Availability of dissolved organic carbon drives differences in microbial nitrogen-cycling processes between two sites with cover crops interseeded into corn

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Abstract

Interseeding cover crops into corn has been proposed as a technique to extend the cover crop growing window, but interseeded cover crops may reduce N availability and compete with corn for available nutrients. To assess N-cycling dynamics in soils where cover crops have been interseeded into corn for one or two years, plots were established in duplicate at two sites in Michigan with differing edaphic properties. Annual ryegrass (Lolium multiflorum Lam.), crimson clover (Trifolium incarnatum L.), oilseed radish (Raphanus sativus L.), or a mixture of ryegrass and clover were interseeded into corn at the V3 or V6 stages of corn growth and compared to control plots that did not receive cover crops. We measured active C and N pools during the growing season and after harvest as well as potential activities of microbially mediated nutrient-cycling processes via extracellular enzymes, nitrification, and denitrification. We found that after two years, interseeded cover crops had little to no effect on active pools of C and N or on microbial nutrient-cycling activities. However, we observed major differences in these parameters between sites, with finer-textured soils exhibiting increased dissolved organic C availability and greater peptidase activity compared to coarser-textured soils. Our results reveal important spatial and temporal trends that suggest greater C availability can lower the potential for N loss while maintaining a rapid flux of N through the N cycle.

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Core Ideas

- Interseeding cover crops into corn did not negatively influence nutrient availability
- Microbial communities had distinct nutrient cycling strategies between two locations with cover crops interseeded into corn
- Soils with more available C had more active N cycles but with less potential for N loss
- Managing interseeded cover crops for maximal benefit requires an awareness of site-specific nutrient dynamics

Abstract

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Abbreviations

BG, β-glucosidase; DEA, denitrification enzyme assay; DOC, dissolved organic carbon; DON, dissolved organic nitrogen; EEA, extracellular enzyme assay; EL, East Lansing; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; N_2O, nitrous oxide; NAG, β-1,4-N-acetyl-glucosaminidase; NH_4^+, ammonium; NO_3^-, nitrate; NPA, nitrification potential assay; RDA, redundancy analysis; SOM, soil organic matter; SV, Saginaw Valley

Introduction

Cover cropping has been promoted as a practice that promotes soil health (Finney et al., 2017; Snapp et al., 2005; Wegner et al., 2018). Studies have shown how integrating cover crops into a rotation increases many ecosystem services, such as protection against erosion, the formation of soil carbon (C), improved water retention, and reductions in nutrient loss (Blanco-Canqui & Ruis, 2020; Daryanto et al., 2018; Schipanski et al., 2014). In turn, cover cropping is also frequently associated with increased yields of the cash crop (Daryanto et al., 2018; Fageria et al., 2005; Marcillo & Miguez, 2017; Smith et al., 2008). Nevertheless, the benefits of cover cropping can take time to manifest, and often depend on how cover crops are managed as well as on climate and soil properties (Abdalla et al., 2019; Kallenbach et al., 2019; Snapp et al., 2005; Xu et al., 2020). Understanding the mechanisms of how cover crops improve soil health can inform the selection of appropriate cover cropping strategies for growers.

Plant productivity is dependent on the timely supply of nitrogen (N) to meet crop demands. Often N is supplied through an input of chemical or organic fertilizer once or twice a year. Maximizing the efficient use of N inputs requires timing N additions with the critical period of plant growth, which often depends on environmental factors that are difficult to control or predict, such as precipitation and temperature (Cassman et al., 2002; G. P. Robertson & Vitousek, 2009). When anthropogenic N additions do not match demand, N accumulates in various forms and is subject to transformations and subsequent loss from the ecosystem (G. P. P. Robertson & Groffman, 2015; Schlesinger, 2009). The soil N cycle also provides plant-available N through biological N fixation and by releasing N contained within plant residues and soil organic matter. In most natural systems, the N provided by the microbial community is the sole source of N for the native vegetation (Galloway et al., 2004). Therefore, internal N cycling in the soil is a double-edged sword. It can provide nutrients to plants during their peak demand, but it can also drive nutrient losses from ecosystems when N is in excess. This presents many opportunities to understand and optimize N cycling to maximize ecosystem services (Van Groenigen et al., 2015).

Cover crops are often specifically chosen to improve N cycling in the soil by both retaining and releasing N. Some cover crops, particularly grasses, are often used as "catch crops" to capture excess N, helping to keep this vital nutrient in the field. Such uses of cover crops are particularly effective in the winter, when excess

moisture in bare soils can result in substantial amounts of N leaching (Hirsh et al., 2021; Komatsuzaki & Wagger, 2015). Leguminous cover crops can also be utilized to provide "N credits" through their symbiotic association with N-fixing bacteria (Ebelhar et al., 1984; Snapp et al., 2005; Tonitto et al., 2006). As N-containing cover crop residues decompose, their N is released for utilization by the cash crop. These benefits are particularly evident in wheat-clover systems (Gaudin et al., 2014; Thorsted et al., 2006). In addition to cover crop selection, the timing of cover crop planting can determine the potential of cover crops to scavenge nutrients and produce biomass (Hashemi et al., 2013; Komainda et al., 2016; Lawson et al., 2015).

