseous losses, even using best management practices, because they are applied in soluble forms that can be rapidly lost if they exceed crop demand. Intensively managed agroecosystems typically have low stocks of soil organic matter (SOM), which exacerbates N losses because microbial processes that can cycle and store N in soil are C limited (Drinkwater and Snapp 2007). In contrast, use of organic N sources, particularly legume cover crops (i.e., non-harvested crops), offers greater promise for reducing N losses (Syswerda et al. 2012, Blesh and Drinkwater 2013), because opportunities for microbial N assimilation are increased by providing a high quality C input to soil (Fisk et al. 2015). Legume cover crops also improve N retention by increasing the temporal diversity of crop rotations and taking up N

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during periods when soil would otherwise be bare. For example, in a meta-analysis, Tonitto et al. (2006) showed that legume cover crops reduced  $\text{NO}_3^-$  leaching by 40%, compared to the absence of a cover crop. A long-term (11 yr) experiment in Michigan, USA revealed that  $\text{NO}_3^-$  leaching was reduced by more than 60% in two management systems with greater biodiversity and legume N sources (Syswerda et al. 2012). In addition, use of legume cover crops as N sources can reduce  $\text{N}_2\text{O}$  emissions compared to bare fallows (Han et al. 2017). Legume cover crops also increase the spatial diversity of agroecosystems when planted in multispecies mixtures. When legumes are combined with grass species, which are even stronger N scavengers, they can further reduce  $\text{NO}_3^-$  leaching (Kaye et al. 2019) and  $\text{N}_2\text{O}$  emissions (Basche et al. 2014). Overall, cover crops are considered central to an ecological approach to agroecosystem management.

Legume cover crops can sequester atmospheric  $\text{CO}_2$  by increasing C inputs to agroecosystems and building stocks of SOM (Poeplau and Don 2015, King and Blesh 2018). In some long-term experiments, management systems with legume cover crops do not have greater net C inputs to soil compared to lower diversity rotations such as continuous grains, yet the diversified systems still accumulate more SOM over time (Drinkwater et al. 1998, Grandy and Robertson 2007). Emerging understandings of soil C stabilization suggest a potential mechanism for these findings (Cotrufo et al. 2013). Legume residues have low C:N ratios and are easily decomposed by microbial communities. As a result, the fraction of legume cover crop residue that becomes SOM may be higher if it increases the relative production of microbial byproducts and necromass, key components of stable SOM pools, for a given C input to soil (Schmidt et al. 2011, Cotrufo et al. 2013). Cover crops also increase the proportion of the year with plant-root-microbe interactions in the rhizosphere, and associated release of root exudates, extracellular enzyme production, and presence of aggregate binding agents, which have previously been linked to accumulation of SOM (Tiemann et al. 2015, Lehmann et al. 2017) and to soil nutrient cycling capacity (Clarholm 1985, Drinkwater and Snapp 2007, Kuzyakov and Blagodatskaya 2015). In particular, legume cover crops can increase fractions of SOM that turnover more quickly than the total SOM pool, especially particulate organic matter (POM). POM pools respond to changes in management on year to decadal scales, correlate with soil N mineralization rates, and are early indicators of longer-term accumulation of SOM (Cambardella and Elliott 1992, Wander 2004, Luce et al. 2016). Legume cover crops can therefore enhance multiple ecosystem functions.

Despite these benefits, farmers continue to rely on inorganic fertilizers partly because they have more predictable outcomes for crop production. Nitrogen inputs from legume cover crops depend on ecological processes and are inherently more variable. There is a need, then, to

increase predictive understanding of legume  $\text{N}_2$  fixation inputs from cover crops. In natural ecosystems, key ecological controls on  $\text{N}_2$  fixation include high levels of soil inorganic N, low availability of non-N nutrients that may limit legume growth or fixation (e.g., P, Fe, K, Mo), preferential grazing of legumes by herbivores, and shade intolerance of legumes compared to other plant functional groups (Vitousek et al. 2013). Biotic interactions can also influence these mechanisms. For example, the percentage of legume N from the atmosphere is mediated by interactions with grasses. Grasses are strong competitors for soil N, reducing soil inorganic N pools and causing the legume to fix more of its N (Li et al. 2009). However, grasses may also compete with legumes for soil nutrients such as P, or resources like light, reducing legume biomass production (Cech et al. 2010, Poffenbarger et al. 2015). In agroecosystems, which typically have lower diversity than natural ecosystems, many of these constraints are alleviated through management. However, nutrients such as P or Fe can limit legume biomass production, and it is well known that inorganic N fertilizers inhibit legume  $\text{N}_2$  fixation because of the greater C cost for acquiring N from symbiotic  $\text{N}_2$  fixation compared to assimilating soil inorganic N (Schipanski et al. 2010, Gelfand and Robertson 2015).

In diversified agroecosystems, SOM pools including POM are central to soil nutrient cycling dynamics, yet most research on  $\text{N}_2$  fixation in agriculture has focused on inorganic N supplied by synthetic fertilizer, while the role of N mineralized from decomposition of SOM remains poorly understood (Schipanski and Drinkwater 2011). Diversified agroecosystems that rely on legumes and other organic N sources have different N cycling dynamics from fields with synthetic fertilizers, which experience episodic inputs of inorganic N that saturate the root zone. Organic and diversified farms have larger N inputs than natural ecosystems, to balance the N removed in harvested crops, but, compared to intensified agroecosystems, inorganic N is released more gradually in the rhizosphere through microbial turnover of SOM. Further, N cycling rates differ among farms with SOM of differential quantity and quality. For instance, agroecosystems with a long organic management history may have low standing pools of inorganic N because of tight coupling between plant and microbial productivity (Paterson 2003), but high mineralization rates due to accumulation of SOM and enhanced nutrient cycling capacity (Berthrong et al. 2013). Given the potential for legumes to build POM pools, and the critical role that SOM pools such as POM play in nutrient cycling, relationships between POM, N mineralization, and legume  $\text{N}_2$  fixation are important to understand.

