

## ARTICLE

## Agroecosystems

# A grass–legume cover crop maintains nitrogen inputs and nitrous oxide fluxes from an organic agroecosystem

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## Abstract

Legume cover crops are central to an ecological nutrient management approach that can reduce nitrogen (N) losses from agriculture. Diversifying cropping systems with a legume–grass cover crop mixture could further reduce N losses by increasing soil N assimilation and synchronizing N mineralization with N uptake by the following crop. We established four winter cover crop treatments (crimson clover, cereal rye, clover–rye mixture, and weedy fallow control) in an organic grain agroecosystem that had been managed for 30 years with a legume cover crop as the only external N source. We hypothesized that the legume–grass mixture would provide similar inputs of biologically fixed N<sub>2</sub> compared with the sole legume, while reducing N<sub>2</sub>O emissions during decomposition following tillage. We measured cover crop aboveground biomass C:N and clover N<sub>2</sub> fixation, soil inorganic N and N<sub>2</sub>O fluxes throughout the corn growing season following cover crop tillage, and corn N assimilation at harvest. Even with a reduced clover seeding rate in mixture, the clover and mixture treatments had similar fixed N inputs, litter N and C:N, and no differences in cumulative N<sub>2</sub>O emissions. During the first peak flux, N<sub>2</sub>O emissions were 2–5 times higher in clover and mixture relative to rye and fallow, with no differences between clover and mixture. There were no treatment differences at the second N<sub>2</sub>O peak, which followed the first major rain event. We contextualized these findings by calculating a 6-year partial N mass balance for this agroecosystem, which was slightly negative ( $-6.8 \pm 0.8 \text{ kg N ha}^{-1} \text{ year}^{-1}$ ) when accounting for historical mean annual N<sub>2</sub>O emissions and nitrate leaching. Overall, N inputs and harvested N exports were approximately in balance for this legume-based crop rotation, suggesting that the legacy of ecological nutrient management has promoted efficient N cycling. However, results from our field experiment indicate that short-term N<sub>2</sub>O flux rates following cover crop incorporation can be high even for a legume–grass mixture. Additional strategies to reduce soil disturbance are therefore needed to further tighten N cycling in organic grain agroecosystems.

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**KEYWORDS**

biological nitrogen fixation, cover crops, functional traits, legume–grass mixture, nitrogen mass balance, nitrous oxide

**INTRODUCTION**

The convergence of global climate change with widespread food, energy, and water crises, along with urgent calls from the international scientific community for swift and significant reductions in global greenhouse gas emissions (IPCC, 2019), is motivating a growing interest in sustainable soil management practices. Excess nitrogen (N) inputs (e.g., synthetic inorganic N fertilizer and manure) to agricultural fields contribute to climate change by increasing nitrous oxide (N<sub>2</sub>O) emissions from soils (Eagle et al., 2020). Globally, N<sub>2</sub>O emissions released directly from agricultural soils were estimated to have increased from 1.6 Tg N year<sup>−1</sup> in 1990 to 1.8 Tg N year<sup>−1</sup> in 2006 (Syakila & Kroeze, 2011) and are projected to increase by 35% from 2005 to 2030 (USEPA, 2012). In the United States, approximately 75% of N<sub>2</sub>O emissions are derived from agricultural soils (Robertson & Vitousek, 2009; USEPA, 2021). Given the significant impact of soil management on N<sub>2</sub>O emissions, it is critical that farmers employ ecological management practices that tighten the N cycle, improve soil quality, and reduce N losses (Drinkwater & Snapp, 2007).

Ecological nutrient management applies principles from ecosystem ecology to manage ecological interactions to achieve both crop production and sustainability (Drinkwater & Snapp, 2007, 2022). Ecological practices provide an alternative to input intensive management, for instance, by replacing synthetic fertilizer N with legume N<sub>2</sub> fixation within crop rotations (Blesh & Drinkwater, 2013). Legumes can be added to crop rotations as cover crops, which are unharvested crops that are typically planted in the fall and terminated in the spring in temperate agroecosystems. Cover crops have potential to increase functional diversity to provide a broad suite of ecosystem services in grain agroecosystems with minimal disruption of typical grain rotations or reductions in yield (King & Blesh, 2018; Rodriguez et al., 2021; Snapp et al., 2005).

Cover crops can reduce N losses from agroecosystems because they recouple carbon (C) and N cycles. For example, cover crops increase the length of time during which photosynthesis is occurring throughout the year, increasing C and N entering soil organic matter (SOM), thereby increasing SOM stocks (King & Blesh, 2018; McDaniel et al., 2014; Poeplau & Don, 2015). Furthermore, legume cover crops can reduce or replace synthetic N fertilizer inputs through biological N<sub>2</sub> fixation (BNF) carried out by

symbiotic bacteria. Compared with synthetic N fertilizer inputs, legume N sources can better balance N inputs to fields with N exported in harvested crops, reducing N surpluses and potential for loss (Blesh & Drinkwater, 2013). Legume biomass inputs provide an energetically favorable substrate that can stimulate microbial activity, increasing both internal N cycling and storage, which can result in lower N losses (leaching and denitrification) compared with synthetic N fertilizer inputs (Drinkwater et al., 1998; Kallenbach et al., 2015; Syswerda et al., 2012).

Despite these benefits compared with synthetic fertilizer N, when compared with other functional types of cover crops that scavenge more soil N, sole stands of legumes can result in greater N losses to the environment (Alluvione et al., 2010; Huang et al., 2004; Millar et al., 2004; White et al., 2017). As a result, there is growing interest in planting mixtures of grasses, such as cereal rye, with cover crops in the legume family to simultaneously enhance multiple ecosystem functions (Blesh, 2017; Hayden et al., 2014; Poffenbarger et al., 2015; Snapp et al., 2005; Wood et al., 2015). In a legume–grass cover crop mixture, the legume provides a N source through BNF, while the grass enhances soil N retention and recycling, and other functions such as weed suppression. Rather than maximizing one function at the cost of others, legume–grass mixtures can supply multiple functions at thresholds that meet management goals for sustainability (Blesh, 2017; Kaye et al., 2019). Additionally, the functions provided by legumes can be enhanced in mixtures with grasses compared with monoculture stands. Legumes generally allocate less photosynthate to BNF if sufficient plant-available N is already present in soil. Through competition for soil N with grasses, legumes respond by increasing the energy-intensive processes needed to fix N<sub>2</sub>, increasing BNF rates per plant (Hogh-Jensen & Schjoerring, 1997; Jensen, 1996; Li et al., 2016). This could result in similar BNF inputs from legumes planted at half rate in a mixture compared with sole legume cover crops, while also providing ecosystem functions from grasses.

Past experiments have found differences in N<sub>2</sub>O emissions between different cover crop functional types, suggesting that legumes produce higher emissions than grasses. For example, in their meta-analysis, Basche et al. (2014) found that out of 106 observations from 26 publications, 40% of fields with cover crops had lower N<sub>2</sub>O emissions than fields with no cover crop, while 60% had increased emissions. Generally, higher N inputs to soil

lead to higher N mineralization rates during decomposition, and thus higher N<sub>2</sub>O losses (e.g., Han et al., 2017). In the context of cover crop N inputs, Basche et al. (2014) found that legumes, which supply new fixed N to agroecosystems, can result in higher N<sub>2</sub>O emissions than nonlegume cover crops that assimilate greater quantities of soil N. However, this meta-analysis only included six observations for agroecosystems with legume cover crops as the only N source (Basche et al., 2014), limiting our ability to generalize these results. Given these limited data and the high variability of N<sub>2</sub>O emissions within treatments and over time, there is a need for further study in organic agroecosystems that depend on legume N sources. Even fewer studies have quantified N<sub>2</sub>O emissions from legume–grass mixtures. By increasing the C:N and molecular diversity of organic compounds entering soil from litter (Finney et al., 2016; Kallenbach et al., 2019), legume–grass mixtures have the potential to slow down N mineralization early in the growing season and improve synchrony between mineralization and crop N assimilation, reducing N losses while still providing substantial N to future crops.