Typically, cover crops are planted after harvest, but in some locations, particularly northern latitudes, this can leave little time for cover crops to grow and establish before winter (CTIC et al., 2020; Komainda et al., 2016). Interseeding can help overcome these narrow cover cropping windows. Interseeding is a practice where cover crops are planted during the growing season between rows of a main crop, such as corn, allowing for a longer growing season for the cover crop. On the other hand, there are some concerns that if planted too early, interseeded cover crops may compete with the main crop for needed nutrients, such as N (Fageria & Baligar, 2005; Snapp et al., 2005; Wachendorf et al., 2006). Therefore, it is important to understand how interseeded cover crops impact the N cycle both during the growing season and after harvest, when cover crop residues are decomposed in the soil.

The soil microbial community is responsible for carrying out the N transformations that make up the soil N cycle (Booth et al., 2005; G. P. P. Robertson & Groffman, 2015). The balance between N supply and microbial demand determines the amount of N that is mineralized from organic matter by the microbial community and made available for plant uptake or immobilized in microbial biomass. Soil microbes also carry out a host of mineral N transformations, such as nitrification, the conversion of ammonium to nitrite and nitrate, and denitrification, which closes the N cycle by converting nitrate to nitrous oxide (N_2O) and/or dinitrogen gas. Cover crops have the potential to influence the soil microbial community by altering the types and diversity of organic inputs to the soil as well as by increasing the amount of residues (Aulakh et al., 1991; Tiemann et al., 2015). For example, C-rich grass residues could increase microbial N demand and lead to N immobilization, while the degradation of N-rich clover residues could either help provide plant available N or lead to greater N losses if degradation does not coincide with plant demand (McKenney et al., 1993, 1995; O'Connell et al., 2015; Steenwerth & Belina, 2008). Ultimately, the impact of cover crops on soil N availability depends on the N transformations carried out by the microbial community. Understanding how cover crops influence the microbially mediated N cycle is therefore essential to maximizing the benefits of cover crops for increasing plant yields and building soil health.

We sought to determine how interseeding cover crops into corn impacts measures of soil health and soil N cycling and provisioning. Since these factors are heavily influenced by environmental and edaphic factors, the experiment was initiated twice at two locations in Michigan, USA, with varying soil and climate. Soils were sampled during the growing season and after harvest over three years. To distinguish legacy effects of cover cropping from year-to-year variation, the experiment was repeated at each site and maintained for two consecutive years for a total of eight site-years. We hypothesized that (1) interseeded cover crops would improve soil health by increasing active soil C and N pools and microbial activity; (2) a mixture of grass and legume interseeded cover crops would provide greater soil health benefits than either grass or legume interseeded alone; (3) soil N provisioning would be greater and potential N losses reduced with interseeded cover crops, with these benefits most pronounced under the grass-legume mixture, as indicated by reductions in concentrations of inorganic forms of N, especially nitrate, and reduced rates of nitrification and denitrification; and (4) soils sampled after consecutive years of interseeding cover crops compared to an initial year would have greater "active" soil C and N pools, greater microbial activity, and more efficient N provisioning.

Methods

Site Description and Experimental Design

The first set of experimental plots were established at our two research locations in central Michigan, USA, in 2017. The first site was at the East Lansing Agronomy Farm (42.7100° N, 84.4663° W). The soils are an Aubbeenaubbee–Capac sandy loam (fine-loamy, mixed, active, mesic Aeric Epiaqualf; fine-loamy, mixed, active, mesic Aquic Glossudalf). Soil organic matter (SOM) was between 2.8 and 2.9%, with pH between 5.8 and 7.6. The second site was located at the Saginaw Valley Research and Extension Center (43.3952° N, 83.6831° W). Soils at this location are a Tappan-Londo loam (fine-loamy, mixed, active, calcareous, mesic Typic Epiaquolls; fine-loamy, mixed, semiactive, mesic Aeric Glossaqualfs), with 3.0% SOM and pH of 7.5. The following year, 2018, a second, identical set of plots was established at each location in a different field. Each set of plots was maintained for two years for a total of eight site years (A. P. Brooker et al., 2020).

Cover crop treatments were established within four blocks at each location for both establishment years. Cover crop treatments included two factors: cover crop species and cover crop seeding time. The four cover crop species were annual ryegrass (*Lolium multiflorum*Lam.), crimson clover (*Trifolium incarnatum* L.), oilseed radish (*Raphanus sativus* L.), and a mixture of ryegrass and clover. Within these cover crop species, a seeding time treatment was applied, where covers were seeded during either the V3 or V6 stage of corn growth. These cover crop treatments were compared to plots that did not receive any cover crop treatment. Since the seeding time treatment could not be applied across all levels of cover crop species (i.e., the no-cover treatment did not have a seeding-time treatment), this resulted in an incomplete-randomized-block design. Cover crop biomass was determined at the time of corn harvest by sampling aboveground biomass of cover crops within 0.25 m^2 quadrats placed randomly in two locations within the plot. Cover crop biomass was oven dried prior to weighing.