The goal of this study was to identify ecosystem-scale controls on legume  $\text{N}_2$  fixation in the context of diversified agroecosystems. Determining patterns in  $\text{N}_2$  fixation across variable soil conditions could inform management of legume N sources, and increase understanding of feedback mechanisms that underpin the efficiency of

legumes demonstrated by long-term studies. In a field experiment on 10 farms spanning a gradient of soil fertility levels due to distinct management legacies, I separated soil samples into two SOM fractions, based on size (>53  $\mu\text{m}$ ) and density, which are indicators of internal nutrient cycling and N availability (light or “free” POM; and POM that is physically protected inside microaggregates; Marriott and Wander 2006b). Here, I used these two POM fractions to assess relationships between N availability from turnover of SOM and legume N<sub>2</sub> fixation (measured using stable isotope methods) across a gradient of SOM levels, holding management constant. I hypothesized that legume N<sub>2</sub> fixation would decrease with increasing levels of N in POM, due to enhanced microbial processing of SOM and increased N mineralization with larger POM pools (Fig. 1). Furthermore, I hypothesized that intra-aggregate POM should account for more variation in this relationship relative to light fraction POM because it turns over more slowly and is considered a better indicator of longer-term changes in soil N cycling capacity due to management (Wander et al. 1994, Schipanski et al. 2010). Finally, I expected interspecific competition in a legume–grass mixture to reduce the effect of POM N on N<sub>2</sub> fixation, due to soil N assimilation by the fibrous root system of the intercropped grass (Fig. 1).

## MATERIALS AND METHODS

### *Experimental design*

I partnered with 10 vegetable farmers in southeastern Michigan to establish an on-farm experiment identifying patterns in N<sub>2</sub> fixation by a legume cover crop, hairy vetch (*Vicia villosa* L.), grown alone and in mixture with a grass cover crop, cereal rye (*Secale cereale* L.), across a soil gradient. The farms had been in organic vegetable production from 3 to 14 yr at the start of the experiment, and analysis of baseline soil properties from each experimental field reflected a soil fertility gradient, which resulted primarily from distinct management histories (Tables 1 and 2). Nine of the 10 fields were Alfisols, and one was a Mollisol. Six of the 10 fields had previous compost inputs from poultry litter or other sources of composted manure. Across farms, total organic C varied from 33.1 to 64.1 Mg/ha, plant-available phosphorus (P) was eight times greater on the highest P site (112 mg/kg) than on the lowest (14.3 mg/kg). The size and N concentration of POM pools also ranged widely, particularly for the protected POM pool, indicating that there were differences in the quality of SOM on the farms. Soil inorganic N pools (NO<sub>3</sub><sup>-</sup>-N + NH<sub>4</sub><sup>+</sup>-N) ranged from 2.6 to 14.4 mg/kg.

Management details for the experiment, planted in one field per farm, were determined collaboratively with farmers. In summer 2015, after analysis of baseline soil samples, farmers planted the field to a crop in the *Amaryllidaceae* family, either onion or garlic. The *Allium*

crop was fertilized with compost inputs on four of the farms, and the remaining farms applied no fertility inputs for this crop. No additional fertility amendments were added to fields for the remainder of the experiment, except for the N inputs from legume N<sub>2</sub> fixation. Following crop harvest, fields were divided into four replicate blocks to establish the experimental treatments for the cover crop along with a no-cover-crop control. The fields ranged in size from 0.05 to 1.4 ha, but the measurements were constrained to a 130 m<sup>2</sup> area to minimize variability in field area across farms. Farmers planted the fields in a mixture of cereal rye (seeding rate of 56 kg/ha) and hairy vetch (seeding rate of 25 kg/ha) between 21 August and 28 August 2015. Vetch seeds were inoculated with N-Dure (Verdesian Life Sciences, Cary, North Carolina, USA) at approximately 4 g/kg seed. Seeds were surface broadcast and lightly incorporated. The cover crop overwintered, and by mid-May 2016, farmers mowed and then rototilled the field to incorporate the cover crop, and planted a primary crop in the *Cucurbitaceae* family between 27 May and 4 June 2016. Following crop harvest, the cover crop mixture was planted for a second season between 30 August and 14 September 2016 using the same method as in the first year. The cover crop was terminated by mowing and rototillage in May 2017 following final sampling.

### *Baseline soil sampling*

A composite, baseline, soil sample from each experimental field was analyzed to determine initial soil conditions and characterize soil fertility status in spring of 2014 or fall 2015 on all participating farms (Tables 1 and 2). Approximately 15–20 soil cores (2 cm diameter by 20 cm depth) were composited per site. I estimated bulk density from the fresh mass of eight cores per field, which was later adjusted for soil moisture. Soil was processed immediately for soil moisture, determined gravimetrically by drying at 105°C for 48 h, and triplicate soil subsamples were sieved to 2 mm for analysis of extractable inorganic N (NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>) with 2 mol/L KCl. The amount of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> in each sample was analyzed colorimetrically on a discrete analyzer (AQ2, Seal Analytical, Mequon, Wisconsin, USA). Triplicate soil subsamples were also sieved to 2 mm for a 7-d anaerobic N mineralization incubation, an indicator of potential N availability from decomposition of organic matter, followed by extraction with 2 mol/L KCl and colorimetric analysis of NH<sub>4</sub><sup>+</sup>. Potentially mineralizable N (N-min) was calculated by subtracting the initial amount of NH<sub>4</sub><sup>+</sup> in the soil from the NH<sub>4</sub><sup>+</sup> released during the 7-d incubation (Drinkwater et al. 1996).

The POM > 53  $\mu\text{m}$  was isolated from triplicate 40-g subsamples of unsieved, air-dried soil using a combined size and density fractionation method. To isolate the light fraction POM (also called free POM), the subsamples were first gently shaken for 1 h in sodium polytungstate (1.7 g/cm<sup>3</sup>), allowed to settle for 16 h, and free