To better understand N cycling dynamics with a legume–grass cover crop mixture, we planted an experiment testing a crimson clover–cereal rye mixture compared with clover and rye grown alone within the organic management system at the Kellogg Biological Station (KBS) Long-Term Ecological Research (LTER) site. The cover crop mixture treatment represents an increase in both species diversity and spatial diversity of cover crops in this cropping system, which had been managed for 30 years with winter cover crops of red clover frost-seeded into winter wheat and annual rye following corn harvest. In this agroecosystem, red clover BNF is the only external N source. Our hypothesis was that the crimson clover–cereal rye mixture would produce the highest biomass due to complementary traits and interactions between rye and clover. We also expected similar legume N<sub>2</sub> fixation inputs between the mixture and clover treatment due to higher rates of BNF when clover was competing with rye for soil N. We hypothesized that the mixture would increase the C:N of litter inputs compared with the clover treatment and slow litter decomposition, improving synchrony between soil inorganic N availability and corn N assimilation, and reducing overall N<sub>2</sub>O emissions throughout the three-month growing season. Although better synchrony may reduce N losses, we expected that corn N assimilation would decline as C:N ratios increased from clover to mixture to rye, reducing potential N mineralization. Finally, we expected the six-year, partial N mass balance for the site to indicate that N inputs from BNF approximately balance N removal in harvested crops given its long history of ecological nutrient management.

## METHODS

### Site description and experimental design

We conducted our experiment from 2019 to 2020 in subplots of the biologically based (i.e., certified organic) cropping system in the Main Cropping System Experiment (MCSE) (replicates 1–4) of the KBS LTER site (latitude/longitude: 42°14'24" N, 85°14'24" W; elevation: 288 m). The site has been managed with a corn, soy, wheat rotation since 1989, with an average temperature of 9.2°C and an average rainfall of 933 mm year<sup>-1</sup>. The site resides on a glacial outwash plain. The soils are well-drained loam, sandy loam, and sandy clay loam in the Kalamazoo and Oshtemo series (Crum & Collins, 1995). Aside from atmospheric deposition, the only N inputs at this site since 1989 have been from legume N<sub>2</sub> fixation by medium red clover (*Trifolium pratense* L.), which is frost-seeded into winter wheat every three years, and soybeans (*Glycine max* L.), which are planted every three years. The rotation also includes a cereal rye (*Secale cereale* M.Bieb) overwintering cover crop planted after corn and incorporated before soybean.

The experiment had four treatments, which were planted on 31 July 2019, in a randomized complete block design with four blocks. Treatments were planted using a grain drill in 3.1 × 12.2 m plots: (1) cereal rye (*S. cereale* L., seeding rate: 100.9 kg ha<sup>-1</sup>), (2) crimson clover (*Trifolium incarnatum* L., seeding rate: 16.8 kg ha<sup>-1</sup>), (3) rye/clover mixture (seeding rate: 50.4 kg ha<sup>-1</sup> rye, 9.0 kg ha<sup>-1</sup> clover), and (4) a weedy fallow control. Seeding rates were determined based on recommendations from Michigan State University Extension. The cover crops overwintered, and all four treatments were rototilled into the soil on 26 May 2020. Viking Organic Seed Corn Brand (O.84-95UP) Variety (A1025726) was planted on 1 June 2020 at a rate of 12,950 seeds ha<sup>-1</sup>.

### N assimilation in cover crop biomass and corn grain

We sampled aboveground biomass (cover crops and weeds) from all treatments prior to tillage on 26 May 2020 in one 0.25-m<sup>2</sup> quadrat placed randomly in each replicate plot avoiding edges. We harvested corn on 28 October 2020. To reduce edge effects, we sampled corn from the middle 8.5 m of the plots. Using a Kincaid 8XP Plot Combine (Kincaid Manufacturing, Haven, KS), we measured grain yield and moisture using the onboard Mirus Harvest Master computer software (Juniper Systems, Logan, UT) from the middle two rows (1.5 m) of each treatment. From the combined bin, we

collected a grain subsample for chemical analysis. Cover crop shoot biomass and corn grain were dried for at least 48 h at 60°C and weighed. Shoot biomass was coarsely ground (<2 mm) in a Wiley mill and corn grain was ground to the consistency of flour before being analyzed for total C and N by dry combustion on a Leco TruMac CN Analyzer (Leco Corporation, St. Joseph, MI). We calculated corn grain and cover crop N assimilation (in kilograms of nitrogen per hectare) by multiplying grain yield or cover crop aboveground biomass (in kilograms per hectare) by %N.

### N<sub>2</sub>O flux from incorporation of cover crops to corn maturity

We used the static chamber method (Kahmark et al., 2018) to measure N<sub>2</sub>O for three months following tillage of all treatments (cover crops and weedy fallow). Stainless steel cylinders (28.5 cm in diameter) fitted with lids sealed by O-rings served as airtight static chambers. In each plot, we measured N<sub>2</sub>O every few days after cover crop incorporation and then every two weeks over 91 days for a total of 13 sampling events. Specifically, we sampled N<sub>2</sub>O more frequently over the first two weeks after tillage to capture the initial flux following soil disturbances from tillage (26 May) and mechanical weed control (8, 9, and 12 June). We also sampled after early season rain events (29 May, 9–12 June, and 22–23 June) to capture emission peaks. Once corn began to grow in late June, we sampled every two weeks for the remainder of the summer, which matches the typical sampling frequency at this experimental site, with the last sampling event occurring on 27 August. This sampling schedule aimed to capture the main episodes of N<sub>2</sub>O flux from tillage and subsequent decomposition of organic matter.

Static chambers were installed in the soil at least a day before each sampling event to reduce the effect of soil disturbance on emissions data. On each sampling date, lids were secured over each chamber between 10 am and noon. Immediately after closing the chamber lids, 10 mL of gas samples were extracted with a syringe from a rubber septum port every 20 min for an hour (0, 20, 40, and 60 min). Samples were later analyzed for N<sub>2</sub>O using a gas chromatograph equipped with an electron capture detector (Agilent, Santa Clara, CA). The internal volume was measured for each chamber and used to calculate the change in headspace N<sub>2</sub>O concentration over 60 min. Each set of data points was screened for nonlinearity and then the

rate of change over time was analyzed using linear regression.

### Soil sampling

In June 2019, we collected composite baseline soil samples (10, 2 × 20 cm cores per plot) prior to planting corn in July. Soil was air-dried and total soil C and N were measured by dry combustion on a Leco TruMac CN Analyzer (Leco Corporation, St. Joseph, MI, USA). Beginning the day after tillage on 27 May 2020, we sampled soil for analysis of inorganic N (NH<sub>4</sub><sup>+</sup> + NO<sub>3</sub><sup>−</sup>) near the static chambers every two weeks for the duration of the 91-day N<sub>2</sub>O sampling period. Within 1 m of each static chamber, we collected six, 2-cm diameter × 10-cm deep soil cores. Samples were homogenized and sieved to 2 mm before extraction with 2 M KCl. Soil moisture was analyzed gravimetrically. Extractions were stored at −20°C and later thawed before analysis for NO<sub>3</sub><sup>−</sup> and NH<sub>4</sub><sup>+</sup> on a discrete analyzer (AQ2; Seal Analytical, Mequon, WI).

### Legume N<sub>2</sub> fixation by natural abundance

We used the natural abundance method (Shearer & Kohl, 1986) to estimate N<sub>2</sub> fixation by crimson clover. In the field, we collected aboveground clover biomass from both the monoculture and mixture treatments, and rye from the monoculture treatment (the non-N<sub>2</sub> fixing reference plant). We dried, weighed, and finely ground (<0.5 mm) each sample and analyzed them for total N and δ<sup>15</sup>N enrichment using a continuous flow PDZ Europa 20-20 isotope ratio mass spectrometer connected to a PDZ Europa ANCA-GSL elemental analyzer (Secron Ltd., Cheshire, UK) at the UC Davis Stable Isotope Facility. We calculated percent N derived from the atmosphere (%Nd<sub>fa</sub>) as:

$$\%Nd_{fa} = 100 \times ((\delta^{15}N_{ref} - \delta^{15}N_{legume}) / (\delta^{15}N_{ref} - B)),$$

where δ<sup>15</sup>N<sub>ref</sub> is the δ<sup>15</sup>N signature of the reference plant (rye), δ<sup>15</sup>N<sub>legume</sub> is the δ<sup>15</sup>N signature of the clover, 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 crimson clover in a N-free medium in a greenhouse following methods in Blesh (2017). We found a mean *B* value of −1.57, which we used in our calculation of %Nd<sub>fa</sub>. To calculate BNF (in kilograms of nitrogen per hectare), we multiplied total aboveground biomass by shoot %N, and then by %Nd<sub>fa</sub>.



