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8   **Running head:** Soil organic matter and N_2 fixation

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10   Feedbacks between nitrogen fixation and soil organic matter increase ecosystem functions in

11   diversified agroecosystems

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Abstract

Nitrogen (N) losses from intensified agriculture are a major cause of global change, due to nitrate

(NO_3^-) export and the eutrophication of aquatic systems as well as emissions of nitrous oxide (N_2O)

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 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 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 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 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

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

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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 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 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 during periods when soil would otherwise be bare. For example, in a meta-analysis, Tonitto et al. (2006) showed that legume cover crops reduced NO_3^- leaching by 40%, compared to the absence of a cover crop. A long-term (11 yrs) experiment in Michigan revealed that 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 N_2O emissions compared to bare fallows (Han et al. 2017). Legume cover crops also increase the spatial diversity of agroecosystems when planted in multi-species mixtures. When legumes are combined with grass species, which are even stronger N scavengers, they can further reduce NO_3^- leaching (Kaye et al. 2019) and N_2O emissions (Basche et al. 2014). Overall, cover crops are considered central to an ecological approach to agroecosystem management.

Legume cover crops can sequester atmospheric 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

92   C inputs to soil compared to lower diversity rotations such as continuous grains, yet the diversified

93   systems still accumulate more SOM over time (Drinkwater et al. 1998, Grandy and Robertson

94   2007). Emerging understandings of soil C stabilization suggest a potential mechanism for these

95   findings (Cotrufo et al. 2013). Legume residues have low C:N ratios and are easily decomposed by

96   microbial communities. As a result, the fraction of legume cover crop residue that becomes SOM

97   may be higher if it increases the relative production of microbial byproducts and necromass – key

98   components of stable SOM pools – for a given C input to soil (Schmidt et al. 2011, Cotrufo et al.

99   2013). Cover crops also increase the proportion of the year with plant root-microbe interactions in

100   the rhizosphere, and associated release of root exudates, extracellular enzyme production, and

101   presence of aggregate binding agents, which have previously been linked to accumulation of SOM

102   (Tiemann et al. 2015, Lehmann et al. 2017) and to soil nutrient cycling capacity (Clarholm 1985,

103   Drinkwater and Snapp 2007, Kuzyakov and Blagodatskaya 2015). In particular, legume cover crops

104   can increase fractions of SOM that turnover more quickly than the total SOM pool, especially

105   particulate organic matter (POM). POM pools respond to changes in management on year to

106   decadal scales, correlate with soil N mineralization rates, and are early indicators of longer-term

107   accumulation of SOM (Cambardella and Elliott 1992, Wander 2004, Luce et al. 2016). Legume

108   cover crops can therefore enhance multiple ecosystem functions.

109   Despite these benefits, farmers continue to rely on inorganic fertilizers partly because they

110   have more predictable outcomes for crop production. Nitrogen inputs from legume cover crops

111   depend on ecological processes and are inherently more variable. There is a need, then, to increase

112   predictive understanding of legume N_2 fixation inputs from cover crops. In natural ecosystems, key

113   ecological controls on N_2 fixation include high levels of soil inorganic N, low availability of non-N

114   nutrients that may limit legume growth or fixation (e.g., P, Fe, K, Mo), preferential grazing of

115   legumes by herbivores, and shade intolerance of legumes compared to other plant functional groups

116   (Vitousek et al. 2013). Biotic interactions can also influence these mechanisms. For example, the

117   percentage of legume N from the atmosphere is mediated by interactions with grasses. Grasses are

118   strong competitors for soil N, reducing soil inorganic N pools and causing the legume to fix more of

119   its N (Li et al. 2009). However, grasses may also compete with legumes for soil nutrients such as P,

120   or resources like light, reducing legume biomass production (Cech et al. 2010, Poffenbarger et al.

121   2015). In agroecosystems, which typically have lower diversity than natural ecosystems, many of

122   these constraints are alleviated through management. However, nutrients such as P or Fe can limit

123   legume biomass production, and it is well known that inorganic N fertilizers inhibit legume N_2

124 fixation because of the greater C cost for acquiring N from symbiotic N_2 fixation compared to

125 assimilating soil inorganic N (Schipanski et al. 2010, Gelfand and Robertson 2015).

126   In diversified agroecosystems, SOM pools including POM are central to soil nutrient cycling

127 dynamics, yet most research on N_2 fixation in agriculture has focused on inorganic N supplied by

128 synthetic fertilizer, while the role of N mineralized from decomposition of SOM remains poorly

129 understood (Schipanski and Drinkwater 2011). Diversified agroecosystems that rely on legumes and

130 other organic N sources have different N cycling dynamics from fields with synthetic fertilizers,

131 which experience episodic inputs of inorganic N that saturate the root zone. Organic and diversified

132 farms have larger N inputs than natural ecosystems, to balance the N removed in harvested crops,

133 but inorganic N is released more gradually in the rhizosphere through microbial turnover of SOM.