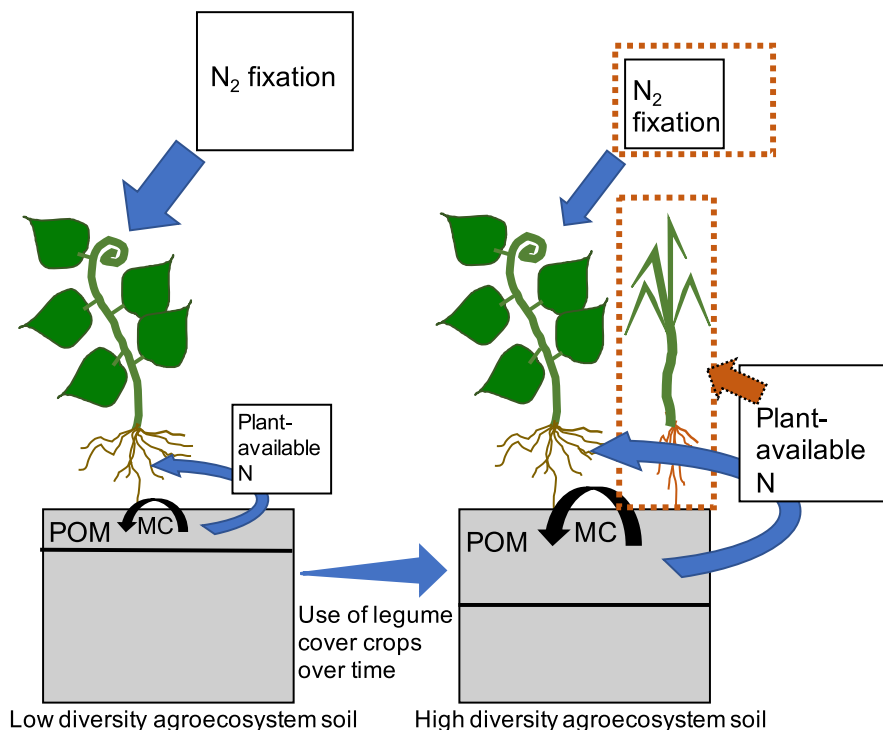


FIG. 1. Conceptual diagram of the hypothesized stabilizing feedback between particulate organic matter (POM) pools and legume N<sub>2</sub> fixation. In soils with a legacy of low plant diversity, POM pools are small and the supply of plant-available N from microbial turnover of soil organic matter (SOM) is small, which leads to high rates of legume N<sub>2</sub> fixation. Regular use of legume cover crops in diversified agroecosystems over time is expected to increase the size of POM pools, which are an energy source for microorganisms. The size of the microbial community (MC) and rates of microbial turnover of POM are therefore also expected to increase. Greater mineralization of N from POM increases the plant-available N pool, and ultimately decreases legume reliance on N<sub>2</sub> fixation, because of the C-cost of acquiring N through the symbiotic relationship with rhizobia. The overall effect is greater N availability for crop assimilation from internal nutrient cycling processes. The right side of the figure also shows the hypothesized moderating effect of intercropping the legume with a grass cover crop (dashed lines). The grass assimilates soil N, drawing down the plant-available N pool and maintaining higher rates of N<sub>2</sub> fixation (i.e., the brown dashed line around the N<sub>2</sub> fixation box).

TABLE 1. Baseline soil properties of each field, prior to planting the experiment on the 10 farms in the study, measured from composite soil cores sampled to 20-cm depth.

Farm	TOC (Mg/ha)	TN (Mg/ha)	N-min (mg·kg <sup>-1</sup> ·week <sup>-1</sup> )	C-min (μg CO <sub>2</sub> -C·g <sup>-1</sup> ·d <sup>-1</sup> )	Free POM (g/kg)	C:N free POM	Protected POM (g/kg)	C:N protected POM	Protected POM N (mg/kg)
1	38.8	4.8	9.9	56.5	3.0	20.4	4.4	12.8	72.5
2	52.8	5.0	11.7	111.6	2.6	24.5	4.3	14.5	55.6
3	64.1	6.2	23.5	84.5	13.9	16.6	11.4	14.1	139.7
4	56.8	5.0	10.1	59.0	5.2	37.3	9.1	13.5	61.6
5	47.0	4.9	14.3	65.5	13.8	16.6	3.7	13.8	31.5
6	47.3	5.1	10.8	45.5	4.3	25.1	3.1	18.9	45.0
7	58.9	5.8	16.9	70.1	14.4	18.6	3.7	18.6	28.7
8	39.8	4.1	14.4	54.8	9.0	19.0	2.2	15.5	33.5
9	50.3	5.1	14.9	53.8	11.7	25.5	3.7	19.7	28.4
10	33.1	3.7	7.0	31.5	5.6	23.2	2.1	17.8	117.0

Notes: C-min, short-term C mineralization; C:N, C-to-N ratio; N-min, potentially mineralizable N; POM, particulate organic matter; TOC, total organic carbon; TN, total soil nitrogen.

POM floating on top of the solution was removed by aspiration. To isolate intra-aggregate POM (i.e., physically protected POM), the remaining soil sample was shaken with 10% sodium hexametaphosphate to disperse

soil aggregates and then rinsed through a 53-μm filter (Marriott and Wander 2006b). The material larger than 53 μm was retained, and the protected POM was separated from sand by decanting. The C and N of both

TABLE 2. Additional baseline soil properties of each field, prior to planting the experiment on the 10 farms in the study, measured from composite soil cores sampled to 20 cm depth.

Farm	NO <sub>3</sub> -N + NH <sub>4</sub> -N (mg/kg)	P (mg/kg)	K (mg/kg)	pH	Sand (%)	Silt (%)	Clay (%)	Soil order
1	3.0	45.0	100.8	7.0	74.0	15.0	11.0	Alfisol
2	2.6	18.3	78.5	6.3	48.0	28.0	24.0	Mollisol
3	13.9	43.0	162.0	7.5	60.5	23.5	16.0	Alfisol
4	5.2	18.0	31.8	7.2	61.5	22.0	16.5	Alfisol
5	13.8	84.0	82.0	7.1	60.0	26.0	14.0	Alfisol
6	4.3	14.3	94.5	7.1	36.5	34.5	29.0	Alfisol
7	14.4	65.0	173.3	7.4	49.5	27.5	23.0	Alfisol
8	9.0	25.0	95.8	6.4	65.5	22.0	12.5	Alfisol
9	11.7	58.0	110.3	6.4	38.0	30.5	31.5	Alfisol
10	5.6	111.5	130.3	7.0	74.0	15.0	11.0	Alfisol

POM fractions were measured on an ECS 4010 CHNSO Analyzer (Costech Analytical Technologies, Valencia, California, USA). Total soil C and N (to 20 cm) were measured by dry combustion on a Leco TruMac CN Analyzer (Leco Corporation, St. Joseph, Michigan, USA). Subsamples of ~100 g of sieved dried soil were analyzed for particle size, pH, Bray-1 P, and K at the A & L Great Lakes Laboratories (Fort Wayne, Indiana, USA). As an indicator of microbial activity and SOM quality, C mineralization (C-min) was determined by a short-term aerobic incubation of rewetted soil (i.e., the flush of CO<sub>2</sub> during a 1-d incubation), using a method modified from Franzluebbers et al. (2000; Appendix S1).

