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Original research article

High quality residues from cover crops favor changes in microbial community and enhance C and N sequestration

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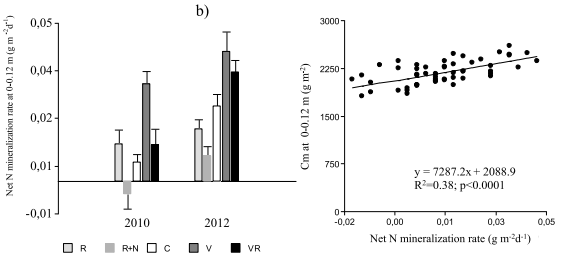
HIGHLIGHTS

- Vetch cover crop led to more Gram + bacteria.
- Vetch–sorghum rotation had the highest Shannon–Weaver diversity index.
- Vetch–sorghum had highest microbial biomass and N mineralization.
- Vetch–sorghum sequestered more C and N in stable SOM fractions.

GRAPHICAL ABSTRACT

Highest net N mineralization rates for vetch and vetch plus rye cover crops (a) and more effective carbon sequestration (Cm) at higher net N mineralization rates(b).



Control without cover crops (C), rye (R), rye + 40N (R+N), vetch (V) and vetch+rye (VR).

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ABSTRACT

The objective of the study was to evaluate the effect of a change in management on the soil microbial community and C sequestration. We conducted a 3-year field study in La Pampa (Argentina) with rotation of sorghum (*Sorghum bicolor*) in zero tillage alternating with rye (*Secale cereale)* and vetch *(Vicia villosa ssp. dasycarpa*). Soil was sampled once a year at two depths. Soil organic matter fractions, dissolved organic matter, microbial biomass (MBC) and community composition (DNA extraction, qPCR, and phospholipid FAME profiles) were determined. Litter, aerial- and root biomass were collected and all material was analyzed for C and N. Results showed a rapid response of microbial biomass to a bacterial dominance independent of residue quality. Vetch had the highest diversity index, while the fertilized treatment had the lowest one. Vetch–sorghum rotation with

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Mineral associated C and N
Particulate C and N

high N mineralization rates and diverse microbial community sequestered more C and N in stable soil organic matter fractions than no-till sorghum alone or with rye, which had lower N turnover rates. These results reaffirm the importance of enhanced soil biodiversity for maintaining soil ecosystem functioning and services. The supply of high amounts of N-rich residues as provided by grass–legume cover crops could fulfill this objective.

1. Introduction

Enhancement of biological health is crucial for recuperating degraded soils, since the living component of soil is essential to ecosystem functions and services (Lehman et al., 2015). Crop rotations, cover crops and minimum tillage (e.g. no-till) are strategies used in agroecosystems to improve soil organic carbon sequestration as one of the most important ecosystem service. These practices have a positive impact on soil biota (Balota et al., 2014; Dick, 1992; Helgason et al., 2010; Lupwayi et al., 2012; Poeplau and Don, 2015; Wardle and Wardle, 1992; Wright, 2008).

However, there are different points of view about how soil microbial biomass and nutrient cycling affect soil C storage. Earlier studies have assumed that a faster nutrient cycling by soil microorganisms resulted in lower soil organic carbon stabilization rates (Jastrow et al., 2006), while more recently Gentile et al. (2010, 2008) concluded that short-term nutrient availability through a more rapid residue cycling would not compromise long-term soil organic matter contents. More recent studies indicated that residues with higher quality (Verchot et al., 2011; Vesterdal et al., 2013) or with nutrient additions (Kirkby et al., 2013) were more efficiently stabilized as soil organic carbon (SOC). Some studies also showed that root-C was more important for the formation of stable C than residue-C (Kong and Six, 2012, 2010; Kong et al., 2011) and Mueller et al. (2015) indicated that the current conceptual models of litter quality impacts on soil carbon sequestration are overly simplistic. An incipient amount of evidence indicates that higher litter quality and more efficient assimilation of residue C by the soil biota would favor more SOC stabilization and ultimately higher SOC pools (Bradford et al., 2013; Cotrufo et al., 2015, 2013).

This conceptual framework leads to the hypothesis that microbes use labile plant constituents more efficiently, and that these would become the main precursors of stable SOC by promoting aggregation (O'Brien and Jastrow, 2013; Six and Paustian, 2014; Verchot et al., 2011) and through strong chemical bonding to the mineral matrix (Barré et al., 2014; Chenu, 1995; Tisdall and Oades, 1982). Thus, C sequestration can be conceptualized as a dynamic function related with higher rates of microbial activity stimulated by adequate levels of high quality litter inputs that allow a dynamic equilibrium between labile mineral and stable organic fractions (Cotrufo et al., 2015; Dungait et al., 2012; Wieder et al., 2014). Despite knowledge on which practices are likely to lead to improved SOC status, a better understanding of the controls on SOM distribution, stabilization, and turnover will help to better target these practices (Smith et al., 2015).