Soil Chemical Properties

Soil samples were taken throughout the year using a 1.9 cm diameter soil probe to 10 cm depth (Table 1). Samples were sieved through a 4 mm mesh. Soil water content was determined gravimetrically. Dissolved organic C (DOC) and total dissolved N were extracted by combining 8 g of field moist soil with 40 mL 0.5 M K_2SO_4 and shaking for 1 hour followed by filtration with Whatman #1 filters. Soil ammonium (NH_4^+) and nitrate (NO_3^-) concentrations were determined colorimetrically in clear 96-well plates. Ammonium determination was done using salicylate and cyanurate color change chemistry (Sinsabaugh et al., 2000), and NO_3^- concentrations were determined by first reducing $NO_3^-$to NO_2^- using nitrate reductase from*Arabidopsis thaliana* (EC 1.7.1.1; NECi, USA) followed by standard procedures for nitrite determination modified for a microplate format (Mulvaney, 1996). DOC and total dissolved N were determined using a Vario TOC select elemental analyzer (Elementar Americas, USA). Dissolved organic N (DON) levels were calculated by subtracting concentrations of NO_3^- and NH_4^+ from total dissolved N concentrations. We estimated microbial biomass using a modified chloroform-fumigation method (Vance et al., 1987). In brief, 1 ml of chloroform was added to 8 g of soil and incubated in sealed tubes for 24 hours. After venting chloroform for one hour under a fume hood, we performed K_2SO_4 extractions and quantified total DOC and total dissolved N as described above. The difference in DOC and total dissolved N levels between fumigated and non-fumigated samples is considered microbial biomass following the application of a chloroform efficiency factor of 0.45.

Table 1 Soil sampling dates. For analysis sampling dates were combined by season as indicated in the table. Two distinct sets of plots were established at each location in 2017 and 2018. For each set of plots, soil samples were taken in the first year of establishment and during the second year of cover cropping

		Soil sampling dates	Soil sampling dates	Soil sampling dates	Soil
		2017 Plots	2017 Plots	2018 Plots	2018
Year	Season	East Lansing	Saginaw Valley	East Lansing	Sagi

		Soil sampling dates	Soil sampling dates	Soil sampling dates	Soil
2017	Growing	July 10	July 17	—	—
	Postharvest	Nov. 7; Dec. 8; Feb. 26; May 1	Nov. 9; Dec. 19; Feb. 27; Apr. 27	—	—
2018	Growing	July 26; Sept. 12	July 27; Sept. 17	—	—
	Postharvest	Nov. 19; Mar. 27	Nov. 2; Mar. 28	Dec. 3; Apr. 16	Nov.
2019	Growing	—	—	Aug. 14	July
	Postharvest	—	—	Nov. 5; Mar. 9; Apr. 22	Oct.

Soil Biological Properties

We used fluorescently labelled substrates to estimate the activity of eight extracellular enzymes: β-glucosidase (BG), β-1,4-N-acetyl-glucosaminidase (NAG), phosphatase, alanine aminopeptidase, arginine aminopeptidase, leucine aminopeptidase, glutamate aminopeptidase, and tyrosine aminopeptidase. BG, NAG, and phosphatase substrates were labelled with 4-methylumbelliferone, and aminopeptidase substrates were labelled with 7-amino-4-methylcoumarin, according to the high-throughput microplate method (German et al., 2011).

Potential rates of denitrification were determined using a modified denitrification enzyme activity (DEA) assay (Groffman et al., 1999). Five grams of soil were placed in airtight jars and combined with 15 mL of a solution containing 4.75 mM KNO_3 and 20 mM glucose. Jars were evacuated and flushed with N_2 three times. To inhibit N_2O reduction, acetylene was added to the headspace of each jar to a final concentration of 10% (v/v). Jars were incubated at 22°C on an orbital shaker. Headspace gas samples were taken after 30 and 90 minutes and placed in pre-evacuated 12 ml gas-tight vials. Gas samples were analyzed for N_2O concentrations using a TRACE 1310 gas chromatograph equipped with electron capture detector (Thermo Fisher Scientific, USA).

Nitrification potential activity (NPA) was determined using the shaken soil-slurry method (Hart et al., 1994). Briefly, soil slurries were prepared by combining 15 g of field moist soil with 100 ml of a solution containing 1 mM phosphate, and1.5 mM NH_4^+, adjusted to pH 7.2. Jars were flushed with O_2 to prevent denitrification and incubated at 22°C on an orbital shaker. Over the course of 24 hours, soil slurry samples were taken four times at evenly spaced intervals. Nitrate concentrations in the soil slurry were determined using the microplate method described above, and changes through time were used to calculate the rate of nitrification.

Statistical Analyses

Because the experimental design, with seeding time and cover crop species in an incomplete factorial, would not allow us to simultaneously analyze these two cover crop effects, we first analyzed the main effect of cover crop species alone and its interactions with season and environment. To determine the effect of seeding time when the cover-crop-species treatment was found to be significant, we omitted the no-cover-crop treatment and performed a second analysis that included the effects of both cover crop species and seeding time as well as their interaction.