#### Legume N<sub>2</sub> fixation

During the cover crop seasons, I established three small plots of 1 m<sup>2</sup> within each replicate block in each field (i.e., 12 small plots per farm) to estimate biological N<sub>2</sub> fixation by the vetch grown in mixture and monoculture based on <sup>15</sup>N natural abundance (Shearer and Kohl 1986). Soon after establishment of the cover crop mixture (e.g., in late September), all vetch plants were hand weeded out of one set of small plots to establish a sole rye treatment, and all rye plants were hand weeded out of another set of plots for sole vetch stands. A third small plot was left in the mixture, and the background field was also planted to the vetch-rye mixture, except for the no-cover-crop control.

Aboveground biomass in vetch and vetch-rye mixture treatments was sampled from the small plots between 25 April and 6 May 2016, and between 9 and 18 May 2017, from a 0.25-m<sup>2</sup> section of each small plot, avoiding plot edges. Plant biomass was cut at the soil surface, separated by species, and dried at 60°C for 48 h. Dried shoot biomass was first ground in a Wiley Mill to 2 mm, and was then pulverized using a cyclone mill and analyzed on a continuous flow Isotope Ratio Mass Spectrometer (Stable Isotope Facility, UC Davis) for total C and

N content, and for δ<sup>15</sup>N. The %N derived from fixation was calculated using the following model:

$$\begin{aligned} \%N \text{ from fixation} \\ = 100 \times ((\delta^{15}N_{\text{ref}} - \delta^{15}N_{\text{legume}})/(\delta^{15}N_{\text{ref}} - B)) \end{aligned}$$

where δ<sup>15</sup>N<sub>ref</sub> is the δ<sup>15</sup>N signature of the reference plant (cereal rye), δ<sup>15</sup>N<sub>legume</sub> is the δ<sup>15</sup>N signature of the legume, and *B* is defined as the δ<sup>15</sup>N signature of a legume when dependent solely on atmospheric N<sub>2</sub>. *B* values were determined by growing vetch in the greenhouse in a N-free medium (Appendix S1). The *B* value for vetch was -0.44. Total N input from vetch N<sub>2</sub> fixation (kg N/ha) was calculated for mixture and monoculture treatments using aboveground biomass, percent N, and percent N from fixation. The <sup>15</sup>N natural abundance method requires sufficient enrichment of the plant-available soil N pool to accurately estimate the percentage of legume N derived from fixation (i.e., from the atmosphere). There was significant separation in δ<sup>15</sup>N values between the legume (vetch) and the reference plant (sole cereal rye) in mixture and monoculture treatments (Appendix S1; Fig. S1). For example, in 2016 the separation in mixture ranged from about 2–6‰ across sites.

I estimated the quantity of fixed N transferred from hairy vetch to cereal rye in the vetch-rye mixture by comparing the δ<sup>15</sup>N of rye in mixture with the δ<sup>15</sup>N of rye in monoculture plots:

$$\begin{aligned} \% \text{ rye N from transfer} = \\ 100 \times ((\delta^{15}N_{\text{rye monoculture}} - \delta^{15}N_{\text{rye mix}})/ \\ (\delta^{15}N_{\text{rye monoculture}} - C)) \end{aligned}$$

where *C* is the δ<sup>15</sup>N value of the integrated fractionation of δ<sup>15</sup>N during N<sub>2</sub> fixation in legume roots and between the roots and sampled shoots of the rye. *C* is typically estimated as the lowest δ<sup>15</sup>N value of grass grown in mixture, or 0, whichever is lower. Here, *C* was set to 0 because all δ<sup>15</sup>N values were positive for cereal rye. Transfer of fixed N from vetch to rye can occur through multiple mechanisms: (1) turnover of vetch root tissue



and assimilation of mineralized N by the neighboring rye; (2) exudation of soluble N compounds by the vetch and uptake by the rye; and (3) transfer of N through mycorrhizal associations. The natural abundance method has limitations for quantifying N transfer between legumes and non-legumes because of the assumption that the transferred N has a similar  $\delta^{15}\text{N}$  value to that measured in the shoots of the legume, which may not be valid (Peoples et al. 2015). However, this approach provides a relative estimate of fixed N assimilated by rye in the mixture treatment across sites.

#### *Final soil analysis*

Between 9 May and 18 May 2017, immediately before terminating the cover crop and the experiment, a final composite soil sample (8–10 cores) was collected from each replicate block on each farm in the cover crop mixture and no cover crop control. The soil samples were analyzed for extractable inorganic N pools, N-min, and C-min, total C and N, Bray-1 P, and free and protected POM fractions as described for the baseline soil sampling. To determine changes in soil fertility and SOM pools with the cover crop mixture across farms, an effect size was calculated by subtracting the final value for each soil parameter measured in the mixture treatment from the final value measured in the no cover crop control at the May 2017 sampling.

#### *Statistical analysis*

Statistical analyses were computed in R (R Foundation for Statistical Computing, Vienna, Austria). To test for differences in vetch  $\text{N}_2$  fixation between mixture and monoculture, one-way ANOVA analyses were performed for each experimental year using the lme4 package (Bates et al. 2015) for linear, mixed-effect models with treatment as a fixed effect, and block nested in farm as a random effect. To test the hypothesis that increasing POM N concentrations would decrease vetch  $\text{N}_2$  fixation, I used linear regression to model vetch  $\text{N}_2$  fixation in mixture and monoculture in 2016 as a function of SOM pools and other properties expected to control legume  $\text{N}_2$  fixation (e.g., soil P, textural properties), excluding two extreme outliers for vetch  $\text{N}_2$  fixation. Model comparisons were made by assessing goodness of fit with the Akaike Information Criterion (AIC). After 2016, one of the participating farmers decided to leave farming, so there were 10 farms in year one (2016) and nine farms in year two (2017). Results are reported as statistically significant at  $\alpha = 0.05$ .