## Nitrogen balance

To help interpret the single-season N cycling dynamics measured in our experiment, we used long-term data from the KBS MCSE to calculate a partial N mass balance (McLellan et al., 2018; Robertson & Vitousek, 2009) for six years (2014–2019, which spanned two full crop rotation cycles) in the organic cropping system. This “partial balance” approach focuses on the main N fluxes managed by farmers (Blesh & Drinkwater, 2013). We also included an estimate of N inputs from atmospheric deposition, which we assumed to be 10.5 kg N ha<sup>-1</sup> year<sup>-1</sup> based on values collected from the southwest Michigan station by the National Atmospheric Deposition Program. To estimate N inputs from BNF, we first calculated total aboveground biomass N of the red clover using the historical shoot biomass data (in kilograms per hectare) from the KBS LTER MCSE data repository (Robertson & Snapp, 2020), which we multiplied by a mean N concentration of 3.4% to calculate total shoot N (in kilograms of nitrogen per hectare). We then multiplied total aboveground N by %Nd<sub>fa</sub>, which we estimated to be 70% based on Wilke’s (2010) study conducted in the biologically based cropping system at KBS. Finally, belowground N inputs from red clover were assumed to be 40% of aboveground N (Hammehle et al., 2018).

Because BNF rates can change over time, particularly with changes in soil fertility, we conducted a sensitivity analysis to understand how a range of red clover BNF rates would affect the partial N balance. Past studies have measured red clover %Nd<sub>fa</sub> ranging from 35% (Heichel et al., 1985) to 90% (Rochester & Peoples, 2005). A more recent study by Schipanski and Drinkwater (2010), also conducted in temperate grain agroecosystems, reported that when grown with grains, red clover had an average %Nd<sub>fa</sub> of 72% across 15 farms. Based on their dataset, we took the mean of the five lowest and five highest observations and used a range of 50–80 %Nd<sub>fa</sub> to conduct a sensitivity analysis for our experimental site.

The primary N exported from the agroecosystem is in the harvested corn, soybeans, and wheat crops. We calculated grain N export using historical yield and grain %N data stored on the KBS LTER data repository (Robertson, 2020a; Robertson & Snapp, 2019). Specifically, we used a mean grain N concentration of 6.4% in soybeans, 1.2% in corn, and 1.7% in wheat. We multiplied the grain %N by grain dry matter yield (in kilograms per hectare) to calculate N removed in harvest (in kilograms of nitrogen per hectare). Because soybeans are legumes, in the soybean years we also accounted for BNF. First, to estimate total biomass N from soybean yield, we assumed that soybeans had an 80 %N harvest index

(David & Gentry, 2000). We then used an estimate of 80 %Nd<sub>fa</sub> (Gelfand & Robertson, 2015) to calculate the amount of soybean N derived from BNF. The partial N balance was then calculated using the following equation:

$$N_{\text{balance}} = (N_{\text{fixed}} + N_{\text{deposited}}) - (NH_{\text{corn}} + NH_{\text{wheat}} + NH_{\text{soybean}}),$$

where  $N_{\text{fixed}}$  is the estimated inputs from red clover BNF,  $N_{\text{deposited}}$  is the estimated atmospheric deposition, and  $NH$  is the N removed during the harvest of each crop over the six-year period (2014–2019).  $N_{\text{balance}}$  was divided by six to estimate the mean annual N balance. We then used historical measurements of annual N<sub>2</sub>O losses and NO<sub>3</sub><sup>-</sup> leaching from this organic cropping system to supplement the partial N mass balance. Finally, we used data from the site assessing changes in soil organic carbon (SOC) and soil organic nitrogen (SON) from deep soil cores (1 m) to interpret the N mass balance results.

## Data analysis

We calculated descriptive statistics (mean, standard error, and interquartile ranges [IQRs]) and checked all variables for normality of residuals and homoscedasticity. We transformed daily and cumulative N<sub>2</sub>O emissions using the natural log function. We used repeated measures ANOVA models to test for differences in N<sub>2</sub>O flux (in grams of nitrous oxide-nitrogen per hectare per day) across treatments for all time points. Models included day as the repeated measure, cover crop treatment as the fixed effect, and replicate as the random effect. For each cover crop treatment, we calculated the area under the curve to estimate mean cumulative N<sub>2</sub>O emissions (in grams of nitrous oxide-nitrogen per hectare) (Gelfand et al., 2016):

$$\text{Cumulative N}_2\text{O emissions} = \sum_{t_0}^{t_{\text{final}}} [(x_t + x_{t+1})/2] \times [(t+1) - t],$$

where  $t_0$  is the initial sampling date,  $t_{\text{final}}$  is the final sampling date,  $x_t$  is N<sub>2</sub>O flux at time  $t$ , and  $x_{t+1}$  is N<sub>2</sub>O flux at the following sampling date.

We determined the effects of cover crop treatments on cumulative N<sub>2</sub>O, total cover crop biomass (in kilograms per hectare), total biomass N (in kilograms of nitrogen per hectare), biomass C:N, clover N (in kilograms of nitrogen per hectare), and BNF (in kilograms of nitrogen per hectare) using separate ANOVA models with cover crop treatment as the fixed effect and replicate as the random effect. When ANOVA models were significant, post hoc

comparisons of least square means were performed using Tukey's honestly significant difference (HSD), reporting results as statistically significant at  $\alpha = 0.05$ . JMP Pro 15 software (SAS Institute, Cary NC) was used to perform all statistical analyses.

## RESULTS

### Cover crop biomass and traits (C:N and BNF)

On average, the clover and mixture treatments had two-fold higher total aboveground biomass (cover crop and weed species) compared with the weedy fallow ( $p = 0.007$ ). However, biomass in the mixture, clover, and rye treatments did not differ significantly, and the rye treatment was not significantly different from fallow (Table 1). Furthermore, clover biomass in the mixture treatment did not differ significantly from clover biomass in the sole clover treatment. Aboveground biomass N content across plots ranged from a low of  $14.2 \text{ kg N ha}^{-1}$  in one fallow plot to a high of  $117.7 \text{ kg N ha}^{-1}$  in one clover plot, with twofold to threefold higher aboveground biomass N assimilation (cover crop and weed species) in the clover and mixture treatments compared with the rye and weedy fallow ( $p < 0.0004$ ). Across treatments, rye biomass was highly correlated with rye biomass N ( $r = 0.95$ ), as were clover biomass and clover biomass N ( $r = 0.99$ ).

We found a significantly lower C:N in treatments with clover, with C:N decreasing from  $40.3 \pm 1.3$  in rye and  $34.8 \pm 1.9$  in fallow to  $25.6 \pm 1.1$  in the mixture and  $21.8 \pm 0.3$  in clover ( $p < 0.0001$ ). The difference between clover and mixture C:N was not significant. In the clover-rye mixture, clover produced more biomass compared with rye and weeds in three of the four replicates, making up an average of 54.2% of the total mixture biomass, while rye made up an average of 27.7% and weeds made up 18.1% (Table 2). Using stable isotope methods, we estimated that the mean clover shoot N

derived from fixation was 43.4% when grown alone and 63.3% when grown in mixture with rye. Total new aboveground N inputs from BNF between the clover (min: 17.8; max:  $45.9 \text{ kg N ha}^{-1}$ ) and mixture (min: 19.8; max:  $40.4 \text{ kg N ha}^{-1}$ ) treatments did not significantly differ ( $p = 0.677$ ) (Table 1, Figure 1).