To model how season impacted cover crop treatment effects, sampling times were grouped by seasons: samples taken between corn emergence and corn harvest were categorized as "growing season", and samples taken after harvest were "post-harvest" (Table 1). Treatment differences in single response variables were determined using linear models and analysis of variance (ANOVA). Linear models were constructed using the*lme4* package in R (Bates et al., 2015). Fixed effects were season, site, and cover crop species, and when cover crop species effects were significant, additional analyses included seeding time as a fixed effect. Random effects were specified as block (nested within site and establishment year) and its interactions with the various fixed effects. In addition, with respect to season, plot was considered a random effect to account for repeated measures over time. We utilized the *lmerTest* package (Kuznetsova et al., 2017) to determine the significance of main effects and interactions by performing Type III tests with numerator degrees of

freedom calculated using the Kenward-Roger method. When main effects or interactions were found to be significant, pairwise comparisons were conducted with the *emmeans*package (Lenth et al., 2019) and Fisher's LSD at $\alpha = 0.05$ (e.g., we looked for significant differences between sites by season when the site-by-season interaction was significant). Since the five peptidase activities were highly correlated with one another, these were summed together to produce a single variable representing total peptidase activity.

We analyzed correlations between variables separately within each site. Correlation matrices between variables were generated using the*cor* function in R to calculate Pearson's correlation coefficients. Significance of Pearson correlation coefficients were based on $\alpha = 0.05$ using the *cor.test* function, as implemented within the *corrplot* package.

We performed a multivariate analysis to determine how soil chemical properties explained variation among samples in biological process rates. We conducted a redundancy analysis (RDA) using the *vegan*package (Oksanen et al., 2007) with all enzyme data, DEA, and NPA as response variables, and all chemical data as explanatory variables.

Results

Cover Crop Biomass

Cover crop biomass varied between cover crop types and seeding times as well as by location and year (Table 2). For example, in the first year of the study at East Lansing (EL), tillage radish seeded at V3 had nearly twice as much biomass as the next highest cover crop (55.9 versus 29.1 g m^{-2}). However, at the same location, this cover crop failed to emerge when seeded at V6 during the first year of the 2018 plots and did not emerge at all in 2019. In general, 2018 had less cover crop biomass compared to other years. Only V3-seeded clover and mixture at Saginaw Valley (SV) had more than 2.0 g m^{-2}biomass. In contrast, cover crop biomass varied from 3.8 to 19.5 g m^{-2} at SV in 2017. The lack of cover crop biomass in 2018 was likely due to a shortfall of precipitation during the period when cover crops were interseeded (Figure 1).

Table 2 Dry weight of aboveground biomass of cover crops at the time of maize harvest

		Cover crop aboveground biomass
		East Lansing
		2017 Plots
Seeding	Crop	2017
		g m^{-2}
V3	Ryegrass	12.22 (7.06)[a]
	Clover	29.13 (6.79)
	Mixture	25.55 (4.31)
	Radish	55.94 (16.57)
V6	Ryegrass	11.32 (5.76)
	Clover	14.01 (2.85)
	Mixture	8.14 (1.81)
	Radish	21.57 (3.7)

[a] Values presented are means with standard error in parentheses (n = 4)

Soil C and N Pools

We measured dissolved organic C (DOC) and microbial biomass C (MBC) to determine how cover crops influenced active pools of soil C and found no significant main or interactive effects of cover cropping (Table

3). Instead, DOC and MBC varied significantly by site and season, with site differences depending on sampling season. DOC was significantly higher at SV in five out of seven seasons (Figure 2a) and MBC was significantly higher at SV in three out of six seasons, but one season was not observed at EL due to loss of samples (Figure 2b). At EL, DOC concentrations were greater during the growing season than after harvest. There was no consistent seasonal pattern in DOC concentrations at SV.

Figure 1 Cumulative precipitation at East Lansing (a) and Saginaw Valley (b) during the maize growing season. Shaded region indicates the period when cover crops were interseeded

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Figure 2 Concentrations of dissolved organic carbon (a) and microbial biomass carbon (b) at East Lansing (EL) and Saginaw Valley (SV) field sites. Error bars represent one standard error (n = 4). Means that significantly differ between locations for each season are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Within each location, significantly different means across seasons are indicated by lowercase letters ($\alpha = 0.05$)

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Soil N pools were largely unaffected by cover cropping, regardless of sampling site or season (Table 3). We found greater ammonium (NH_4^+) concentrations at EL compared to SV at all seasons except one, but the magnitude of the site difference varied by season (Figure 3a). Soil nitrate (NO_3^-) concentrations did not differ by cover crop and were not consistently different between the two locations (Figure 3b). Within each location, NO_3^- concentrations tended to be greater during growing seasons. Dissolved organic N (DON) was impacted by site and season, although the seasonal effects varied by site (Figure 3c). At EL, DON was significantly higher during the growing season compared to post-harvest, while at SV, post-harvest levels of DON were significantly greater in two out of three years. Microbial biomass N (MBN) was significantly affected by cover crop (Table 3), with significantly greater MBN in the mixture (34.3 ± 1.4 mg MBN-N kg^{-1} soil), radish (33.6 ± 1.3 mg MBN-N kg^{-1} soil), and no-cover (33.5 ± 1.8 mg MBN-N kg^{-1} soil) treatments compared to clover (29.4 ± 1.2 mg MBN-N kg^{-1} soil). MBN was consistently higher at SV than EL and, with no significant site-by-season interaction, was not driven by seasonality effects (Figure 3d).