## RESULTS

### *Biological $\text{N}_2$ fixation in mixture and monoculture*

Using stable isotope methods, the mean percentage of vetch N from fixation across all farms was high in both cover crop seasons, ranging from 76% to 92%, and was

significantly higher in mixture than in monoculture (Fig. 2). In 2017, the mean percentage of vetch N from fixation was higher than in 2016, and in 2017 vetch produced approximately one-half as much biomass compared to the 2016 vetch cover crop. The difference in vetch biomass production between years was reflected in mean total aboveground N accumulation in the treatments (Fig. 2), which was 103 and 109 kg/ha in vetch mixture and monoculture in 2016, respectively, but 69 and 56 kg/ha in mixture and monoculture in 2017. In 2016, total mean N assimilation by rye in mixture was 51.5 kg N/ha (range of 16.6–111.7 kg N/ha across sites), with an estimated 4.6 kg/ha of that N derived from vetch  $\text{N}_2$  fixation, on average. In 2017, the rye grown in mixture assimilated 39.9 kg N/ha, on average, with an estimated 7.6 kg/ha of the N transferred from vetch. Overall, total N accumulation between the mixture and monoculture treatments was not significantly different in either year; however, total N supply from  $\text{N}_2$  fixation was significantly greater in the sole legume stand due to greater legume biomass than in the mixed stand.

Across farms, aboveground vetch  $\text{N}_2$  fixation in 2016 ranged from 1.4 to 177 kg N/ha in mixture and from 16 to 232 kg N/ha in monoculture, and was highly correlated with vetch aboveground biomass ( $R^2 = 0.94$  in mixture and 0.74 in monoculture). In 2017, vetch  $\text{N}_2$  fixation in mixture varied from 1.5 to 78 kg N/ha, and from 3 to 126 kg N/ha in monoculture, across individual farms. The relationships between legume shoot biomass and total fixed N in shoots were similarly strong in 2017 ( $R^2 = 0.92$  in mixture and 0.86 in monoculture).

### *Controls on $\text{N}_2$ fixation across the soil gradient*

Linear regression models using the baseline soil properties as predictors and vetch  $\text{N}_2$  fixation in 2016 as the dependent variable showed that two of the soil properties were significant predictors of the amount of shoot N fixed (in kg N/ha) by vetch (Appendix S1: Table S1). N concentration in the free POM pool was not a significant predictor of  $\text{N}_2$  fixation ( $P = 0.294$  for sole vetch;  $P = 0.421$  for vetch in mixture), however, I found a significant and negative correlation between the N concentration in the protected POM pool and vetch  $\text{N}_2$  fixation in mixture with a regression coefficient of  $-44.16$  ( $P = 0.002$ ), and also in monoculture ( $-41.39$ ,  $P = 0.026$ ). The amount of shoot N fixed by vetch was positively correlated with plant available P concentration across the farm sites ( $P = 0.006$  in mixture, and  $P < 0.001$  in monoculture). Together, these soil properties explained 42% (sole vetch) and 47% (vetch mixture) of the variation in vetch  $\text{N}_2$  fixation. For vetch in mixture, the best fit model had soil P, and the C:N ratio of the protected POM pool (9.32,  $P < 0.001$ ) rather than POM N concentration. This model explained 54% of the variation in vetch  $\text{N}_2$  fixation in mixture; however, the C:N of the protected POM was only a marginally

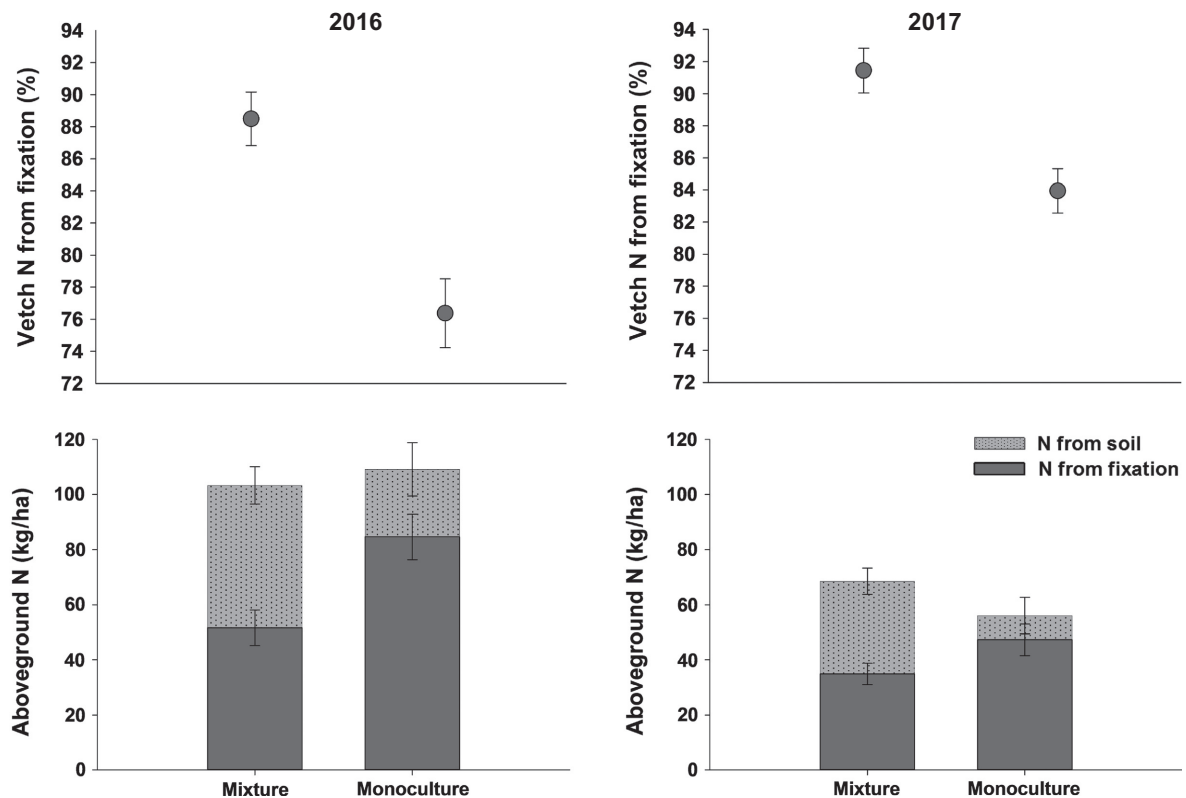


Fig. 2. Top panels show the percentage of vetch N derived from fixation. Bottom panels show total N assimilation in vetch–rye mixture biomass (mixture) and in vetch biomass (monoculture), separated into N derived from vetch N<sub>2</sub> fixation and N from the soil. Values are means  $\pm$  SE.

significant predictor of vetch N<sub>2</sub> fixation in monoculture ( $P = 0.05$ ).