Management can also influence soil microbial community composition, although there are very few studies that studied different agroecosystems (Araújo et al., 2014; Figuerola et al., 2012). High quality residue input might favor bacterial dominance in microbial community structure and low quality residues are supposed to favor fungi (Bossuyt et al., 2001; Kramer et al., 2012). Fungi might be more efficient in C sequestration due to their higher C use efficiency (Frey et al., 1999; Six et al., 2006), although shifts in the fungal or bacterial dominance are not always in line with modifications in agricultural management (Strickland and Rousk, 2010). Moreover it is still a challenge to understand the relationship between microbial community structure or diversity and litter decomposition and nutrient cycling processes (Cleveland et al., 2014; McGuire and Treseder, 2010).

The objective of the present study was to evaluate the effect of different quality residues on the composition and diversity of the soil microbial community and its impact on element cycling and C and N sequestration after a change of soil management to no-till and cover crops.

2. Materials and methods

The study was conducted on a *petrocalcic Paleustoll* (USDA, NRCS, 2010) with a calcium carbonate hardpan at depths between 0.6 and 1.5 m, located in INTA Experimental Station at Anguil, La Pampa, Argentina (S36° 36′ 37.95″; W63° 58′ 48.22″). Mean annual precipitation is 700 mm occurring mostly during spring and autumn. The soil is a sandy loam with clay + silt content of 43%, 2% of organic matter, 0.2% of total N, bulk density of 1.2 g cm^{-3}, pH 5.8 and P Bray 8 mg kg^{-1}. Land use prior to the experiment consisted in annual forage crops under conventional tillage. The experiment started in 2010 with a 3-year rotation of sorghum (*Sorghum bicolor*) planted in zero tillage during the summer season alternating with rye and vetch cover crops (*Secale Cereale* and *Vicia villosa sp. dasycarpa*) during the winter months. Treatments were rye + vetch (RV), rye (R), vetch (V), rye fertilized with 40 kg ha^{-1} of urea–nitrogen (R+N), and a control without cover crop (C). A completely randomized block design was established with 4 replicates and a plot size of 500 m^2. Cover crops were seeded end of March at a density of 200 seeds m^{-2} for both R and V, and a 60/40 proportion of rye and vetch for RV treatment. All treatments

were fertilized with 20 kg ha^{-1} of phosphorus as triple superphosphate broadcast before seeding, simultaneous with broadcast urea in the R + N treatment. Cover crops were killed beginning of October by application of a mixture of glyphosate (3 l ha^{-1}) and 2,4-dichlorophenoxiacetic acid (0.4 kg ha^{-1}). Sorghum was planted in all plots during mid- November at a density of 150,000 plants ha^{-1} and a row spacing of 0.52 m.

Soil samples were taken in December 2010 and 2012 in a 0.25 m^2 area between sorghum lines, replicated 3 times in each plot, and 7 subsamples were collected in this area with a tubular auger of 0.032 m diameter at 0–0.06 and 0.06–0.12 m depth. Subsamples were mixed and homogenized in the field, and separated for soil organic matter fractions, dissolved organic matter, soil microbial biomass and microbial community composition (DNA and lipid extraction) determinations.

2.1. Soil organic matter content and particle size fractions

Soil fraction separation was based on complete soil dispersion followed by wet sieving (Noellemeyer et al., 2006 adapted from Cambardella and Elliott, 1994). The soil suspension obtained was wet sieved through a 53-μm sieve for 3 min (Fritsch Analysette Spartan Vibratory 3). Soil fractions collected were placed in metal jars in oven at 60 °C until complete drying. Dry weight of the fraction >53 μm (particulate organic C; Cp) was recorded, and the weight difference with the original sample (50 g) was used to calculate weight of fraction <53 μm (mineral associated C; Cm). Carbon and N analysis were performed by dry combustion with a CN auto analyzer (LECO—TrueSpec®).