Table 3 Type III ANOVA table of fixed effects for soil chemical properties

	DOC	DOC	DOC	MBC	MBC	MBC	NH_4^+	NH_4^+	NH_4^+
	F	df	P-value	F	df	P-value	F	df	P-value
Site (St)	45.86	1	<0.001 ***	11.87	1	0.003 **	119.12	1	<0.001 ***
Season (Sn)	26.61	6	<0.001 ***	7.89	5	<0.001 ***	7.28	6	<0.001 ***
Crop (C)	0.37	4	0.826	0.81	4	0.520	1.75	4	0.147
St × Sn	14.77	6	<0.001 ***	17.73	5	<0.001 ***	5.00	6	<0.001 **
St × C	0.46	4	0.766	0.16	4	0.959	0.19	4	0.945
Sn × C	0.49	24	0.981	0.59	20	0.922	0.76	24	0.792
St × Sn × C	0.38	24	0.997	0.69	20	0.840	1.28	24	0.171

Table 3 (cont'd)

	NO_3^-	NO_3^-	NO_3^-	DON	DON	DON	MBN	MBN	MBN
	F	df	P-value	F	df	P-value	F	df	P-value
Site (St)	0.43	1	0.520	62.02	1	<0.001 ***	59.63	1	<0.001 ***
Season (Sn)	37.63	6	<0.001 ***	30.46	6	<0.001 ***	24.70	5	<0.001 ***
Crop (C)	1.86	4	0.126	0.60	4	0.666	2.81	4	0.031 *
St × Sn	10.10	6	<0.001 ***	24.95	6	<0.001***	1.34	5	0.254
St × C	1.92	4	0.115	0.51	4	0.728	1.35	4	0.259
Sn × C	0.55	24	0.958	0.95	24	0.537	0.85	20	0.657
St × Sn × C	0.70	24	0.851	0.70	24	0.857	0.77	20	0.747

Asterisks next to P-values indicate thresholds of significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

Figure 3 Concentrations of ammonium (a), nitrate (b), dissolved organic nitrogen (c), and microbial biomass N (d) at East Lansing (EL) and Saginaw Valley (SV) field sites. Error bars represent one standard error (n = 4). Means that significantly differ between locations for each season are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Within each location, significantly different means across seasons are indicated by lowercase letters ($\alpha = 0.05$)

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Soil Biological Activity

As with soil chemical parameters, cover cropping did not account for a significant amount of variation in extracellular enzyme activity (EEA), but we did observe significant site, season, and site-by-season effects (Table 4). EEA potential rates significantly differed between locations for most enzymes (Figure 4). BG and NAG activities tended to be significantly greater at EL than SV in most seasons (Figure 4a, b). Conversely, peptidase rates were more than twice as high at SV than EL in all seasons (Figure 4c). Phosphatase activities were significantly greater at EL in two out of seven seasons but were significantly higher at SV in one season (Figure 4d). Although activities varied significantly over time at both locations, there were no consistent seasonal trends.

Nitrification potential activity (NPA) and denitrification potential (DEA) both differed by site and season, with significant site-by-season effects (Table 5). NPA rates were significantly greater at SV than EL across all seasons except the 2018 growing season (Figure 5a). Conversely, DEA was significantly greater at EL than SV in most seasons (Figure 5b).

Table 4 Type III ANOVA table of fixed effects for extracellular enzyme activities

	BG	BG	BG	NAG	NAG	NAG	Peptidase	Peptidase	Peptidase	Phosphatase
	F	df	P-value	F	df	P-value	F	df	P-value	F
Site (St)	31.02	1	<0.001 ***	71.15	1	<0.001 ***	159.26	1	<0.001 ***	1.87
Season (Sn)	21.44	6	<0.001 ***	8.23	6	<0.001 ***	22.10	6	<0.001 ***	36.97
Crop (C)	0.78	4	0.541	1.00	4	0.412	0.24	4	0.916	0.75
St × Sn	30.93	6	<0.001 ***	11.23	6	<0.001 ***	13.50	6	<0.001 ***	63.12
St × C	0.48	4	0.753	0.27	4	0.898	0.49	4	0.740	0.70
Sn × C	1.08	24	0.368	0.96	24	0.513	0.79	24	0.744	0.65
St × Sn × C	1.37	24	0.118	1.28	24	0.170	0.75	24	0.793	0.64

Asterisks next to P-values indicate thresholds of significance (*, P < 0.05; **, P < 0.01; ***, P < 0.001)

Table 5 Type III ANOVA table of fixed effects for potential nitrification and denitrification

	NPA	NPA	NPA	DEA	DEA	DEA
	F	df	P-value	F	df	P-value
Site (St)	76.09	1	<0.001 ***	42.64	1	<0.001 ***
Season (Sn)	37.45	6	<0.001 ***	23.86	4	<0.001 ***
Crop (C)	0.38	4	0.822	0.36	4	0.833
St × Sn	5.66	6	<0.001 ***	5.94	4	<0.001 ***
St × C	2.16	4	0.083	1.66	4	0.173
Sn × C	0.86	24	0.655	1.04	16	0.411
St × Sn × C	0.67	24	0.878	0.23	16	0.999