There was also a significant, negative relationship between the N concentration of the protected POM pool and the percentage of vetch shoot N derived from fixation (Fig. 3). The slope of this relationship was steeper for sole vetch than for vetch grown in mixture (Fig. 3). For sole vetch, the percentage of N from fixation also exhibited a significant and negative relationship with N-min, although it was weaker than the relationship with POM (Appendix S1: Fig. S2;  $R^2 = 0.11$ ;  $P = 0.04$ ), and there was no relationship between N-min and percentage of N from fixation for vetch in mixture ( $P = 0.617$ ). In contrast to the positive relationships between soil P and the amount of fixed N in vetch shoots in mixture and monoculture (i.e., driven by biomass production), there was no relationship between soil P and the percentage of vetch aboveground N derived from fixation (Appendix S1: Fig. S3) for either mixture ( $P = 0.793$ ) or monoculture treatments ( $P = 0.612$ ).

#### *Changes in labile organic matter pools with a legume–grass mixture*

Finally, I asked how two seasons of the overwintering vetch–rye cover crop mixture influenced the indicators

of soil fertility measured at baseline (Fig. 4). There was a significant increase in mean C and N mineralization rates compared to the no cover crop control across farms. The mean size of both POM pools (free and protected POM) also increased, as did the N concentration of the protected POM pool (shown as a significant decrease in C:N ratio). At the final soil sampling across farm sites, there was a strong positive relationship between protected POM N and N-min (Fig. 5), where POM N accounted for one-half of the variation in potentially mineralizable N. There were also weaker, but significantly positive relationships between both POM pools (in g/kg dry soil) and N-min (free POM,  $R^2 = 0.33$ ;  $P < 0.0001$ ; and protected POM,  $R^2 = 0.16$ ,  $P = 0.007$ ). Mean soil NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> concentrations, and plant available P concentration, did not change with the cover crop.

#### DISCUSSION

Strategic management of plant diversity in agroecosystems for increased ecosystem functioning (Storkey et al. 2015, Finney and Kaye 2017, Blesh 2018) could help alleviate the widespread environmental costs of intensified agriculture. Use of legume cover crops can build soil organic C and N (Drinkwater

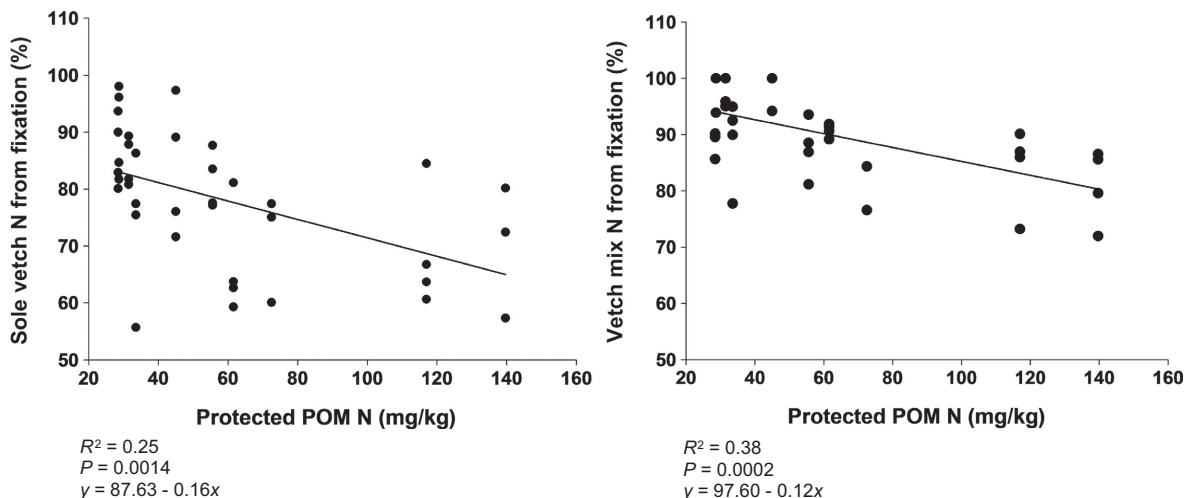


FIG. 3. Regression relationships for N concentration in the protected POM pool and the percentage of vetch shoot N derived from fixation, for sole vetch (left) and vetch grown in mixture with rye (right).

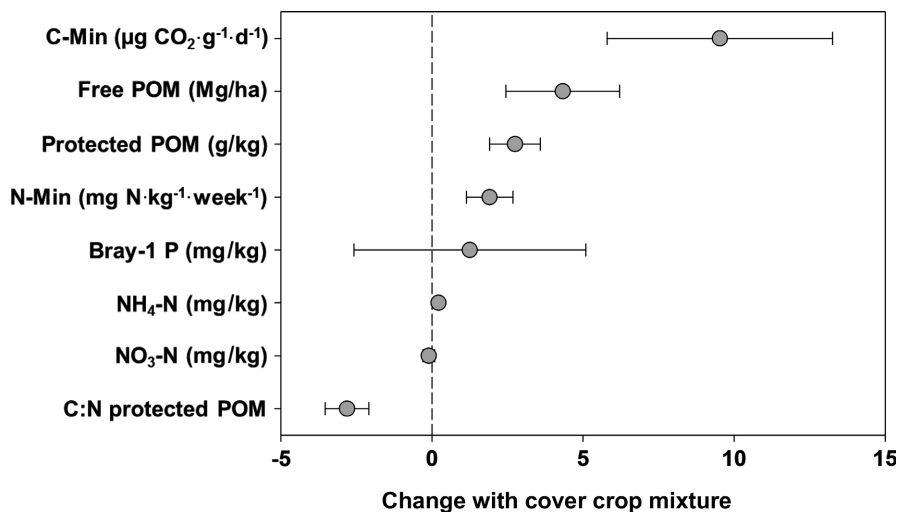


FIG. 4. Change across nine farms in properties that reflect soil nutrient cycling dynamics, calculated as the difference between the vetch-rye cover crop mixture treatment and the no cover crop control, shown in their measured units as means with 95% confidence intervals. Min, mineralization.