134 Further, N cycling rates differ among farms with SOM of differential quantity and quality. For

135 instance, agroecosystems with a long organic management history may have low standing pools of

136 inorganic N because of tight coupling between plant and microbial productivity (Paterson 2003),

137 but high mineralization rates due to accumulation of SOM and enhanced nutrient cycling capacity

138 (Berthrong et al. 2013). Given the potential for legumes to build POM pools, and the critical role

139 that SOM pools such as POM play in nutrient cycling, relationships between POM, N

140 mineralization, and legume N_2 fixation are important to understand.

141   The goal of this study was to identify ecosystem-scale controls on legume N_2 fixation in the

142 context of diversified agroecosystems. Determining patterns in N_2 fixation across variable soil

143 conditions could inform management of legume N sources, and increase understanding of feedback

144 mechanisms that underpin the efficiency of legumes demonstrated by long-term studies. In a field

145 experiment on 10 farms spanning a gradient of soil fertility levels due to distinct management

146 legacies, I separated soil samples into two SOM fractions, based on size (> 53 μm) and density,

147 which are indicators of internal nutrient cycling and N availability (light or "free" POM; and POM

148 that is physically-protected inside microaggregates) (Marriott and Wander 2006b). Here, I used

149 these two POM fractions to assess relationships between N availability from turnover of SOM and

150 legume N_2 fixation (measured using stable isotope methods) across a gradient of SOM levels,

151 holding management constant. I hypothesized that legume N_2 fixation would decrease with

152 increasing levels of N in POM, due to enhanced microbial processing of SOM and increased N

153 mineralization with larger POM pools (Fig. 1). Furthermore, I hypothesized that intra-aggregate

154 POM should account for more variation in this relationship relative to light fraction POM because it

155 turns over more slowly and is considered a better indicator of longer-term changes in soil N cycling

156 capacity due to management (Wander et al. 1994, Schipanski et al. 2010). Finally, I expected

157 interspecific competition in a legume-grass mixture to reduce the effect of POM N on N_2 fixation,

158 due to soil N assimilation by the fibrous root system of the intercropped grass (Fig. 1).

159

160 **Materials and methods**

161 *Experimental design*

162   I partnered with 10 vegetable farmers in southeastern Michigan to establish an on-farm

163 experiment identifying patterns in N_2 fixation by a legume cover crop, hairy vetch (*Vicia villosa* L.),

164 grown alone and in mixture with a grass cover crop, cereal rye (*Secale cereale* L.), across a soil

165 gradient. The farms had been in organic vegetable production from three to 14 years at the start of

166 the experiment, and analysis of baseline soil properties from each experimental field reflected a soil

167 fertility gradient, which resulted primarily from distinct management histories (Table 1, Table 2).

168 Nine of the 10 fields were Alfisols, and one was a Mollisol. Six of the 10 fields had previous

169 compost inputs from poultry litter or other sources of composted manure. Across farms, total

170 organic C varied from 33.1 to 64.1 Mg ha^{-1}, plant available phosphorus (P) was 8 times greater on

171 the highest P site (112 mg kg^{-1}) than on the lowest (14.3 mg kg^{-1}). The size and N concentration of

172 POM pools also ranged widely, particularly for the protected POM pool, indicating that there were

173 differences in the quality of SOM on the farms. Soil inorganic N pools (NO_3^--N + NH_4^+-N) ranged

174 from 2.6-14.4 mg kg^{-1}.

175   Management details for the experiment, planted in one field per farm, were determined

176 collaboratively with farmers. In summer 2015, after analysis of baseline soil samples, farmers

177 planted the field to a crop in the *Amaryllidaceae* family—either onion or garlic. The *Allium* crop

178 was fertilized with compost inputs on four of the farms, and the remaining farms applied no fertility

179 inputs for this crop. No additional fertility amendments were added to fields for the remainder of the

180 experiment, except for the N inputs from legume N_2 fixation. Following crop harvest, fields were

181 divided into four replicate blocks to establish the experimental treatments for the cover crop along

182 with a no-cover crop control. The fields ranged in size from 0.05 to 1.4 ha, but the measurements

183 were constrained to a 130 m^2 area to minimize variability in field area across farms. Farmers

184 planted the fields in a mixture of cereal rye (seeding rate of 56 kg ha^{-1}) and hairy vetch (seeding rate

185 of 25 kg ha^{-1}) between August 21- August 28, 2015. Vetch seeds were inoculated with N-Dure

186 (Verdesian Life Sciences, Cary, NC) at approximately 4 g kg^{-1} seed. Seeds were surface broadcast

187 and lightly incorporated. The cover crop overwintered, and by mid-May, 2016, farmers mowed and

188 then rototilled the field to incorporate the cover crop, and planted a primary crop in the

189 *Cucurbitaceae* family between May 27 - June 4, 2016. Following crop harvest, the cover crop

190 mixture was planted for a second season between August 30 - September 14, 2016 using the same

191 method as in year one. The cover crop was terminated by mowing and rototillage in May, 2017,

192 following final sampling.