Asterisks next to P-values indicate thresholds of significance (*, P < 0.05; **, P < 0.01; ***, P < 0.001)

Figure 4 Activities of extracellular enzymes: β-glucosidase (a), N-acetyl-glucosaminidase (b), peptidase (c), and phosphatase (d) at East Lansing (EL) and Saginaw Valley (SV) field sites. Error bars are one standard error (n = 4). Means that significantly differ between locations for each season are indicated by asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001). Within each location, significantly different means across seasons are indicated by lowercase letters (α = 0.05)

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Figure 5 Potential rates of nitrification (a) and denitrification (b) at East Lansing (EL) and Saginaw Valley (SV) field sites. Error bars are standard error (n = 4). Means that significantly differ between locations for each season are indicated by asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001). Within each location, significantly different means across seasons are indicated by lowercase letters (α = 0.05)

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Correlations between Soil Biological and Chemical Properties

DOC was correlated with enzyme activities at both locations, but these relationships were stronger at SV (Figure 6). At EL, DOC was inversely correlated to BG and NPA, while at SV, DOC was positively correlated with peptidase, phosphatase, and NPA. DOC was inversely related to BG and NAG activity at SV. There were fewer significant relationships between soil N and enzyme activities at EL than SV. NPA and NH_4^+ as well as DON and peptidase were strongly correlated at SV but not at EL. Among enzyme activities, we found that BG and peptidase were strongly positively correlated at EL but possessed a significant inverse relationship at SV. At both locations, NPA and peptidase activities were positively correlated.

Figure 6 Correlation plots of soil chemical and biological factors at East Lansing (a) and Saginaw Valley (b). Color scale represents the Pearson correlation coefficient, the significance of which is indicated by asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001)

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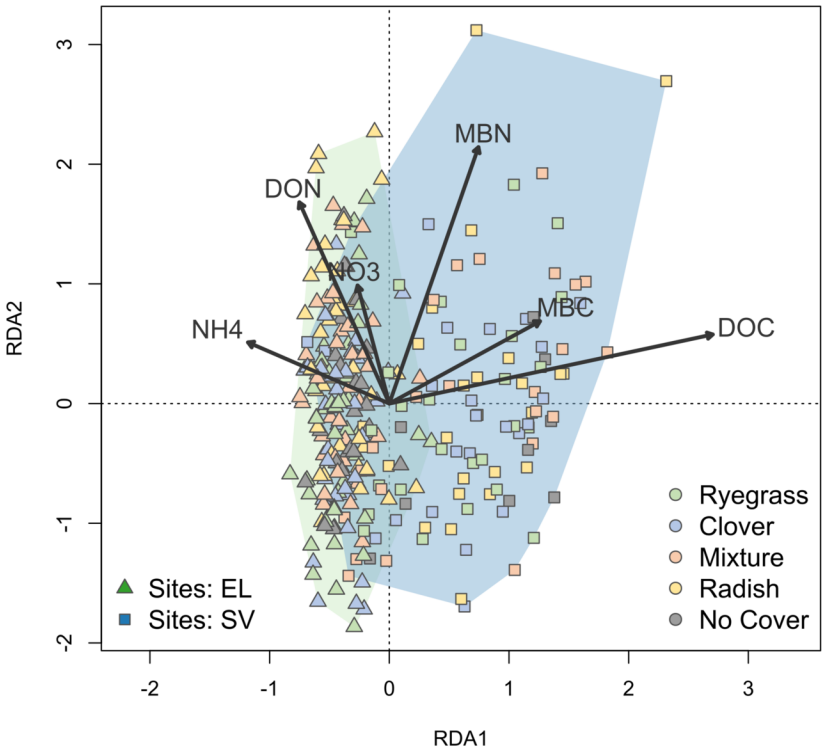
RDA Analysis

We used RDA to assess the treatment effects on all microbially mediated nutrient-cycling processes together and to determine which soil characteristics were most important in driving differences between the two locations (Figure 7). A total of 28% of the variation in biological process rates can be explained by the environmental variables included in our analysis, with 89% of this variation being expressed on the first two axes of the RDA. The two sites differentiate from one another along the first RDA axis. This axis is most strongly positively correlated with DOC, while it is negatively correlated with NH_4^+. Total N and MBN account for much of the variation explained by RDA2, but these environmental factors are not highly correlated with any of the response factors.