2.2. Soil microbial biomass and dissolved organic matter

Soil subsamples were extracted using the fumigation–extraction method (Voroney, 2006) with a ratio soil to extractant (K_2SO_4, 0.5 M) of 1:2. Microbial biomass carbon (MBC) was determined according to Vance et al. (1987). MBC was calculated according to following the equation $MBC = E_C/0.45$, where E_C is the difference between organic C extracted from the K_2SO_4 extracts of fumigated and non-fumigated soils, both expressed as μgC g^{-1} oven dry soil (Wu et al., 1990). The same K_2SO_4 soil extract was used to determine MBN by Kjeldahl digestion. MBN was calculated as the difference between the extractable total nitrogen of fumigated and non-fumigated soil, using the equation $MBN = E_N/0.54$, where E_N is the difference between the amounts of total N extracted from the K_2SO_4 extract of fumigated and non-fumigated soil both expressed as μgN g^{-1} oven dry soil (Brookes et al., 1985). The obtained values were transformed into MBC and MBN mass using the average bulk density for each depth.

Dissolved organic matter was quantified according to Chantigny et al. (2006). Extracts were obtained from 25 g of field moist soil shaken in 50 ml $CaCl_2$ 5 mM and filtered using 0.4 μm polycarbonate filters (ISOPORE Membrane Filters, Millipore). Sample moisture was determined with gravimetric method. Extracts were stored at −20 °C until analysis. Dissolved organic carbon (DOC) and nitrogen (DON) were determined by dichromate reduction (Vance et al., 1987) and Kjeldahl (Brookes and Joergensen, 2005) methods respectively.

2.3. Residue quality and net N mineralization rates

Litter on the soil surface was collected in 0.25 m^2 area with three replicates per plot at the early stages of sorghum (December) each year. C and N analyses were performed by dry combustion with a CN auto analyzer (LECO—TrueSpec®). Litter quality was expressed as C to N ratio. Crop aerial biomass, nitrate (0–0.20 m), soil temperature and roots (0–0.12 m) were determined at sowing and harvest of cover crops and sorghum with three replicates per plot. Crop aerial biomass was collected in a 0.25 m^2 area, oven-dried to constant weight at 60 °C. For root determination, 3 transects were established perpendicular to and in-between two crop rows in each plot, and soil samples were taken with a tubular auger (0.032 m diameter) at 4 points of each transect, coinciding the first and last point with a crop row. Samples were wet-sieved through a 500 μm mesh sieve, applying pressure with tap water. Roots retained by the sieve were oven-dried to constant weight at 60 °C and weighed. Total C and N determinations were made with a CN auto analyzer (LECO—TrueSpecR CN). The sum of roots in and between crop rows was considered total root biomass. Roots in crop rows correspond to average weight divided cylinder section ($\pi * \text{auger radio}^2 * 2$ samples) affected by the percentage of the area occupied by rows, while roots between rows were calculated in the same way but affected by the area of between-rows. N-nitrate was determined on field moist samples according to Cataldo et al. (1975). With this information, we calculated nitrogen-mineralization by applying the N budget equation for soil–plant systems (Powlson and Barraclough, 1993) where mineralized N is equal to N absorbed by plant and roots + N-nitrate at harvest—N-nitrate at planting – N fertilizer – N decomposition of residues. No gaseous N losses or rhizo-deposition inputs were included. Net N mineralization rates were calculated as the difference between N mineralization and NBM (0–0.12 m) according to Binkley and Vitousek (1989) and rates were expressed in term of days.

2.4. DNA extraction and real-time PCR quantification

Soil DNA was extracted from soil samples taken in 2010 and 2012, using Power Soil™ kit (MO BIO Laboratories Inc., USA) according to the manufacturer's instructions. We used real-time quantitative PCR on total DNA to determine total fungi and bacteria abundances. Standard curves were run in triplicate with at least five 10-fold serial dilutions of a plasmid containing

a full-length copy of the 16S and 18S rRNA genes from *E.coli*. Primer sets were R534 5′-ATTACCGCG GCTGCTGG3′ and 338P 5′-C CTACGGGAGGCAGCAG-3′ for 16S rRNA (Muyzer et al., 1993) and FU18S1 5′GGAAACTCACCAGGTCCAGA-3′ y SSU-1536 5′-ATTGCAATGCYCTATCCCCA-3′ for 18S rRNA (Borneman and Hartin, 2000; Plassart et al., 2008). qPCR assays were carried out in a 25 μl total reaction volume containing 12.5 μl of SYBR Green PCR Master Mix (Biodynamics SRL, Argentina), 0.5 μl of each forward and reverse primers (10 mM),0.5 μl of bovine serum albumin (10 μg ml^{-1}) and 10 ng ml^{-1} of soil DNA samples. Primer annealing temperature was optimized for PCR specificity in temperature-gradient PCR assays for each set of primers, using DNA Engine Opticon 2 System (MJ Research, USA). Optimal conditions for PCR were defined as 5 min at 94 °C, 35 cycles of 94 °C for 20 s, 55 and 56 °C annealing temperature for bacterial and fungal assays respectively for 20 s, 72 °C for 30 s and 82 °C for 3 s for both sets of primers.