193

194 *Baseline soil sampling*

195 A composite, baseline soil sample from each experimental field was analyzed to determine

196 initial soil conditions and characterize soil fertility status in spring of 2014 or fall 2015 on all

197 participating farms (Table 1, Table 2). Approximately 15-20 soil cores (2 cm diameter by 20 cm

198 depth) were composited per site. I estimated bulk density from the fresh weight of 8 cores per field,

199 which was later adjusted for soil moisture. Soil was processed immediately for soil moisture,

200 determined gravimetrically by drying at 105 °C for 48 hours, and triplicate soil subsamples were

201 sieved to 2 mm for analysis of extractable inorganic N (NO_3^- and NH_4^+) with 2 M KCl. The amount

202 of NH_4^+ and NO_3^- in each sample was analyzed colorimetrically on a discrete analyzer (AQ2, Seal

203 Analytical, Mequon, WI). Triplicate soil subsamples were also sieved to 2 mm for a 7-day

204 anaerobic N mineralization incubation, an indicator of potential N availability from decomposition

205 of organic matter, followed by extraction with 2 M KCl and colorimetric analysis of NH_4^+.

206 Potentially mineralizable N (N-min) was calculated by subtracting the initial amount of NH_4^+ in the

207 soil from the NH_4^+ released during the 7-day incubation (Drinkwater et al. 1996).

208 The POM > 53 μm was isolated from triplicate 40 g subsamples of unsieved, air-dried soil

209 using a combined size and density fractionation method. To isolate the light fraction POM (also

210 called free POM), the subsamples were first gently shaken for 1 h in sodium polytungstate (1.7 g

211 cm^{-3}), allowed to settle for 16 h, and free POM floating on top of the solution was removed by

212 aspiration. To isolate intra-aggregate POM (i.e., physically-protected POM), the remaining soil

213 sample was shaken with 10% sodium hexametaphosphate to disperse soil aggregates and then

214 rinsed through a 53 μm filter (Marriott and Wander 2006b). The material larger than 53 μm was

215 retained, and the protected POM was separated from sand by decanting. The C and N of both POM

216 fractions were measured on an ECS 4010 CHNSO Analyzer (Costech Analytical Technologies,

217 Inc.; Valencia, CA). Total soil C and N (to 20 cm) were measured by dry combustion on a Leco

218 TruMac CN Analyzer (Leco Corporation, St. Joseph, MI). Subsamples of ~100g of sieved dried soil

219 were analyzed for particle size, pH, Bray-1 P, and K at the A & L Great Lakes Laboratories, Inc.

220 (Fort Wayne, IN). As an indicator of microbial activity and SOM quality, C mineralization (C-min)

221 was determined by a short-term aerobic incubation of rewetted soil (i.e., the flush of CO_2 during a

222 1-day incubation), using a method modified from Franzluebbers et al. (2000) (Appendix S1).

223

224 *Legume N_2 fixation*

225 During the cover crop seasons, I established three small plots of 1 m^2 within each replicate

226 block in each field (i.e., 12 small plots per farm) to estimate biological N_2 fixation by the vetch

227 grown in mixture and monoculture based on ^{15}N natural abundance (Shearer and Kohl 1986). Soon

228 after establishment of the cover crop mixture (*e.g.,* in late September), all vetch plants were hand-

229 weeded out of one set of small plots to establish a sole rye treatment, and all rye plants were hand-

230 weeded out of another set of plots for sole vetch stands. A third small plot was left in the mixture,

231 and the background field was also planted to the vetch-rye mixture, except for the no cover crop

232 control.

233 Aboveground biomass in vetch and vetch-rye mixture treatments was sampled from the

234 small plots between 25 April - 6 May 2016, and between 9 - 18 May 2017, from a 0.25 m^2 section

235 of each small plot, avoiding plot edges. Plant biomass was cut at the soil surface, separated by

236 species, and dried at 60 °C for 48 h. Dried shoot biomass was first ground in a Wiley Mill to 2mm,

237 and was then pulverized using a cyclone mill and analyzed on a continuous flow Isotope Ratio Mass

238 Spectrometer (Stable Isotope Facility, UC Davis) for total C and N content, and for $\delta^{15}N$. The %N

239 derived from fixation was calculated using the following model:

240

241 $$\%N \text{ from fixation} = 100*((\delta^{15}N_{ref} - \delta^{15}N_{legume}) / (\delta^{15}N_{ref} - B))$$

242

243 where $\delta^{15}N_{ref}$ is the $\delta^{15}N$ signature of the reference plant (cereal rye), $\delta^{15}N_{legume}$ is the $\delta^{15}N$ signature

244 of the legume, and B is defined as the $\delta^{15}N$ signature of a legume when dependent solely on

245 atmospheric N_2. B values were determined by growing vetch in the greenhouse in a N-free medium

246 (Appendix S1). The B value for vetch was -0.44. Total N input from vetch N_2 fixation (kg N ha^{-1})

247 was calculated for mixture and monoculture treatments using aboveground biomass, % N, and %N

248 from fixation. The ^{15}N natural abundance method requires sufficient enrichment of the plant-

249 available soil N pool to accurately estimate the % of legume N derived from fixation (i.e., from the

250 atmosphere). There was significant separation in $\delta^{15}N$ values between the legume (vetch) and the

251 reference plant (sole cereal rye) in mixture and monoculture treatments (Appendix S1: Fig. S1). For

252 example, in 2016 the separation in mixture ranged from about 2 - 6 ‰ across sites.