### Daily $\text{N}_2\text{O}$ emissions following tillage

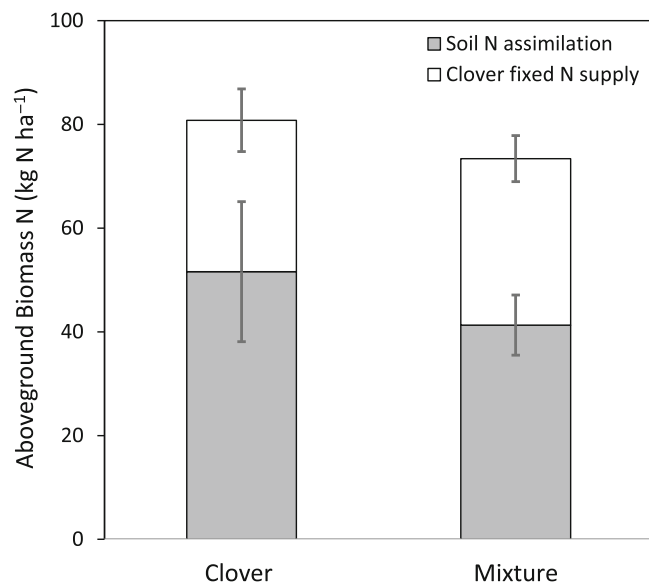
We found a significant effect of sampling day ( $p < 0.0001$ ) and cover crop treatment ( $p < 0.001$ ) on daily  $\text{N}_2\text{O}$  flux, with no interaction between day and treatment ( $p = 0.346$ ). During the first peak, eight days after tillage,  $\text{N}_2\text{O}$  emissions were fivefold higher in the mixture treatment ( $18.0 \pm 5.6 \text{ g N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$ ) compared with the rye treatment ( $3.6 \pm 1.0 \text{ g N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$ ) ( $p = 0.049$ ). Emissions from the clover treatment ( $11.3 \pm 5.2 \text{ g N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$ ) were not significantly higher than the rye treatment. Eleven days after tillage,  $\text{N}_2\text{O}$  emissions were fivefold higher in mixture ( $9.4 \pm 2.6 \text{ g N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$ ) than in rye ( $1.8 \pm 0.4 \text{ g N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$ ) ( $p = 0.018$ ), and at 12 days, emissions were four times higher in clover ( $5.9 \pm 1.1 \text{ g N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$ ) compared with rye ( $1.5 \pm 0.6 \text{ g N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$ ) ( $p = 0.018$ ). By the 15th day, emissions from both clover ( $4.4 \pm 1.3 \text{ g N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$ ) and mixture ( $7.2 \pm 1.6 \text{ g N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$ ) treatments were

**TABLE 2** Species composition, separated by rye, clover, and weeds, of the mixture treatments by block replicate and means and SE (in parentheses) by species.

| Replicate | % Rye | % Clover | % Weeds |
|-----------|-------|----------|---------|
| 1         | 7.5   | 71.9     | 20.7    |
| 2         | 29.2  | 51.8     | 19.0    |
| 3         | 26.7  | 57.9     | 15.5    |
| 4         | 47.3  | 35.1     | 17.5    |
| Mean      | 27.7  | 54.2     | 18.1    |
| (SE)      | (7.1) | (6.6)    | (0.9)   |

**TABLE 1** Means and SE (in parentheses) for aboveground biomass, aboveground biomass nitrogen (N), and biological  $\text{N}_2$  fixation (BNF) by species across treatments.

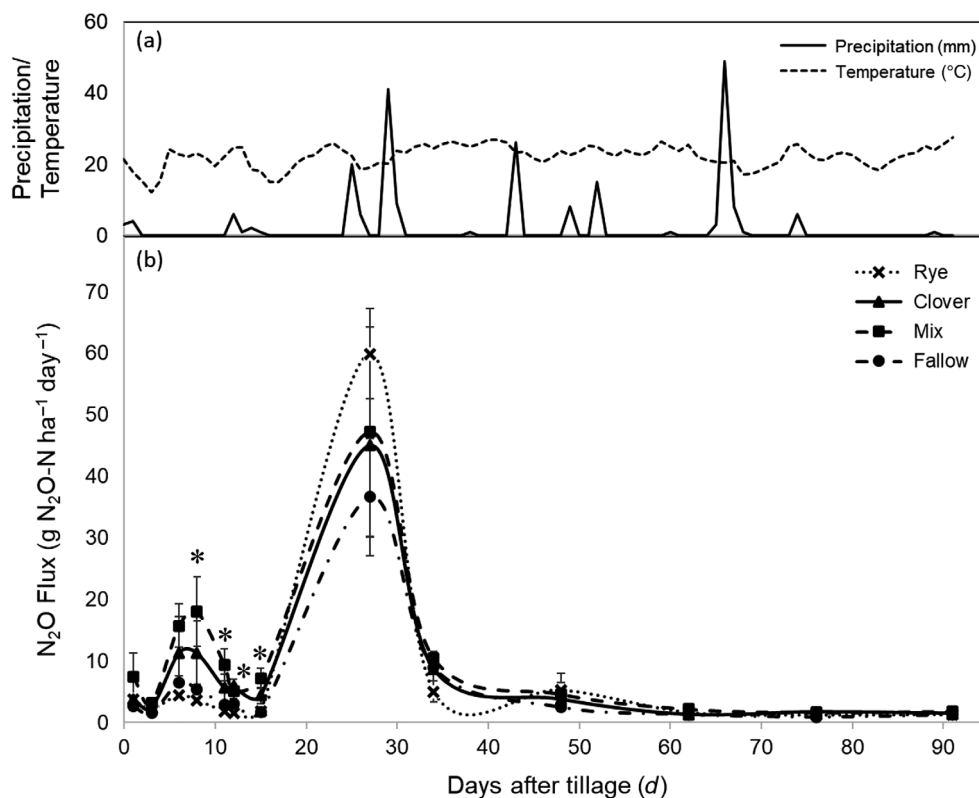
| Treatment | Total                           |                                     | Clover                          |                                     |                               | Rye                             |                                     | Weeds                           |                                     |
|-----------|---------------------------------|-------------------------------------|---------------------------------|-------------------------------------|-------------------------------|---------------------------------|-------------------------------------|---------------------------------|-------------------------------------|
|           | Biomass ( $\text{kg ha}^{-1}$ ) | Biomass N ( $\text{kg N ha}^{-1}$ ) | Biomass ( $\text{kg ha}^{-1}$ ) | Biomass N ( $\text{kg N ha}^{-1}$ ) | BNF ( $\text{kg N ha}^{-1}$ ) | Biomass ( $\text{kg ha}^{-1}$ ) | Biomass N ( $\text{kg N ha}^{-1}$ ) | Biomass ( $\text{kg ha}^{-1}$ ) | Biomass N ( $\text{kg N ha}^{-1}$ ) |
| Rye       | 2842.8 (212.2)                  | 31.9 (1.4)                          |                                 |                                     |                               | 2367.7 (161.8)                  | 25.4 (0.5)                          | 475.2 (89.9)                    | 6.5 (1.1)                           |
| Clover    | 3972.1 (579.7)                  | 80.8 (13.5)                         | 2963.9 (654.8)                  | 67.5 (14.0)                         | 29.2 (6.0)                    |                                 |                                     | 1008.2 (90.4)                   | 13.3 (1.2)                          |
| Mix       | 4219.1 (297.2)                  | 73.4 (5.8)                          | 2310.0 (380.7)                  | 50.6 (7.0)                          | 32.1 (4.4)                    | 1148.9 (300.9)                  | 13.1 (3.6)                          | 760.3 (43.3)                    | 9.6 (0.6)                           |
| Fallow    | 2005.8 (387.9)                  | 26.0 (6.6)                          |                                 |                                     |                               |                                 |                                     | 2005.8 (387.9)                  | 26.0 (6.6)                          |



**FIGURE 1** Aboveground biomass nitrogen (mean  $\pm$  SE) in the clover and mixture treatments separated between N assimilated from the soil (including clover and weeds in the clover treatment and clover, rye, and weeds in the mixture treatment [gray bars]) and N derived from clover biological nitrogen fixation (white bars).

significantly higher than rye ( $1.9 \pm 0.4$  g  $\text{N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$ ) and fallow ( $1.7 \pm 0.3$  g  $\text{N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$ ) ( $p = 0.007$ ). We did not find any other significant differences between treatments for the remainder of the 91-day  $\text{N}_2\text{O}$  sampling period (Figure 2b).