Melting curve and gel electrophoresis analyses were performed to confirm that the amplified products were the appropriate size. We implemented the one-point calibration method proposed by Brankatschk et al. (2012) as an alternative method for absolute quantification from qPCR data that allowed reducing the data variation coefficient. The template concentration of a sample (Ns) was estimated from the cycle thresholds of the sample (CTs) and the standard (CTst) and their respective efficiencies (Es and Est) according to following equation: $Ns = Nst * (Est^{CTst}/Es^{CTs})$. With this information, we calculated bacterial and fungal relative abundances (Fierer et al., 2005) and the fungal to bacterial ratio.

2.5. Phospholipid fatty acids methyl ester extraction and analysis

Analysis of FAME profiles was performed to detect changes in soil microbial community composition in response to three years of no-till and cover crop implementation. The extraction used 8 g of soil in 20 mL of MeOH/$CHCl_3$/Buffer (2/1/0.8). After decantation and separation the organic phase was dried under N_2 flux and suspended in $CHCl_3$ (3 × 1 mL) and purified by chromatography in a silica column. The purified methanol phase was methylated by adding 1 mL of MeOH/Tol (1/1), 1 mL of KOH 0.2 M (MeOH), 2 mL of H_2O, and 0.3 mL of AcOH 1.0 M and heated during 15 min at 37 °C. After adding 50 μL of internal standard solution 10% P/V (Methyl nonadecanoate, C19:0, Sigma), the organic phase was extracted and kept in a freezer at −18 °C until analysis. A final volume of 30 μL was used for chromatography (GCMS Clarus 600, Perkin Elmer) in scan mode from m/z = 50 to m/z = 400, and data were acquired with TurboMass 5.4.2 Software. For the identification of peaks we used library spectra from NIST MS Search 2.0 and retention time and spectra from standard methylated phospholipids from bacteria (BAME, Sigma).

Fatty acid methyl esters described here use the standard nomenclature for lipid markers, A:Bω*C*, isomers are indicated with the suffixes *c* (cis) or *t* (trans) and cyclopropyl group with cy. Methyl branching groups are described by the prefixes *i* (iso) and a (anteiso). The microbial community composition was characterized according to 1. Branched-chain fatty acids indicative of Gram-positive bacteria (i15:0, a15:0, i16:0 and i17:0); 2. Monounsaturated fatty acids (16:1ω7*c*, 18:1ω9*c* and 18:1ω9t) plus hydroxy fatty acids (α14:0, β14:0 y α16:0) and cyclopropyl fatty acids (cy17:0) all indicative of Gram-negative bacteria; 3. Total bacteria represented the sum of the abundances of Gram-positive and Gram-negative biomarkers and saturated fatty acids 15:0 and 17:0 indicative of general bacteria; 4. Polyunsaturated 18:2ω6*c* was indicative of fungal biomass. Fatty acid concentrations (expressed as μgg $soil^{-1}$) were calculated and their percentages expressed in terms of the total fatty acid weights. The fungal sum divided by the bacterial sum represents the fungal to bacterial ratio (Frostegård and Bååth, 1996).

2.6. Shannon–Weaver index

The Shannon–Weaver biodiversity index was calculated as (Σp*i* ln p*i*); where p*i* is the peak area of *i*th peak over the area of all peaks for each treatment (Kaur et al., 2005).

2.7. Statistical analyses

Analyses of variance (ANOVA) were carried out using mixed linear model procedure for time-repeated measurements of longitudinal data. Means were compared using Fisher's test at a significance level of 0.05 (α). Multiple linear regressions analyses were used to predict best models to predict soil organic matter fractions using a stepwise elimination process. We also performed single linear regressions between the parameters that contributed to the multiple regression models with the purpose to compare treatments. Shannon–Weaver index means were compared with a Students *t*-test and a confidence interval of 95% was also calculated for each treatment. All statistical analyses were carried out using InfoStat software (Di Rienzo et al., 2009).