253   I estimated the quantity of fixed N transferred from hairy vetch to cereal rye in the vetch-rye

254 mixture by comparing the $\delta^{15}N$ of rye in mixture with the $\delta^{15}N$ of rye in monoculture plots:

255   % rye N from transfer = $100[(\delta^{15}N_{rye\ monoculture} - \delta^{15}N_{rye\ mix})/(\delta^{15}N_{rye\ monoculture} - C)]$

256 where C is the $\delta^{15}N$ value of the integrated fractionation of $\delta^{15}N$ during N_2 fixation in legume roots

257 and between the roots and sampled shoots of the rye. C is typically estimated as the lowest $\delta^{15}N$

258 value of grass grown in mixture, or 0, whichever is lower. Here, C was set to 0 because all $\delta^{15}N$

259 values were positive for cereal rye. Transfer of fixed N from vetch to rye can occur through

260 multiple mechanisms: (i) turnover of vetch root tissue and assimilation of mineralized N by the

261 neighboring rye; (ii) exudation of soluble N compounds by the vetch and uptake by the rye; and (iii)

262 transfer of N through mycorrhizal associations. The natural abundance method has limitations for

263 quantifying N transfer between legumes and non-legumes because of the assumption that the

264 transferred N has a similar $\delta^{15}N$ value to that measured in the shoots of the legume, which may not

265 be valid (Peoples et al. 2015). However, this approach provides a relative estimate of fixed N

266 assimilated by rye in the mixture treatment across sites.

267

268 *Final soil analysis*

269   Between 9 May - 18 May 2017, immediately before terminating the cover crop and the

270 experiment, a final composite soil sample (8-10 cores) was collected from each replicate block on

271 each farm in the cover crop mixture and no cover crop control. The soil samples were analyzed for

272 extractable inorganic N pools, N-min and C-min, total C and N, Bray-1 P, and free and protected

273 POM fractions as described for the baseline soil sampling. To determine changes in soil fertility and

274 SOM pools with the cover crop mixture across farms, an effect size was calculated by subtracting

275 the final value for each soil parameter measured in the mixture treatment from the final value

276 measured in the no cover crop control at the May 2017 sampling.

277

278 *Statistical analysis*

279   Statistical analyses were computed in R (The R Foundation for Statistical Consulting,

280 Vienna, Austria). To test for differences in vetch N_2 fixation between mixture and monoculture,

281 one-way ANOVA analyses were performed for each experimental year using the *lme4* package for

282 linear, mixed-effect models with treatment as a fixed effect, and block nested in farm as a random

283 effect. To test the hypothesis that increasing POM N concentrations would decrease vetch N_2

284 fixation, I used linear regression to model vetch N_2 fixation in mixture and monoculture in 2016 as

285 a function of SOM pools and other properties expected to control legume N_2 fixation (e.g., soil P,

286 textural properties), excluding two extreme outliers for vetch N_2 fixation. Model comparisons were

287 made by assessing goodness of fit with the Akaike Information Criterion (AIC). After 2016, one of

288 the participating farmers decided to leave farming, so there were 10 farms in year one (2016) and

289 nine farms in year two (2017). Results are reported as statistically significant at $\alpha = 0.05$.

290

291 **Results**

292 *Biological N_2 fixation in mixture and monoculture*

293 Using stable isotope methods, the mean percentage of vetch N from fixation across all farms

294 was high in both cover crop seasons, ranging from 76 – 92%, and was significantly higher in

295 mixture than in monoculture (Fig. 2). In 2017, the mean % of vetch N from fixation was higher than

296 in 2016, and in 2017 vetch produced approximately half as much biomass compared to the 2016

297 vetch cover crop. The difference in vetch biomass production between years was reflected in mean

298 total aboveground N accumulation in the treatments (Fig. 2), which was 103 and 109 kg ha^{-1} in

299 vetch mixture and monoculture in 2016, respectively, but 69 and 56 kg ha^{-1} in mixture and

300 monoculture in 2017. In 2016, total mean N assimilation by rye in mixture was 51.5 kg N ha^{-1}

301 (range of 16.6 – 111.7 kg N ha^{-1} across sites), with an estimated 4.6 kg ha^{-1} of that N derived from

302 vetch N_2 fixation, on average. In 2017, the rye grown in mixture assimilated 39.9 kg N ha^{-1}, on

303 average, with an estimated 7.6 kg ha^{-1} of the N transferred from vetch. Overall, total N

304 accumulation between the mixture and monoculture treatments was not significantly different in

305 either year; however, total N supply from N_2 fixation was significantly greater in the sole legume

306 stand due to greater legume biomass than in the mixed stand.

307 Across farms, aboveground vetch N_2 fixation in 2016 ranged from 1.4 to 177 kg N ha^{-1} in

308 mixture and from 16 to 232 kg N ha^{-1} in monoculture, and was highly correlated with vetch

309 aboveground biomass ($R^2 = 0.94$ in mixture, and 0.74 in monoculture). In 2017, vetch N_2 fixation in

310 mixture varied from 1.5 to 78 kg N ha^{-1}, and from 3 to 126 kg N ha^{-1} in monoculture, across

311 individual farms. The relationships between legume shoot biomass and total fixed N in shoots were

312 similarly strong in 2017 ($R^2 = 0.92$ in mixture and 0.86 in monoculture).