Figure 7 Redundancy analysis ordination of soil biological properties constrained to the variation exhibited within soil chemical properties. Black vectors correspond to soil chemical properties. The blue polygon encompasses the samples taken from Saginaw Valley, and the green polygon encompasses those from East Lansing



Discussion

The overall benefits of cover crops to soil health and soil C are well known (Daryanto et al., 2018; Fageria et al., 2005; Snapp et al., 2005). Cover crops can help to build soil C stocks, thereby improving soil health, and this is often a stated goal of utilizing cover crops (CTIC et al., 2020). However, within any particular location, the soil-health benefits of cover crops will depend on site-specific factors and may take time to manifest. For example, across the four plot locations in this study, we did not observe consistent effects of cover crops on any of the soil chemical or biological indicators of soil health. The lack of significant effects is likely due to a combination of low amounts of C inputs supplied by cover crops (Table 2) and the relatively short duration of the study. Other studies have also failed to detect significant differences in various soil C metrics after only a few years of cover cropping. In one study, there were no differences in SOM after 5 years of cover cropping, but detectable effects did emerge by 7 years, with this effect dependent on main cropping system type and management strategy (Wegner et al., 2015, 2018). Another study in Michigan also found that 5 years was insufficient time to produce significant differences in SOC (Ladoni et al., 2016), but in a similar experiment, differences in SOC were apparent after 12 years between a management system with cover crops and one without (Syswerda et al., 2011). The increase in soil C over time is likely linked to persistent inputs of cover crop biomass year after year. Long-term studies show that soil C concentrations are positively related to increases in C inputs (Barbera et al., 2012; Mazzoncini et al., 2011). Across all years of our study, cover crop biomass inputs were relatively modest, especially when compared to the contributions of biomass C from the corn main crop (Table 2). Cover crop biomass was typically between 10 to 200 kg ha^{-1}. Thus, compared to corn residues, which can be measured in megagrams per hectare, the cover crop biomass made a modest contribution to soil C inputs. A primary reason for the low biomass was likely a lack of precipitation during the key growing phase for the cover crops after interseeding, especially in 2018 (A. Brooker et al., 2020). The timing of precipitation for interseeded cover crops is particularly important: Precipitation occurring later in the season is of less benefit to interseeded cover crops because the closure of the corn canopy shades out emerging cover crops.

The strongest effects we observed on all measured parameters were between seasons and sites (Table 3). In general, seasonal trends were more pronounced at EL. For instance, all soil N pools were usually higher during the growing season. Greater amounts of N during the growing season are typical of agricultural systems, where anthropogenic N additions make up the overwhelming majority of N input, with less than half being taken up by the crop (Fageria & Baligar, 2005; G. P. Robertson & Vitousek, 2009). In contrast, seasonal patterns in inorganic N were not as pronounced at SV. Although NO_3^- concentrations tended to be higher during the growing season at SV, levels of soil NH_4^+ did not follow a consistent seasonal pattern. In addition, soil NH_4^+ was consistently lower at SV compared to EL at every sampling date. Greater microbial N demand at SV may have helped to maintain lower levels of inorganic N throughout the year compared to EL. Microbial immobilization of inorganic N can vary greatly between soils and is driven largely by soil organic C content (Barrett & Burke, 2000). When C is available, net N immobilization by microbes occurs, reducing the concentration of dissolved inorganic N (Aulakh et al., 1991; Hume et al., 2002; McKenney et al., 1995). We found higher DOC concentrations at SV compared to EL in most seasons (Figure 2a), and MBN was also greater at SV (Figure 3d), indicating more N in the microbial pool. While higher DOC levels may have driven microbial immobilization at SV, C limitation at EL could have hampered the ability of microbes to utilize N when it was available, allowing mobile forms of inorganic N to build up in the soil and become susceptible to loss.

DOC was higher and subject to less temporal variation at SV, suggesting a steady supply of DOC. Ultimately, this is due to more DOC being released through the degradation of crop residues and/or SOM (Kalbitz et al., 2000; Neff & Asner, 2001), but it is not immediately evident whether these site differences are caused by variable efficiencies in degradation dynamics or by differences in stocks of SOM. Enzyme activities were significantly different between sites. Further, at SV, enzyme activities were both positively and negatively correlated with DOC, indicating a complex relationship between DOC availability and the regulation of extracellular enzyme production. It is also possible that DOC levels were maintained through rapid turnover

of MBC. MBC concentrations were not always higher at SV compared to EL, but they did tend to exhibit greater variability, with season-to-season changes larger than the total pool of DOC. While some of MBC turnover becomes stabilized as necromass (Buckeridge et al., 2022; Miltner et al., 2012), a substantial portion can feed back into the pool of DOC (Blazewicz et al., 2014; Shahbaz et al., 2017). Greater DOC availability may thus lead to increased microbial activity, stimulating a virtuous cycle where C is actively cycled back and forth between DOC and MBC.

Regardless of its source, the availability of DOC was strongly correlated with differences in potential nutrient cycling activities between the microbial communities at the two locations. The strength of this relationship outweighed the contribution of inorganic N and microbial biomass to site differences (Figure 7). Differences in DOC could have driven distinct microbial nutrient-acquisition strategies at each location. For instance, we found that BG and peptidase activities were strongly positively correlated at EL but strongly negatively correlated at SV (Figure 6b). This inverse relationship was unexpected because extracellular enzyme activities tend to increase together (Sinsabaugh et al., 2008, 2009); however, others have also described a negative pattern between peptidase and glucosidase activities at the field scale (Weedon et al., 2014). These distinct relationships between enzyme activities could signify differences in nutrient limitation (Chen et al., 2014; Mooshammer et al., 2014). Stoichiometric decomposition theory describes how microorganisms shift their strategies for C- and nutrient acquisition to overcome differences in the stoichiometry of available resources and the relatively narrow C:N requirements of biomass (Sinsabaugh & Shah, 2012). Increased C availability at SV stimulated microbial N demand, resulting in a shift in allocation of microbial resources towards acquisition of N by producing peptidases (Allison & Vitousek, 2005; Geisseler et al., 2009). Indeed, we found that DOC availability and peptidase activities were tightly correlated at SV but not at EL (Figure 6). This agrees with other studies that have also shown a relative increase in peptidase activity associated with DOC and have attributed this to increased microbial N demand (Bowles et al., 2014).