Across treatments, there was a significant correlation between soil inorganic N ( $\text{NO}_3^- + \text{NH}_4^+$ ) and daily  $\text{N}_2\text{O}$  flux six days after tillage ( $r = 0.53$ ), but not at any other sampling date. At the second peak, which occurred 27 days after tillage, there were no significant differences in soil inorganic N or  $\text{N}_2\text{O}$  flux between cover crop treatments. We found the highest variability in  $\text{N}_2\text{O}$  flux between replicates at this peak for all treatments (e.g.,  $45.1 \pm 15.1$  g  $\text{N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$  for the clover treatment) compared with any other sampling date. For the remainder of the sampling period, background  $\text{N}_2\text{O}$  flux was low, with minimal variability within treatments and no differences between treatments (Figure 2b). It rained 26 mm on days 25–26 after tillage, right before sampling day 27. It then rained another 50 mm on days 29–30 after tillage, ahead of the sampling point on day 34, which did not have pulse emissions. It rained significantly again



**FIGURE 2** (a) Precipitation and mean air temperature over the course of the study period. (b) Net nitrous oxide ( $\text{N}_2\text{O}$ ) flux (g  $\text{N}_2\text{O-N ha}^{-1} \text{ day}^{-1}$ ) from the soil (mean  $\pm$  SE) over 91 days following tillage on 28 May 2020 ( $d = 0$ ). An asterisk indicates days when we found significant differences between cover crop treatments ( $p < 0.05$ ).

66 (49 mm) and 67 (8 mm) days post-tillage, ahead of the sampling event on day 76 (Figure 2a).

Across soil inorganic N measurements, taken every one to two weeks for the 91-day sampling period, we found a significant effect of sample date ( $p < 0.0001$ ) and a significant effect of treatment ( $p < 0.0001$ ), but no interaction effect ( $p = 0.635$ ). Across all sampling points, mean soil inorganic N concentrations were higher in the clover ( $8.3 \pm 0.7$  mg N kg soil<sup>-1</sup>) and mixture ( $7.2 \pm 0.8$  mg N kg soil<sup>-1</sup>) treatments compared with the rye ( $5.1 \pm 0.6$  mg N kg soil<sup>-1</sup>) and fallow ( $4.6 \pm 0.6$  mg N kg soil<sup>-1</sup>) treatments (Figure 3).

## Cumulative N<sub>2</sub>O emissions

We found no significant differences in the mean cumulative N<sub>2</sub>O emissions (across the 91-day measurement period) among treatments ( $p = 0.688$ ) (Table 3). There was a wider range of cumulative emissions for the clover (min: 1088; max: 3057 g N<sub>2</sub>O-N ha<sup>-1</sup>) and mixture (min: 932; max: 3257 g N<sub>2</sub>O-N ha<sup>-1</sup>) treatments than for rye (min: 1688; max: 2198 g N<sub>2</sub>O-N ha<sup>-1</sup>) and weedy fallow (min: 1143; max: 1925 g N<sub>2</sub>O-N ha<sup>-1</sup>) treatments. Yield-scaled emission estimates (in grams of nitrous oxide per hectare per gram of grain per hectare) also showed no significant differences between treatments ( $p = 0.113$ ), but the mean of the mixture (904.8) was more than two times higher than the rye (492.7), clover (438.3), or fallow (303.6).

## N<sub>2</sub>O emissions for the full crop rotation

Given that N<sub>2</sub>O fluxes vary throughout the three-year crop rotation, it is important to compare the magnitude of the emissions during the corn growing season to N<sub>2</sub>O fluxes for a complete crop rotation. Based on the field-scale data in the biologically based cropping system in the MCSE at KBS where the cover crop experiment was conducted, N<sub>2</sub>O fluxes were highest during soybean years, followed by corn years, with wheat producing the lowest levels of N<sub>2</sub>O (Robertson, 2020b; Table 4). The mean cumulative annual N<sub>2</sub>O flux from 2015 to 2020 was  $2.0 \pm 0.4$  kg N<sub>2</sub>O-N ha<sup>-1</sup> year<sup>-1</sup>.

## Corn yield and quality

Corn yields in the experimental subplots were impacted by multiple factors likely related to their location on the edge of the whole plots in the long-term cropping systems study. In particular, lower quality soil, higher weed

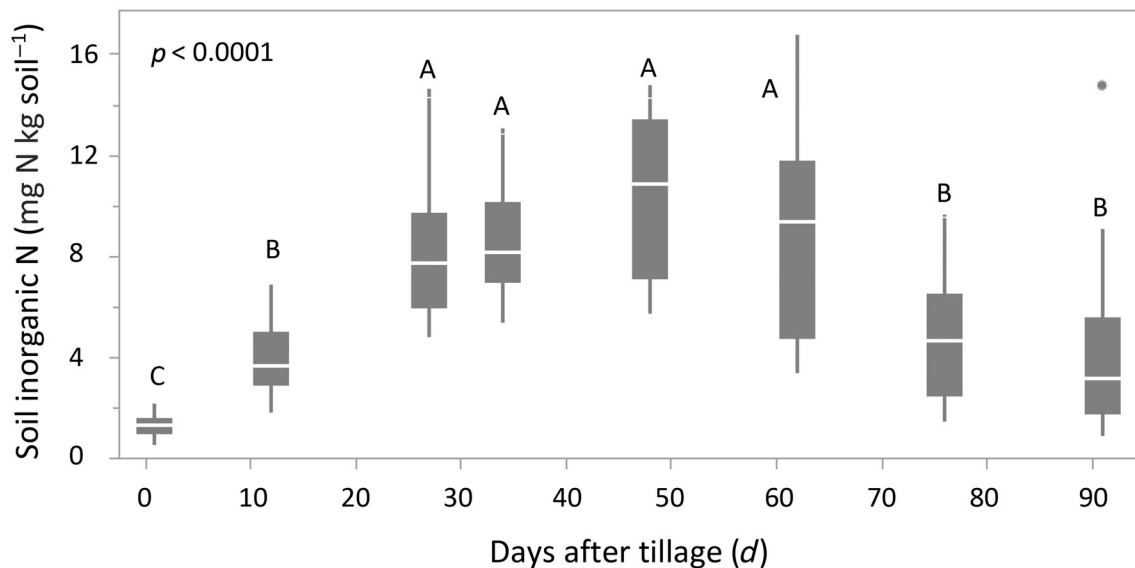
pressure, and poor contact between corn seed and soil (especially from the use of rototillage in this experiment) reduced corn establishment in the subplots and led to lower yields than the whole plot yields in the MCSE at KBS, which averaged 6712 kg ha<sup>-1</sup> (Plumhoff et al., 2022). Although corn yields were lower than expected, we found differences by cover crop treatment with significantly higher yields from the weedy fallow ( $5026 \pm 492$  kg ha<sup>-1</sup>) and clover ( $4304 \pm 222$  kg ha<sup>-1</sup>) treatments than the mixture treatment ( $2914 \pm 490$  kg ha<sup>-1</sup>), while rye ( $4057 \pm 274$  kg ha<sup>-1</sup>) was the same as the other treatments ( $p = 0.010$ ). Similarly, corn grain N was higher in the fallow ( $54.6 \pm 4.8$  kg N ha<sup>-1</sup>) and clover ( $56.4 \pm 2.7$  kg N ha<sup>-1</sup>) treatments than in the mixture ( $36.7 \pm 6.5$  kg N ha<sup>-1</sup>), while rye ( $45.4 \pm 3.0$  kg N ha<sup>-1</sup>) was the same as the other treatments ( $p = 0.033$ ). The C:N ratio of the corn grain was significantly higher in fallow ( $40.5 \pm 2.2$ ) than in clover ( $33.8 \pm 0.4$ ), while the mixture ( $35.2 \pm 1.0$ ) and rye ( $39.2 \pm 1.0$ ) did not differ from the other treatments ( $p = 0.016$ ). We found more variable C:N values across replicates in the fallow than in the other treatments. Corn grain %N was significantly higher following clover ( $1.31 \pm 0.02\%$ ) than rye ( $1.12 \pm 0.03\%$ ) or fallow ( $1.09 \pm 0.06\%$ ) ( $p = 0.009$ ). We found similar corn %N between the clover and mixture ( $1.25 \pm 0.03\%$ ); however, the mixture treatment did not differ significantly from rye and fallow treatments (Figure 4).