313

314 *Controls on N_2 fixation across the soil gradient*

315   Linear regression models using the baseline soil properties as predictors, and vetch N_2

316 fixation in 2016 as the dependent variable, showed that two of the soil properties were significant

317 predictors of the amount of shoot N fixed (in kg N ha^{-1}) by vetch (Appendix S1: Table S1). N

318 concentration in the free POM pool was not a significant predictor of N_2 fixation ($P = 0.294$ for sole

319 vetch; $P = 0.421$ for vetch in mixture), however, I found a significant and negative correlation

320 between the N concentration in the protected POM pool and vetch N_2 fixation in mixture with a

321 regression coefficient of -44.16 ($P = 0.002$), and also in monoculture (-41.39, $P = 0.026$). The

322 amount of shoot N fixed by vetch was positively correlated with plant available P concentration

323 across the farm sites ($P = 0.006$ in mixture, and $P < 0.001$ in monoculture). Together, these soil

324 properties explained 42% (sole vetch) and 47% (vetch mixture) of the variation in vetch N_2 fixation.

325 For vetch in mixture, the best fit model had soil P, and the C:N ratio of the protected POM pool

326 (9.32, $P < 0.001$) rather than POM N concentration. This model explained 54% of the variation in

327 vetch N_2 fixation in mixture; however, the C:N of the protected POM was only a marginally

328 significant predictor of vetch N_2 fixation in monoculture ($P = 0.05$).

329   There was also a significant, negative relationship between the N concentration of the

330 protected POM pool and the % of vetch shoot N derived from fixation (Fig. 3). The slope of this

331 relationship was steeper for sole vetch than for vetch grown in mixture (Fig. 3). For sole vetch, the

332 % N from fixation also exhibited a significant and negative relationship with N-min, although it was

333 weaker than the relationship with POM (Appendix S1: Fig. S2; $R^2 = 0.11$; $P = 0.04$), and there was

334 no relationship between N-min and % N from fixation for vetch in mixture ($P = 0.617$). In contrast

335 to the positive relationships between soil P and the amount of fixed N in vetch shoots in mixture

336 and monoculture (i.e., driven by biomass production), there was no relationship between soil P and

337 the % of vetch aboveground N derived from fixation (Appendix S1: Fig. S3) for either mixture ($P =$

338 0.793) or monoculture treatments ($P = 0.612$)

339

340 *Changes in labile organic matter pools with a legume-grass mixture*

341   Finally, I asked how two seasons of the overwintering vetch-rye cover crop mixture

342 influenced the indicators of soil fertility measured at baseline (Fig. 4). There was a significant

343 increase in mean C and N mineralization rates compared to the no cover crop control across farms.

344 The mean size of both POM pools (free and protected POM) also increased, as did the N

345 concentration of the protected POM pool (shown as a significant decrease in C:N ratio). At the final

346 soil sampling across farm sites, there was a strong positive relationship between protected POM N

347 and N-min (Fig. 5), where POM N accounted for half of the variation in potentially mineralizable

348 N. There were also weaker, but significantly positive relationships between both POM pools (in g

349 kg dry soil^{-1}) and N-min (free POM, R^2=0.33; P<0.0001; and protected POM, $R^2 = 0.16$, $P =$

350 0.007). Mean soil NH_4^+ and NO_3^- concentrations, and plant available P concentration, did not

351 change with the cover crop.

352

353 **Discussion**

354   Strategic management of plant diversity in agroecosystems for increased ecosystem

355 functioning (Storkey et al. 2015, Finney and Kaye 2017, Blesh 2018) could help alleviate the

356 widespread environmental costs of intensified agriculture. Use of legume cover crops can build soil

357 organic C and N (Drinkwater et al. 1998, King and Blesh 2018), thereby supplying N to crops while

358 also sequestering atmospheric CO_2, and reducing NO_3^- leaching to aquatic systems and N_2O

359 emissions. Given that legume N_2 fixation varies with soil properties and interspecific interactions,

360 predictive understanding of this variation is needed to inform managing legumes as a N source for

361 crop production. Physiological controls on N_2 fixation have been fairly well characterized, but

362 ecological controls (i.e., controls influenced by interactions between legumes and other organisms,

363 or between legumes and the environment) are less well understood (Vitousek et al. 2013),

364 particularly in diversified agroecosystems. For example, numerous greenhouse and field

365 experiments have shown that increasing inorganic N reduced nodulation and legume N_2 fixation

366 (e.g., Van Kessel and Hartley 2000, Goss et al. 2002, Salvagiotti et al. 2008). However, there

367 remains a lack of studies across gradients of SOM levels (cf. Schipanski et al. 2010, Blesh 2018)—a

368 critical gap because diversified agroecosystems do not have pulse additions of inorganic N but

369 instead have more gradual release of N through decomposition of SOM (Schipanski and Drinkwater

370 2011). This study shows a strong stabilizing feedback between N in intra-aggregate SOM (protected

371 POM) and N_2 fixation, due to increased N mineralization from enhanced microbial activity fostered

372 by greater organic matter inputs to soil. These results increase understanding of ecosystem-scale

373 rates and regulation of N_2 fixation across working agroecosystems.