et al. 1998, King and Blesh 2018), thereby supplying N to crops while also sequestering atmospheric  $\text{CO}_2$ , and reducing  $\text{NO}_3^-$  leaching to aquatic systems and  $\text{N}_2\text{O}$  emissions. Given that legume  $\text{N}_2$  fixation varies with soil properties and interspecific interactions, predictive understanding of this variation is needed to inform managing legumes as a N source for crop production. Physiological controls on  $\text{N}_2$  fixation have been fairly well characterized, but ecological controls (i.e., controls influenced by interactions between legumes and other organisms, or between legumes and the environment) are less well understood (Vitousek et al. 2013), particularly in diversified agroecosystems. For example, numerous greenhouse and field experiments have shown that increasing inorganic N reduced

nodulation and legume  $\text{N}_2$  fixation (e.g., Van Kessel and Hartley 2000, Goss et al. 2002, Salvaggiotti et al. 2008). However, there remains a lack of studies across gradients of SOM levels (cf. Schipanski et al. 2010, Blesh 2018), a critical gap because diversified agroecosystems do not have pulse additions of inorganic N but instead have more gradual release of N through decomposition of SOM (Schipanski and Drinkwater 2011). This study shows a strong stabilizing feedback between N in intra-aggregate SOM (protected POM) and  $\text{N}_2$  fixation, due to increased N mineralization from enhanced microbial activity fostered by greater organic matter inputs to soil. These results increase understanding of ecosystem-scale rates and regulation of  $\text{N}_2$  fixation across working agroecosystems.



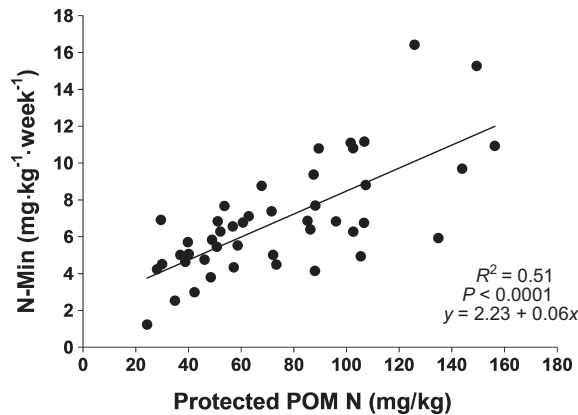


FIG. 5. Regression relationship between the N concentration in the protected POM pool and the rate of potentially mineralizable N across farm sites at the final sampling time.

As predicted, with increasing N concentrations in protected POM across the gradient, I found a significant and negative relationship with both the percentage of vetch N from fixation and total fixed N in shoots. There was also a significantly positive relationship between POM N concentration and N-min and a stabilizing feedback between N-min and % N from fixation for vetch in monoculture, supporting the hypothesis that POM increases N mineralization from microbial processing of SOM. These results highlight a potential mechanism by which legume N sources in diversified agroecosystems better reduce surplus N and N losses (Drinkwater et al. 1998, Blesh and Drinkwater 2013), by responding to changes in SOM following transitions from conventional to diversified management (Fig. 1). Protected POM N moderated vetch N<sub>2</sub> fixation in sole and mixed stands, while light fraction POM was not a significant predictor of BNF. This was somewhat surprising because both POM pools are considered indicators of soil fertility. A related experiment on a subset of the same farms showed that the free POM pool was negatively related to legume biomass within different cover crop mixtures, in addition to finding a negative relationship between protected POM N and both legume biomass and shoot N fixed (Blesh 2018). However, results from this study supported the hypothesis that the feedback with protected POM N would be stronger. Free POM turns over relatively quickly and reflects recent C inputs to soil (Wander 2004). Consequently, intra-aggregate POM may be a more robust indicator of soil nutrient cycling capacity because it turns over more slowly and can better reflect longer-term changes in SOM due to management (von Lützow et al. 2006, Marriott and Wander 2006a). Schipanski et al. (2010) measured soybean N<sub>2</sub> fixation across a soil gradient and found weak evidence that the percentage of soybean N from fixation was regulated by soil N availability from POM because soil N availability was secondary to the effect of soil texture on N<sub>2</sub> fixation. In their study, differences in soil texture were

confounded with management history and the resulting fertility gradient. In this study, although textural properties varied across farms (e.g., from 11% to 32% clay), there were no significant relationships between percent clay and percentage of N from fixation. Furthermore, across farms in this study, there were no significant relationships between sand or clay content and the measured POM pools, emphasizing the role of management in driving the amount of POM in soils (Marriott and Wander 2006b).

The hypothesis that the relationship between POM N and N<sub>2</sub> fixation would be influenced by biotic interactions was also supported. The slope of the relationship between POM N and the percentage of vetch N from fixation was steeper for the sole vetch treatment. Furthermore, the regression relationships between N-min and percent N from fixation were significant only for sole vetch and not for vetch in mixture (Appendix S1: Fig. S2). This suggests that the effect of N availability from turnover of POM was moderated by growing vetch in mixture with rye (Fig. 3). A previous study testing different cover crop mixture treatments on eight of these farms also found negative correlations between legume biomass production in mixtures (for winter pea and crimson clover) and the protected POM N pool; however, in that experiment soil properties did not predict the percentage of N from fixation (i.e., the N<sub>2</sub> fixation rate) for any species (Blesh 2018). Another study focused on intercropped red clover also found no relationship between labile soil N pools and the percentage of N from fixation (Schipanski and Drinkwater 2011). The lack of relationships with percentage of N from fixation in these other studies could be due in part to intercropping with non-legume species. Here, the intercropped rye competed with the vetch for soil inorganic N, taking up an average of 51.5 kg N/ha in 2016 (with an estimated 46.9 kg/ha of that N derived from soil), which also caused vetch to fix N at a higher rate in mixture (Fig. 2). In contrast to percentage of N from fixation, the effect of POM N on total fixed N in aboveground vetch biomass (in kg N/ha) was stronger in mixture than monoculture (Appendix S1: Table S1). This likely occurred because rye was the stronger competitor in mixture at high soil N levels. For instance, across farms, rye aboveground biomass (both total and as a percentage of mixture biomass) increased with the protected POM N concentration in soil ( $F = 10.56$ ,  $P = 0.002$  and  $F = 16.84$ ,  $P = 0.0002$ , respectively), which reduced vetch biomass, the primary driver of total fixed N inputs (Herridge et al. 2008).