## Agroecosystem N balance

We constructed a partial N mass balance (i.e., focused on the largest N flows driven by management) for the previous six years to capture two complete cycles of the corn-soy-winter wheat rotation. From 2014 to 2019, we found a total N import from BNF and atmospheric deposition of  $313.4 \pm 9.1$  kg N ha<sup>-1</sup> and an N export from harvested crops of  $232.6 \pm 11.5$  kg N ha<sup>-1</sup> with a net balance of  $80.9 \pm 11.3$  kg N ha<sup>-1</sup>. The mean annual net N balance therefore had a small N surplus of  $13.5 \pm 1.9$  kg N ha<sup>-1</sup> year<sup>-1</sup>. To account for potential error in our assumption for red clover %Nd<sub>fa</sub>, we conducted a sensitivity analysis ranging between 50 and 80 %Nd<sub>fa</sub>. The low-end estimate of 50 %Nd<sub>fa</sub> changed the total net N import to  $260.3 \pm 7.0$  kg N ha<sup>-1</sup> over 6 years, resulting in a mean balance of  $4.6 \pm 1.7$  kg N ha<sup>-1</sup> year<sup>-1</sup>. When BNF was estimated at the high end of the range (80 %Nd<sub>fa</sub>), the mean balance was  $17.9 \pm 2.0$  kg N ha<sup>-1</sup> year<sup>-1</sup> (Table 5).

Historically at this site, across all three crops in rotation (corn-soy-wheat), an average of  $2.2$  kg N ha<sup>-1</sup> year<sup>-1</sup> was lost to N<sub>2</sub>O every year (Gelfand et al., 2016) and  $19.0 \pm 0.8$  kg N ha<sup>-1</sup> year<sup>-1</sup> was lost via leaching (Syswerda et al., 2012). When accounting for these potential losses





**FIGURE 3** Box plot of soil inorganic N ( $\text{NH}_4^+ + \text{NO}_3^-$ ) concentration throughout the study period. The following statistics are shown: median (white line); interquartile range (IQR) (gray box);  $1.5 \times \text{IQR}$  (gray lines); outlier (dot). Letters indicate dates that are significantly different from one another (using a Tukey's honestly significant difference test).

**TABLE 3** Cumulative  $\text{N}_2\text{O}$  by treatment.

| Treatment | Mean $\text{N}_2\text{O}$<br>(g $\text{N}_2\text{O-N ha}^{-1} \text{ year}^{-1}$ ) | CV    |
|-----------|--|-------|
| Rye       | 1962.9   | 10.52 |
| Clover    | 1926.4   | 44.08 |
| Mixture   | 2252.9   | 43.69 |
| Fallow    | 1481.1   | 22.01 |

Abbreviations: CV, coefficient of variation;  $\text{N}_2\text{O}$ , nitrous oxide.

**TABLE 4** Annual cumulative nitrous oxide ( $\text{N}_2\text{O}$ ) emissions in the biologically based cropping system.

| Year         | Crop  | Cumulative<br>$\text{N}_2\text{O}$ emissions<br>(kg $\text{N}_2\text{O-N ha}^{-1} \text{ year}^{-1}$ ) |
|--------------|-------|--|
| 2015         | Soy   | 2.11   |
| 2016         | Wheat | 1.60   |
| 2017         | Corn  | 1.22   |
| 2018         | Soy   | 3.45   |
| 2019         | Wheat | 0.63   |
| 2020         | Corn  | 2.86   |
| Mean<br>(SE) |       | 1.98<br>(0.38)   |

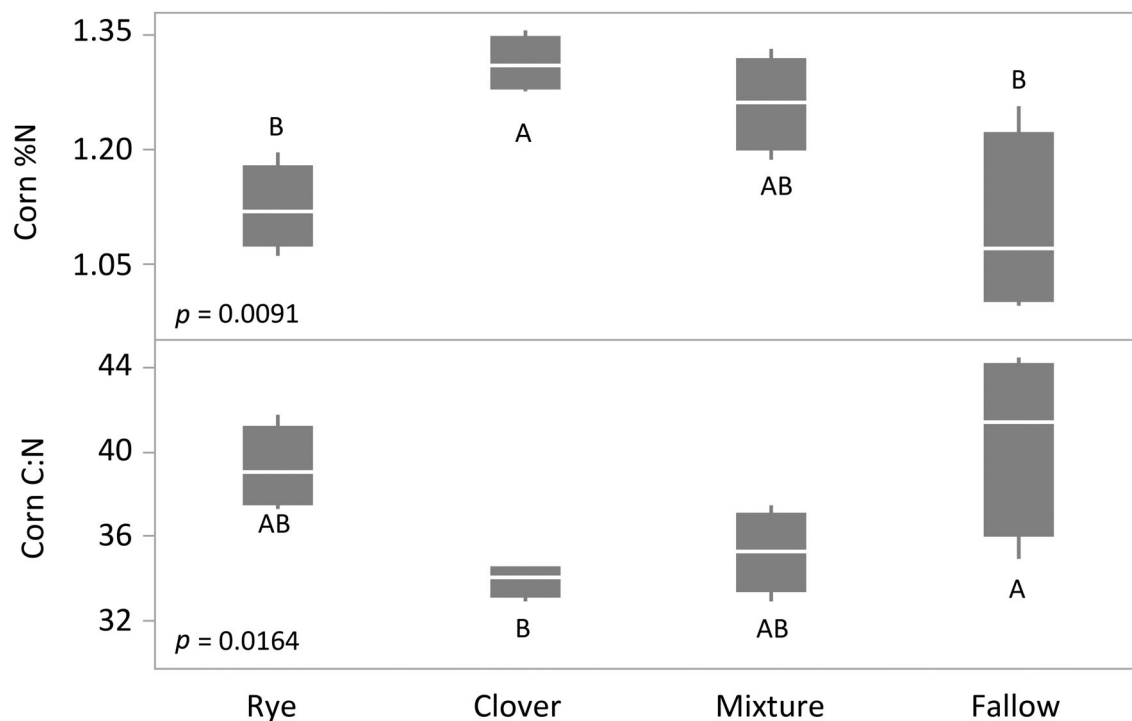
Note: Data from Robertson (2020b) spanning two full crop rotations.

(21.2 kg N  $\text{ha}^{-1} \text{ year}^{-1}$ ), the N balance across replicate plots of this cropping system ranged from negative (−12.8 kg N  $\text{ha}^{-1} \text{ year}^{-1}$ ) to neutral (0.7 kg N  $\text{ha}^{-1} \text{ year}^{-1}$ ). The mean annual N balance was therefore slightly negative

(−6.8 kg N  $\text{ha}^{-1} \text{ year}^{-1}$ ) when accounting for prior measurements of  $\text{N}_2\text{O}$  emissions and  $\text{NO}_3^-$  leaching from this cropping system.

## DISCUSSION

Increasing agroecosystem functional diversity with legume cover crops can improve the sustainability of soil nutrient management by building labile fractions of SOM and reducing N surplus (Blesh, 2019; Blesh & Drinkwater, 2013; Drinkwater & Snapp, 2007). To advance understanding of N cycling dynamics in legume-based cropping systems, we tested the hypothesis that planting a more functionally diverse legume–grass cover crop mixture would provide BNF inputs similar to the sole legume cover crop, while reducing  $\text{N}_2\text{O}$  emissions during the following growing season and increasing corn N assimilation compared with the sole grass cover crop. New N inputs from BNF did not differ between crimson clover and clover–rye mixture treatments, supporting our hypothesis that interspecific interactions in mixture could lead to a similar N supply while also increasing cover crop functional diversity. As expected, the partial N balance we calculated showed that red clover BNF inputs approximately balanced harvested N exports for the two most recent crop rotation cycles, although small N surpluses reflected the potential for N losses from this agroecosystem. Results from our experiment showed high short-term rates of  $\text{N}_2\text{O}$  flux following cover crop incorporation, with high spatial and temporal variability; however, the cover crop mixture



**FIGURE 4** Corn grain percent nitrogen and carbon to nitrogen ratio. The following statistics are shown: median (white line); interquartile range (IQR) (gray box);  $1.5 \times$  IQR (gray lines). Letters indicate categories that are significantly different from one another (using a Tukey's honestly significant difference test).

did not reduce  $\text{N}_2\text{O}$  emissions compared with the clover treatment or increase corn N assimilation compared with the rye treatment. To reduce these pulse  $\text{N}_2\text{O}$  emissions in cover-cropped, organic agroecosystems, additional management strategies to reduce tillage are needed.