374   As predicted, with increasing N concentrations in protected POM across the gradient, I

375 found a significant and negative relationship with both the % of vetch N from fixation, and total

376 fixed N in shoots. There was also a significantly positive relationship between POM N

377 concentration and N-min, and a stabilizing feedback between N-min and % N from fixation for

378 vetch in monoculture, supporting the hypothesis that POM increases N mineralization from

379 microbial processing of SOM. These results highlight a potential mechanism by which legume N

380 sources in diversified agroecosystems better reduce surplus N and N losses (Drinkwater et al. 1998,

381 Blesh and Drinkwater 2013), by responding to changes in SOM following transitions from

382 conventional to diversified management (Fig. 1). Protected POM N moderated vetch N_2 fixation in

383 sole and mixed stands, while light fraction POM was not a significant predictor of BNF. This was

384 somewhat surprising because both POM pools are considered indicators of soil fertility. A related

385 experiment on a subset of the same farms showed that the free POM pool was negatively related to

386 legume biomass within different cover crop mixtures, in addition to finding a negative relationship

387 between protected POM N and both legume biomass and shoot N fixed (Blesh 2018). However,

388 results from this study supported the hypothesis that the feedback with protected POM N would be

389 stronger. Free POM turns over relatively quickly and reflects recent C inputs to soil (Wander 2004).

390 Consequently, intra-aggregate POM may be a more robust indicator of soil nutrient cycling capacity

391 because it turns over more slowly and can better reflect longer-term changes in SOM due to

392 management (Marriott and Wander 2006a, von Lützow et al. 2006). Schipanski et al. (2010)

393 measured soybean N_2 fixation across a soil gradient, and found weak evidence that the % of

394 soybean N from fixation was regulated by soil N availability from POM because soil N availability

395 was secondary to the effect of soil texture on N_2 fixation. In their study, differences in soil texture

396 were confounded with management history and the resulting fertility gradient. In this study,

397 although textural properties varied across farms (e.g., from 11-32% clay), there were no significant

398 relationships between % clay and % N from fixation. Furthermore, across farms in this study, there

399 were no significant relationships between sand or clay content and the measured POM pools,

400 emphasizing the role of management in driving the amount of POM in soils (Marriott and Wander

401 2006b).

402   The hypothesis that the relationship between POM N and N_2 fixation would be influenced

403 by biotic interactions was also supported. The slope of the relationship between POM N and the %

404 of vetch N from fixation was steeper for the sole vetch treatment. Furthermore, the regression

405 relationships between N-min and % N from fixation were significant only for sole vetch and not for

406 vetch in mixture (Appendix S1: Fig. S2). This suggests that the effect of N availability from

407 turnover of POM was moderated by growing vetch in mixture with rye (Fig. 3). A previous study

408 testing different cover crop mixture treatments on eight of these farms also found negative

409 correlations between legume biomass production in mixtures (for winter pea and crimson clover)

410 and the protected POM N pool; however, in that experiment soil properties did not predict the % of

411　N from fixation (i.e., the N_2 fixation rate) for any species (Blesh 2018). Another study focused on

412　intercropped red clover also found no relationship between labile soil N pools and the % N from

413　fixation (Schipanski and Drinkwater 2011). The lack of relationships with % N from fixation in

414　these other studies could be due in part to intercropping with non-legume species. Here, the

415　intercropped rye competed with the vetch for soil inorganic N, taking up an average of 51.5 kg N

416　ha^{-1} in 2016 (with an estimated 46.9 kg ha^{-1} of that N derived from soil), which also caused vetch to

417　fix N at a higher rate in mixture (Fig. 2). In contrast to % N from fixation, the effect of POM N on

418　total fixed N in aboveground vetch biomass (in kg N ha^{-1}) was stronger in mixture than monoculture

419　(Appendix S1: Table S1). This likely occurred because rye was the stronger competitor in mixture

420　at high soil N levels. For instance, across farms, rye aboveground biomass (both total, and as a % of

421　mixture biomass) increased with the protected POM N concentration in soil (F = 10.6, P = 0.002

422　and F = 16.8, P = 0.0002, respectively), which reduced vetch biomass—the primary driver of total

423　fixed N inputs (Herridge et al. 2008).

424　　　　Beyond the regulatory effect of N from turnover of POM, legume biomass production across

425　farms, and thus total N_2 fixed, was limited by P and increased with greater soil P concentrations

426　(Appendix S1: Fig. S2). Together, POM N and soil P concentrations explained 42-54% of the

427　variation in vetch N_2 fixation (Appendix S1: Table S1), similar to earlier findings with different

428　legume cover crop mixtures on these farms (Blesh 2018). However, there was no evidence that the

429　N_2 fixation process itself is regulated by P availability (i.e., there was no relationship between soil P

430　concentration and the % of plant N from fixation), which supports other studies (Vitousek et al.