Beyond the regulatory effect of N from turnover of POM, legume biomass production across farms, and thus total N<sub>2</sub> fixed, was limited by P and increased with greater soil P concentrations (Appendix S1: Fig. S2). Together, POM N and soil P concentrations explained 42–54% of the variation in vetch N<sub>2</sub> fixation (Appendix S1: Table S1), similar to earlier findings with different legume cover crop mixtures on these farms (Blesh 2018). However, there was no evidence that the N<sub>2</sub> fixation process itself is regulated by P availability

(i.e., there was no relationship between soil P concentration and the percentage of plant N from fixation), which supports other studies (Vitousek et al. 2002). Although the model  $R^2$  values were relatively high, especially for data from working agroecosystems, there remains substantial unexplained variation that could result from several factors. First, additional variation in  $N_2$  fixation could result from physiological controls at smaller scales, such as soil  $O_2$  availability (Layzell and Hunt 1990). Second, rhizobial strains differ in their effectiveness, and may have differed in vetch nodules across farms, because background soil populations often occupy nodules despite inoculation (Thies et al. 1991). Consequently, applying one  $B$  value across sites, to account for isotopic fractionation, may not fully meet the assumptions of the natural abundance method. This method also assumes that the reference plant (rye) was not affected by free living  $N_2$  fixation in the rhizosphere, a topic of renewed attention in agroecosystem research on perennial grasses (Roley et al. 2018, Smercina et al. 2019). However, given the experimental design, in which the reference plants were in small plots nested within a larger field inoculated with rhizobia, and given that the farm management histories often included legumes in crop rotation, this assumption is likely valid.

Analysis of SOM pools and fluxes following 2 yr of the cover crop mixture showed that, on average, the cover crop increased the size of both POM pools, and rates of microbial activity (C-min and N-min; Fig. 4). Further, POM N and N-min had a strong relationship across sites. These dynamics would potentially enhance the stabilizing feedback identified here and increase N cycling efficiency over time. The modeling of vetch  $N_2$  fixation as a function of baseline soil properties, to determine if the POM gradient resulting from distinct management histories regulated  $N_2$  fixation, focused on vetch  $N_2$  fixation in 2016, because soil properties started changing quickly in response to the cover crop mixture treatment (Fig. 4). This increase in soil N cycling may also help explain the large reduction in vetch biomass in the second year. These changes in soil properties with the cover crop support the hypothesis that the mechanism driving the stabilizing feedback was increased microbial activity and turnover of N with larger pools of intra-aggregate POM.

Results from this experiment suggest that protected POM represents an ecologically meaningful SOM fraction that regulates  $N_2$  fixation in diversified agroecosystems because it is a key source of inorganic N through N mineralization. The POM that is occluded in aggregates often has a low C:N ratio, suggesting capacity for both mineralization and stabilization of SOM (Marriott and Wander 2006a, Hatton et al. 2012, Cotrufo et al. 2013). Occlusion within microaggregates can stabilize SOM by making it physically inaccessible to decomposers (von Lützow et al. 2006). Although accumulation of SOM with legumes is frequently observed (Fornara and Tilman 2008, McDaniel et al. 2014), POM that is protected

in soil aggregates may exhibit faster turnover rates in diversified agroecosystems than is typically assumed based on experiments finding mean residence times of 10–80 yr for occluded POM (Collins et al. 2000, Puget et al. 2000, Dorodnikov et al. 2011). Legume cover crops and cover crop mixtures increase the presence of living roots in agroecosystems. Root C inputs can contribute to aggregation and stabilization of SOM, but roots may also cause priming effects and other rhizosphere interactions that increase turnover of stable SOM pools, notably by root growth through aggregates or in hotspots with large rhizodeposits (Clarholm 1985, Pateron 2003, Kuzyakov and Blagodatskaya 2015). For instance, plants can increase N mineralization in the rhizosphere (Cheng 2009) by adjusting levels of soluble C compounds released through root exudates (Hamilton and Frank 2001). In this study, the increased N and C mineralization rates with the cover crop mixture relative to the no cover crop control; the negative relationships observed between protected POM N and vetch  $N_2$  fixation, and between N-min and  $N_2$  fixation; and the positive relationship between POM N and N-min, all suggest that cover crops access N from SOM pools that are considered stable (i.e., protected for decades or longer; Wander 2004, von Lützow et al. 2006) through interactions with microorganisms in the rhizosphere. This finding supports other studies reporting that intra-aggregate POM is related to N mineralization rates on organic and diversified farms (e.g., Willson et al. 2001, Marriott and Wander 2006a), which tend to have greater root presence in space and/or time depending on functional diversity of crop rotations.

There is an urgent need to develop more sustainable agroecosystems to avoid crossing critical biophysical thresholds, including degradation of ecosystem processes on which agriculture depends (IPCC 2013). Central to realizing this goal is increasing plant diversity in agroecosystems, particularly diversity of plant functional traits including  $N_2$  fixation (Isbell et al. 2017, Blesch 2018). More on-farm studies, across even wider gradients of soil and management conditions, are needed to further develop and refine predictive models of N inputs from legume  $N_2$  fixation. Legume cover crops represent a feasible opportunity for increasing agroecosystem biodiversity and ecosystem functions because they can be grown between cultivation of primary crops. Results from this on-farm experiment reveal stabilizing feedbacks that help explain the smaller N losses measured in agroecosystems with legume N sources. Over time, agroecosystems with regular use of legume cover crops will likely reach a new steady state for SOM, at which point legume  $N_2$  fixation rates may be lower, or legumes may be less competitive in cover crop mixtures, suggesting that farmers could adapt their management towards other functional groups of cover crops. Cover crops could be adopted more broadly with appropriate policy incentives to increase agroecosystem diversity and meet environmental sustainability and food security goals.

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## SUPPORTING INFORMATION

Additional supporting information may be found online at: <http://onlinelibrary.wiley.com/doi/10.1002/eap.1986/full>

## DATA AVAILABILITY

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.70q4744>.