### Managing legume N sources in an organic agroecosystem

Compared with soluble N fertilizers, managing agroecosystems with  $\text{N}_2$  fixing legumes can reduce N losses by improving synchrony between microbial mineralization of organic N and crop N uptake (Drinkwater & Snapp, 2007). However, the timing and rate of N mineralization from organic nutrient sources can be difficult to predict, creating uncertainty for farmers. Increasing the complexity of litter inputs with a cover crop mixture might increase the molecular diversity of organic compounds entering soil. This greater spatial and biochemical heterogeneity could slow initial decomposition rates by increasing the cost of metabolism while increasing C use efficiency of the microbial community (and SOM persistence) by promoting a microbial community with more diverse traits (Kallenbach et al., 2019; Lehmann et al., 2020).

However, in our study, the functionally diverse cover crop mixture had a similar aboveground biomass C:N

and total N as the clover grown alone, and these treatments also had similar soil inorganic N concentrations and  $\text{N}_2\text{O}$  emissions throughout the growing season. While the mixture treatment had a significantly lower corn grain N assimilation than clover, both treatments resulted in the same corn quality (i.e., grain N concentration measured as %N and C:N). These findings suggest that the clover-rye mixture did not alter cover crop residue composition in ways that would affect agroecosystem N cycling dynamics following its incorporation by tillage. Our experiment had strong clover establishment and growth, representing an average of 54.2% of the total mixture biomass, while rye only represented 27.7%. Planting the cover crops on 31 July may have resulted in lower rye establishment and competitiveness due to hot, dry conditions. With more clover biomass than rye in the mixture, C:N and total biomass N were similar between the mixture and clover treatments. It is therefore possible that in other contexts mixtures might have a stronger effect on these N cycling processes (e.g., based on mixture composition and cover crop traits at incorporation).

In addition, prior research focused on rates of N release from plant litter has neglected important plant-microbe-soil interactions that regulate overall soil N availability. A legume-grass cover crop mixture can occupy more niche space belowground through complementary root traits, which can support a more diverse

**TABLE 5** Partial nitrogen balance in the biologically based cropping system at Kellogg Biological Station.

| %Nd <sub>fa</sub> | N import<br>(kg N ha <sup>-1</sup> ) | N export<br>(kg N ha <sup>-1</sup> ) | Net N balance<br>(kg N ha <sup>-1</sup> ) | Annual balance<br>(kg N ha <sup>-1</sup> year <sup>-1</sup> ) |
|-------------------|--------------------------------------|--------------------------------------|---|---|
| 50                | 260.34<br>(6.98)                     | 232.55<br>(11.51)                    | 27.78<br>(10.02)                          | 4.63<br>(1.67)  |
| 70                | 313.41<br>(9.05)                     | 232.55<br>(11.51)                    | 80.86<br>(11.29)                          | 13.48<br>(1.88)   |
| 80                | 339.94<br>(10.11)                    | 232.55<br>(11.51)                    | 107.39<br>(12.02)                         | 17.90<br>(2.00)   |

Note: Estimated for two rotation cycles of corn–soy–wheat (2014–2019). Means and SE (in parentheses) for a sensitivity analysis for red clover biological N<sub>2</sub> fixation with a low-end estimate of 50 %Nd<sub>fa</sub>, our estimate from the literature of 70 %Nd<sub>fa</sub>, and a high-end estimate of 80 %Nd<sub>fa</sub>.

Abbreviations: %Nd<sub>fa</sub>, percent nitrogen derived from fixation; N, nitrogen.

microbial community compared with single species cover crops, potentially increasing soil N supply and cycling efficiency (Jilling et al., 2018; Kallenbach et al., 2019). Furthermore, cover crops also increase the presence of living roots in the soil compared with fallows, which provide more continuous labile C inputs that could expand both microbial and plant access to N from turnover of multiple SOM pools through rhizosphere priming (Jilling et al., 2018; Kallenbach et al., 2019).

Both the clover and mixture treatments increased soil inorganic N and N<sub>2</sub>O emissions during the first peak after tillage compared with rye and the weedy fallow. This suggests that the two treatments with legumes supported higher N mineralization and N availability during this brief period after a major soil disturbance. However, these treatment differences did not continue after the initial peak. The second peak in N<sub>2</sub>O emissions, 27 days after tillage, was the same across all treatments, including for rye and the weedy fallow. This suggests that the C and N supplied by organic matter pools that have accumulated through long-term cover crop use in this cropping system were a more important driver of N mineralization rates throughout the growing season rather than the short-term addition of the cover crop mixture.

Our study provides evidence that crimson clover in mixture with rye upregulated BNF, supporting our hypothesis that the mixture would supply BNF inputs comparable to clover grown alone, even at half the clover seeding rate. The mixture also had higher mean biomass (3458 kg ha<sup>-1</sup>) than the clover (2964 kg ha<sup>-1</sup>) for the two cover crop species we planted (clover and rye). Therefore, we found greater cover crop biomass production and a high proportion of clover biomass in mixture, similar residue chemistry between mixture and clover treatments, and higher %Nd<sub>fa</sub> of clover in mixture (63.3% vs. 43.4%). Taken together, these factors likely explain the similar N<sub>2</sub>O emissions between the clover and mixture treatments in this experiment. Additionally, baseline soil samples we analyzed for a companion study across all the annual cropping systems at KBS showed that multiple SOM pools in the organic

system were significantly larger than in the conventional management system, including particulate organic matter fractions as well as rates of potentially mineralizable N, both of which reflect soil N supplying capacity (Plumhoff et al., 2022). This long-term management history, which has led to relatively high N availability from decomposition of SOM, may in part explain why we found a low BNF rate in the sole crimson clover in our experiment, considering that clover usually has a %Nd<sub>fa</sub> closer to 70 (Blesh et al., 2019; Schipanski & Drinkwater, 2010).

When managing cover crops to reduce N losses, another important consideration is potential trade-offs between N losses and crop yield and N assimilation. We found that corn quality (%N) was similar between mixture and clover, and that the clover treatment had significantly higher corn %N than the rye and weedy fallow, suggesting that legume cover crops can improve grain N assimilation compared with grass cover crops, which recycle soil N but do not supply an external N source. The high proportion of clover in mixture likely resulted in sufficient new N inputs to maintain corn quality comparable to sole clover. Further, the mixture may hold promise for enhancing a broader suite of ecosystem functions (Blesh, 2017; Finney & Kaye, 2016) in the long term due to greater quantity and diversity of plant residue inputs to the soil (Lehmann et al., 2020).

## N<sub>2</sub>O emissions during the growing season following cover crop incorporation

Across all cover crop treatments, we found temporal trends in N<sub>2</sub>O emissions during the corn growing season that indicate asynchrony between N mineralization and corn N assimilation during the first month of the experiment, followed by tighter synchrony for the remaining two months of the experiment. Most emissions occurred during the first month after tillage when crop N demand was low, but N was being mineralized during cover crop decomposition, increasing the size of the soil inorganic N

pool and thus  $\text{NO}_3^-$  available for denitrification. The first  $\text{N}_2\text{O}$  peak occurred during a two-week period after the cover crop biomass was tilled into the soil and when corn had just been planted. The second peak occurred four weeks after tillage, following the first significant rainfall.