431　2002). Although the model R^2 values were relatively high, especially for data from working

432　agroecosystems, there remains substantial unexplained variation that could result from several

433　factors. First, additional variation in N_2 fixation could result from physiological controls at smaller

434　scales, such as soil O_2 availability (Layzell and Hunt 1990). Second, rhizobial strains differ in their

435　effectiveness, and may have differed in vetch nodules across farms, because background soil

436　populations often occupy nodules despite inoculation (Thies et al. 1991). Consequently, applying

437　one B-value across sites, to account for isotopic fractionation, may not fully meet the assumptions

438　of the natural abundance method. This method also assumes that the reference plant (rye) was not

439　affected by free living N_2 fixation in the rhizosphere, a topic of renewed attention in agroecosystem

440　research on perennial grasses (Roley et al. 2018, Smercina et al. 2019). However, given the

441　experimental design, in which the reference plants were in small plots nested within a larger field

442   inoculated with rhizobia, and given that the farm management histories often included legumes in

443   crop rotation, this assumption is likely valid.

444   Analysis of SOM pools and fluxes following two years of the cover crop mixture showed

445   that, on average, the cover crop increased the size of both POM pools, and rates of microbial

446   activity (C-min and N-min; Fig. 4). Further, POM N and N-min had a strong relationship across

447   sites. These dynamics would potentially enhance the stabilizing feedback identified here and

448   increase N cycling efficiency over time. The modeling of vetch N_2 fixation as a function of baseline

449   soil properties – to determine if the POM gradient resulting from distinct management histories

450   regulated N_2 fixation – focused on vetch N_2 fixation in 2016, because soil properties started

451   changing quickly in response to the cover crop mixture treatment (Fig. 4). This increase in soil N

452   cycling may also help explain the large reduction in vetch biomass in the second year. These

453   changes in soil properties with the cover crop support the hypothesis that the mechanism driving the

454   stabilizing feedback was increased microbial activity and turnover of N with larger pools of intra-

455   aggregate POM.

456   Results from this experiment suggest that protected POM represents an ecologically

457   meaningful SOM fraction that regulates N_2 fixation in diversified agroecosystems because it is a

458   key source of inorganic N through N mineralization. The POM that is occluded in aggregates often

459   has a low C:N ratio, suggesting capacity for both mineralization and stabilization of SOM (Marriott

460   and Wander 2006a, Hatton et al. 2012, Cotrufo et al. 2013). Occlusion within micro-aggregates can

461   stabilize SOM by making it physically inaccessible to decomposers (von Lützow et al. 2006).

462   Although accumulation of SOM with legumes is frequently observed (Fornara and Tilman 2008,

463   McDaniel et al. 2014), POM that is protected in soil aggregates may exhibit faster turnover rates in

464   diversified agroecosystems than typically assumed based on experiments finding mean residence

465   times of 10-80 years for occluded POM (Collins et al. 2000, Puget et al. 2000, Dorodnikov et al.

466   2011). Legume cover crops and cover crop mixtures increase the presence of living roots in

467   agroecosystems. Root C inputs can contribute to aggregation and stabilization of SOM, but roots

468   may also cause priming effects and other rhizosphere interactions that increase turnover of stable

469   SOM pools, notably by root growth through aggregates or in hotspots with large rhizodeposits

470   (Clarholm 1985, Paterson 2003, Kuzyakov and Blagodatskaya 2015). For instance, plants can

471   increase N mineralization in the rhizosphere (Cheng 2009) by adjusting levels of soluble C

472   compounds released through root exudates (Hamilton and Frank 2001). In this study, the increased

473   N and C mineralization rates with the cover crop mixture relative to the no cover crop control; the

474 negative relationships observed between protected POM N and vetch N_2 fixation, and between N-
475 min and N_2 fixation; and the positive relationship between POM N and N-min, all suggest that
476 cover crops access N from SOM pools that are considered stable (i.e., protected for decades or
477 longer; Wander 2004; von Lützow et al. 2006) through interactions with microorganisms in the
478 rhizosphere. This finding supports other studies reporting that intra-aggregate POM is related to N
479 mineralization rates on organic and diversified farms (e.g., Willson et al. 2001, Marriott and
480 Wander 2006a), which tend to have greater root presence in space and/or time depending on
481 functional diversity of crop rotations.

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

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504

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670

671 **Data Availability**

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

673

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. Abbreviations: 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.

Farm	TOC	TN	N-min	C-min	Free POM	C:N Free POM	Protected POM	C:N Protected POM	Protected POM N
	$Mg\ ha^{-1}$	$Mg\ ha^{-1}$	$mg\ kg^{-1}\ wk^{-1}$	$\mu g\ CO_2\text{-}C\ g^{-1}\ d^{-1}$	$g\ kg^{-1}$		$g\ kg^{-1}$		$mg\ kg^{-1}$
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

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.