Greater peptidase activity can result in more available DON at SV (Schimel & Bennett, 2004). As may be expected, we found that peptidase activity and DON were positively related at SV but not at EL (Figure 6). In addition, we found that DON tended to be higher post-harvest at SV (Figure 3c). Compared to corn stover, cover crop residues typically have much lower C:N ratios, especially clover residue, and the degradation of these N-rich substrates can increase soluble N levels during the fall and winter (Abdalla et al., 2019; McKenney et al., 1995). Nevertheless, as with other pools of N, we saw no cover crop effects on the availability of DON at SV. This leaves SOM as the most likely source of DON at SV. SOM is a rich source of N in some soils, and microorganisms often mineralize SOM to obtain needed N (Craine et al., 2007; Moorhead & Sinsabaugh, 2006). Inputs of corn stover could therefore provide an influx of C that stimulates the microbial community to increase N mining from SOM, leading to seasonal increases in DON after harvest (Shahbaz et al., 2017).

NPA was higher at SV (Figure 5a), potentially explaining the lower concentrations of NH_4^+, but interestingly, the correlation between NH_4^+ and NPA was strongly positive (Figure 6). If NPA was the primary process responsible for the net consumption of NH_4^+, there should be an inverse relationship. Rather, it appears that nitrifiers are responding to an increase in NH_4^+concentrations. The high potential activity of peptidases at SV could be driving a mineralization process that creates a niche for nitrifiers. Other studies have found nitrification to be well correlated with N mineralization (Booth et al., 2005; Liang et al., 2014; Ouyang et al., 2016; Steenwerth & Belina, 2008), and it has been suggested accordingly that N mineralization is a better determinant of nitrification substrate supply than concentrations of NH_4^+(Norton & Ouyang, 2019; Stark & Hart, 1997). In support of this possibility, both locations had significant positive correlations between NPA and peptidase activity. While potential peptidase activity does not measure N mineralization *per se* , the depolymerization of extracellular proteins and polypeptides is the rate-limiting step in the process of N mineralization (Schimel & Bennett, 2004). Depolymerization is all the more influential in tightly coupled systems where the demand for N is high and where rapid immobilization maintains a small pool of inorganic N, such as at SV. Only a few studies have explicitly looked at the relationship between potential rates of peptidase activity and nitrification (Schloter et al., 2003; Zaman et al., 1999). Often, no correlation or a negative correlation is found, but these studies are usually performed during the growing season and

are confounded by treatments and field-management practices that provide fertilizer or DON, underscoring the importance of understanding microbial activity and nutrient-cycling dynamics throughout the entire year within agricultural soils. Our study, therefore, highlights the spatial and temporal dynamics that can influence the relationships between N-cycling processes.

A more active N cycle at SV may or may not lead to increased N losses. For example, NPA is linked to N loss via the production of NO_3^-, which is the dominant form of N lost in agricultural ecosystems (G. P. Robertson & Vitousek, 2009). Nitrate is highly mobile and can be easily leached through the soil profile; in addition, NO_3^- is susceptible to loss via denitrification. Although we did not measure*in situ* fluxes of N into and out of the system, we found that DEA and inorganic N levels, including NO_3^-, tended to be lower at SV than EL (Figure 5b). This suggests minimal N loss pathways at SV, where despite the more active fluxes between soil N pools, the greater DOC content drove microbial demand for N. On the other hand, N availability at EL was driven by exogenous inputs, with higher DEA and concentrations of inorganic N suggesting greater potential for nutrient loss.

Conclusions

Interseeded cover crops did not produce consistent changes to soil nutrient pools or microbial activities in the two-year cover cropping treatments utilized in this study. The lack of a strong cover crop effect may have been due in part to the relatively modest cover crop biomass additions that were a result of low precipitation during key moments for cover crop growth. Instead, differences between site and season were far more pronounced. The microbial communities had distinct nutrient cycling strategies between the two locations observed in the study, including patterns of extracellular enzyme activity. We suggest that resource availability and relative nutrient demand drove these differences in nutrient transformations between the two sites, with C availability being associated with a more active N cycle and lower concentrations of inorganic N. These large and consistent differences in N cycling between locations indicates the importance of having site-specific management recommendations to improve N provisioning. For example, the contrasting seasonal patterns of organic N availability suggest that the N contained within cover crop residues will be mineralized differently depending on microbial demand and the strength of various N-cycling processes. Additional research is needed to further describe the mechanisms underlying the relationship between available C and microbial N-cycling processes and how practices such as cover cropping impact these interactions.

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