Soil inorganic N concentrations were significantly higher during the second  $\text{N}_2\text{O}$  peak, which produced 2–3 times higher emissions than the first peak. At 27 days post-tillage, this second  $\text{N}_2\text{O}$  peak occurred following a rewetting event (26 mm; 25–26 days after tillage). The first rainfall after a lengthy dry period, during which labile C and N pools accumulate, can lead to high rates of denitrification if nitrous oxide reductase (*nosZ*) enzyme activity is low (Robertson & Groffman, 2015). Even though the soil inorganic N concentration remained high for another 35 days, we did not see additional peaks in  $\text{N}_2\text{O}$  emissions following subsequent rewetting events 29 and 66 days after tillage. We hypothesize that lower  $\text{N}_2\text{O}$  emissions during this period were in part due to improved synchrony between N supply and corn growth and N assimilation, reducing soil N availability for denitrification following rain events.

In a global meta-analysis of grain cropping systems, Han et al. (2017) reported an average annual  $\text{N}_2\text{O}$  flux of 2.3–3.1 kg N ha<sup>-1</sup> year<sup>-1</sup> for cropping systems with inorganic fertilizer additions. At our study site in Michigan, Gelfand et al. (2016) reported mean  $\text{N}_2\text{O}$  emissions of 2.2 kg N ha<sup>-1</sup> year<sup>-1</sup> in a study spanning 1991–2011. Relative to the mean annual  $\text{N}_2\text{O}$  flux estimate at KBS of 1.98 kg N ha<sup>-1</sup> year<sup>-1</sup> from 2015 to 2020, encompassing two full crop rotations, the cumulative flux we measured post-tillage following the mixture was 14% higher (2.25 kg N ha<sup>-1</sup> year<sup>-1</sup>) while clover and rye produced 97%–99% of the emissions and weedy fallow produced 75% of expected annual emissions at this site. Although we may have missed some emissions later in the growing season when we sampled every two weeks, given that the emissions we measured were close to or slightly less than the annual average at this site, we believe we captured the majority of the  $\text{N}_2\text{O}$  flux by conducting frequent measurements following tillage and the first major rainfall. These ephemeral flux rates were high but not sustained over the three-month measurement period during the corn growing season.

## Interpreting the partial N balance with historical data

One benefit of conducting this experiment at a long-term cropping systems site is the ability to interpret the N cycling dynamics we measured from wheat harvest in July 2019, through corn harvest in October 2020, using data spanning

a longer period. We found a positive mean partial N mass balance of 13.5 kg N ha<sup>-1</sup> year<sup>-1</sup> (Table 5) when accounting for the primary N fluxes that are influenced by farm management practices (N inputs from BNF and N exports through harvested grains). Assuming that SOM stocks are close to steady state, partial N balances are a robust indicator of N that is vulnerable to environmental losses based on data that are relatively easy to collect (McLellan et al., 2018; Robertson & Vitousek, 2009; Zimnicki et al., 2020). The small N surplus we found here is slightly higher than the mean N balance reported in a previous study using this approach across multiple farms in the Midwest with legume N sources (i.e., 3.7 kg N ha<sup>-1</sup> year<sup>-1</sup>; Blesh & Drinkwater, 2013). This suggests that this agroecosystem has some potential for N losses, which is supported by the historical measurements of  $\text{N}_2\text{O}$  emissions and  $\text{NO}_3^-$  leaching reported for this site. These losses were lower than losses from the fertilizer-based cropping systems but higher than the treatments in successional communities (Gelfand et al., 2016; Syswerda et al., 2012). However, when including prior measured rates of  $\text{N}_2\text{O}$  flux and  $\text{NO}_3^-$  leaching (21.2 kg N ha<sup>-1</sup> year<sup>-1</sup> total) in our balance, it became negative (−6.8 kg N ha<sup>-1</sup> year<sup>-1</sup>). Furthermore, these estimates may still miss important fluxes that would make the balance even more negative, especially the loss pathway of total denitrification ( $\text{N}_2\text{O} + \text{N}_2$ ), which is important to consider but difficult to quantify.

There are several possible N sources that could account for the apparent missing N in our balance. First, it is possible that we underestimated red clover BNF inputs. When accounting for potential  $\text{N}_2\text{O}$  and  $\text{NO}_3^-$  leaching losses and applying the high-end BNF rate to red clover in our sensitivity analysis (80 %Ndfa), we found that the N balance across replicate plots ranged from slightly positive (4.3 kg N ha<sup>-1</sup> year<sup>-1</sup>) to negative (−9.84 kg N ha<sup>-1</sup> year<sup>-1</sup>), with a mean of −3.3 kg N ha<sup>-1</sup> year<sup>-1</sup>, which is approximately in balance. It is also possible that there is associative  $\text{N}_2$  fixation that is an unaccounted-for N source (Smercina et al., 2019), or that we underestimated below-ground biomass N inputs from the clover cover crop. Alternatively, low soil phosphorus (P) levels (i.e., a mean Bray-1 P concentration of  $9.31 \pm 1.85$  mg P kg<sup>-1</sup>; Bressler & Blesh, 2022) may limit BNF rates in this cropping system (Sulieman & Tran, 2017; Vitousek et al., 2013), which could also help explain the low %Ndfa we measured for sole crimson clover in our experiment and suggests that it is more likely that our partial N mass balance overestimated red clover  $\text{N}_2$  fixation. There is a need for more frequent measures of red clover BNF in this agroecosystem to understand whether rates are changing over time.

Second, it is possible that  $\text{NO}_3^-$  leaching losses from this cropping system have declined over time. Since Syswerda et al.'s (2012) leaching study ended in 2006, continued



organic management of the site using cover crops may have improved soil quality enough to increase soil N retention (Plumhoff et al., 2022). An analysis of deep soil cores (0–100 cm) collected from this organic management system in 2013 demonstrated gains of  $500 \pm 100 \text{ kg C ha}^{-1} \text{ year}^{-1}$  to the SOC stock and gains of  $39 \pm 8 \text{ kg N ha}^{-1} \text{ year}^{-1}$  to the SON stock over a 25-year period (Córdova et al., In Review). These findings show that more than two decades of organic management, which increased crop rotation functional diversity with legume N sources, cover crops, and winter wheat, increased SOC and SON stocks in both surface and deep soils. These findings suggest that SOM is not in a steady state, but that soil N is still accumulating, and N losses may therefore be smaller than past measurements. It is therefore also possible that increasing soil N stocks account for some of the “missing” N in the partial N balance.

## Management implications

Although legume cover crops can reduce N losses compared with inorganic N fertilizers, they can still produce higher  $\text{N}_2\text{O}$  emissions than other cover crop functional types due to BNF inputs and N-rich residues that can decompose quickly. We hypothesized that a legume–grass mixture would better couple C and N cycling processes to further tighten N cycling and reduce  $\text{N}_2\text{O}$  emissions compared with a sole legume. Overall, we did not find significant differences in cumulative  $\text{N}_2\text{O}$  emissions over the corn growing season following any of the treatments. After a long history of ecological nutrient management at this site, a six-year, partial N mass balance indicated that N inputs from BNF approximately balance N removal in harvested crops, with a growing SOM pool over time. The restoration of SOM pools over 30 years in this organic agroecosystem was likely a more important driver of N cycling dynamics overall compared with increasing the diversity of the overwintering cover crop in a one-year study. While overall emissions were not affected by cover crop functional type, short-term emissions following cover crop incorporation were higher in both treatments with legumes—which had similar aboveground biomass N, BNF, and soil inorganic N concentrations—compared with the rye and weedy fallow treatments. This suggests that additional management strategies are needed for continued gains in N cycling efficiency, particularly those that reduce soil disturbance through tillage. Furthermore, with longer term use, cover crop mixtures of complementary functional types have the potential to enhance multiple agroecosystem benefits such as reducing weed and pest pressure, increasing pollinator habitat, reducing nutrient leaching, and maintaining and building SOM.

## AUTHOR CONTRIBUTIONS

The authors contributed equally to this manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

## DATA AVAILABILITY STATEMENT

Data (Bressler & Blesh, 2023) are available from University of Michigan Deep Blue Data: <https://doi.org/10.7302/zqyt-gz14>.

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