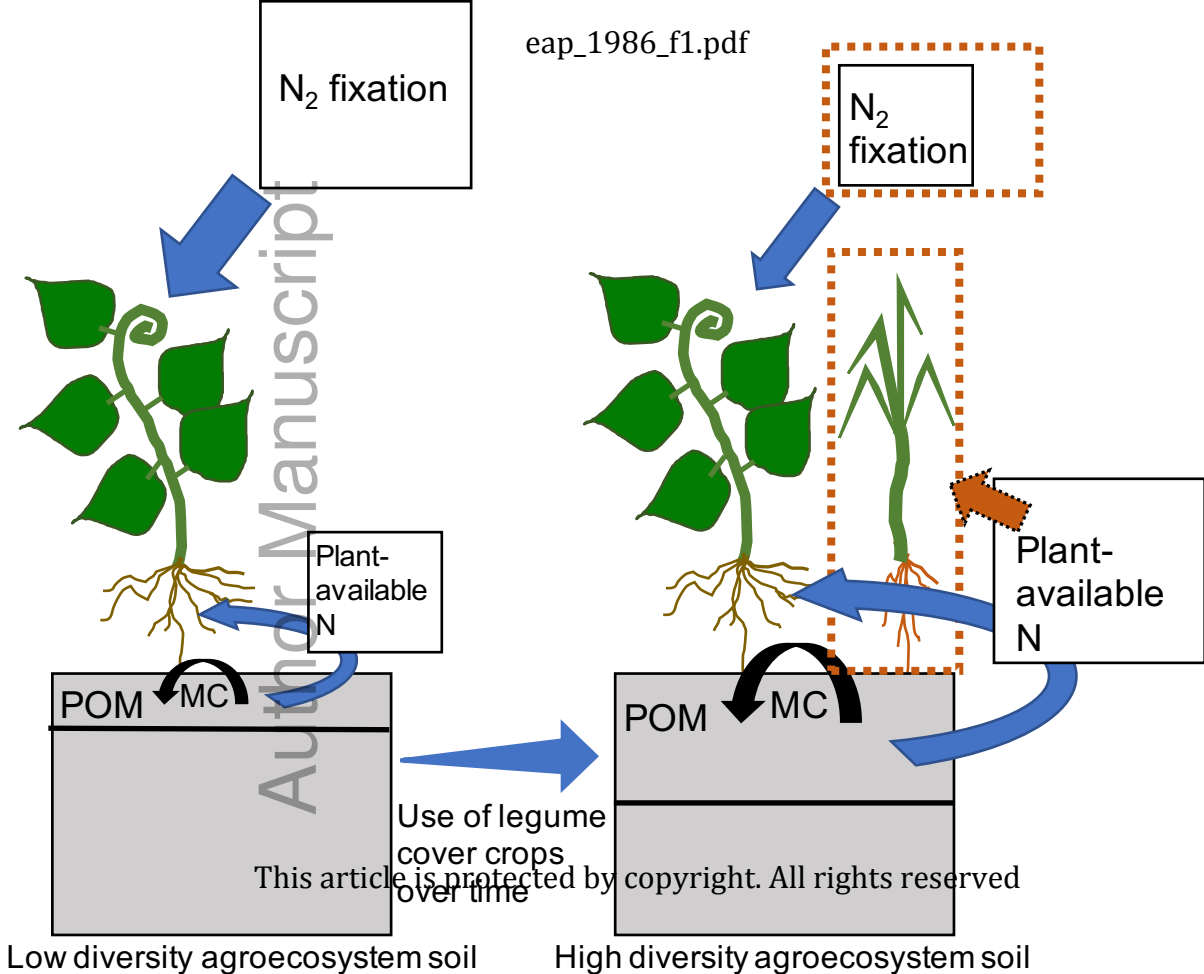| Farm	NO$_3$-N + NH$_4$-N	P	K	pH	sand	silt	clay	Soil order
	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}		%	%	%	
1	3.0	45.0	100.8	7.0	74.0	15.0	11.0	Alfisol
2	2.6	18.3	78.5	6.3	48.0	28.0	24.0	Mollisol
3	13.9	43.0	162.0	7.5	60.5	23.5	16.0	Alfisol
4	5.2	18.0	31.8	7.2	61.5	22.0	16.5	Alfisol
5	13.8	84.0	82.0	7.1	60.0	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

Figure legends.

Figure 1. Conceptual diagram of the hypothesized stabilizing feedback between particulate organic matter (POM) pools and legume N_2 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 SOM is small, which leads to high rates of legume N_2 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_2 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_2 fixation (i.e., the brown dashed line around the N_2 fixation box).

Figure 2. Top panel: Percentage of vetch N derived from fixation. Bottom panel: Total N assimilation in vetch-rye mixture biomass (mixture) and in vetch biomass (monoculture), separated into N derived from vetch N_2 fixation and N from the soil. Values are means +/- standard error.

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

Figure 4. Mean 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 with 95% confidence intervals.

Figure 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.

N$_2$ fixation

Plant-available N

POM MC

Low diversity agroecosystem soil

N$_2$ fixation

Plant-available N

POM MC

High diversity agroecosystem soil

Use of legume cover crops over time

R² = 0.25
P=0.0014
y= 87.63 - 0.16*x*

R² = 0.38
P=0.0002
y= 97.60 - 0.12*x*

Mean change with cover crop mixture