3. Results

3.1. Residue quality of different cover crops and sorghum

At the beginning of the experiment residue amount varied between 303 and 378 g DM m^{-2} with a C to N ratio of 38. Cover crop inclusion represented an increase of 50 to 70% of residue accumulation on soil surface after three years, compared to

Table 1
Plant residues on soil surface, cover crop roots at 0–0.012 m and their C to N ratios under no-till. Treatments were control without cover crops (C), rye (R), rye + 40N (R + N), vetch (V) and vetch + rye (VR). ± *Values indicate standard errors.*

Treatment	Residues (g DM m^{-2})		$C/N_{residues}$		Roots (g DM m^{-2})		C/N_{roots}	
2010								
C	205 ± 46	b	30 ± 2	c	71 ± 40	i	38 ± 3	ab
R	699 ± 46	a	43 ± 3	b	408 ± 23	g	31 ± 2	bc
R + N	647 ± 46	a	45 ± 4	b	346 ± 26	g	34 ± 2	b
V	643 ± 46	a	25 ± 2	cd	292 ± 23	g	18 ± 2	e
VR	675 ± 46	a	40 ± 3	b	566 ± 23	f	37 ± 2	ab
2011								
C	790 ± 86	c	61 ± 6	a	147 ± 33	hi	36 ± 2	ab
R	1268 ± 86	a	56 ± 5	a	628 ± 36	ef	36 ± 2	ab
R + N	1193 ± 86	a	61 ± 6	a	739 ± 38	cd	41 ± 2	a
V	981 ± 86	bc	38 ± 3	b	336 ± 34	g	18 ± 2	e
VR	1111 ± 86	ab	41 ± 3	b	701 ± 33	de	37 ± 2	ab
2012								
C	403 ± 91	b	40 ± 2	b	276 ± 71	gh	32 ± 3	bc
R	917 ± 91	a	38 ± 2	b	843 ± 41	bc	29 ± 2	c
R + N	869 ± 91	a	42 ± 2	b	905 ± 41	ab	27 ± 2	cd
V	748 ± 91	a	22 ± 1	d	652 ± 41	def	23 ± 2	d
VR	912 ± 91	a	28 ± 2	c	967 ± 41	a	29 ± 2	c
Time	<0.0001		<0.0001		<0.0001		<0.0001	
Treatment	<0.0001		<0.0001		<0.0001		<0.0001	
$T \times T$	0.3187		<0.0001		0.0022		<0.0001	

only a 20% increase in sorghum monoculture (control) under zero tillage. Differences between treatments were observed in the second year with higher values in rye treatments. Maximum residue levels observed in 2011 were associated to sorghum remnant of 910–1035 g MS m^{-2} (Table 1). Residue quality varied between treatments and years ($p < 0.0001$). The first year, VR, R and R + N treatments showed similar C to N ratios (40, 43 and 45 respectively) but higher values than V and C (25 and 30). Nevertheless, from the second year V and VR accumulated residues with better quality than R, R + N and control.

Root residues at 0–0.12 m also showed differences according to treatment and year ($p < 0.0022$) (Table 1). Sorghum monoculture had the lowest values of root biomass (between 71 and 276 g DM m^{-2}), and cover crop inclusion represented an extra input of roots with the highest values in rye treatments that ranged between 292 and 967 g DM m^{-2}, but also important amounts in V (292–652 g DM m^{-2}). Root quality in control was similar to rye treatments ranging from 27 to 41. While V had the lowest C to N ratios with values of 18 in 2010 and 2011 that increased significantly to 23 in 2012, rye treatments showed a significant decrease in C/N ratio the third year.

3.2. Soil organic matter pools and N mineralization rates

Total C increased at the end of the experiment at with higher values in V treatment 0–0.06 m ($p < 0.0015$). All treatments showed an increase in total C at 0.06–0.12 m depth ($p = 0.0003$). Total N showed an increase the third year in R + N, V and VR at 0–0.06 m depth ($p = 0.0992$) and in V treatment at 0.06–0.12 m and only the control showed a decrease at this depth ($p = 0.0894$) (Table 2).

Microbial biomass C accounted for 1–1.6% of the total soil C while MBN constituted 0.07 to 0.15% of total N. Both variables showed a rapid response to cover crops reaching highest values in VR at 0–0.06 m and in V at the deeper soil interval. Cover crop treatments also showed higher values of DOC than control mainly the first year at 0–0.06 m depth (0.0011) and all treatments evidenced decreases not only in this parameter but also in DON in the last year and in both depths ($p < 0.0001$) without detectable differences between treatments (Table 2). Particulate organic carbon represented 25% of total carbon in both depths. In general, particulate C (Cp) decreased in the third year at 0.06–0.12 m ($p = 0.0229$). Similar results were observed with particulate N at 0–0.06 m (0.0188). Soil organic C associated with mineral particles (Cm) was different between treatments and time at 0–0.06 m ($p = 0.0002$). Cm values in V, VR and R + N treatments increased at the end with differences between organic (V, VR) and inorganic (R + N) N source used. In addition, treatments with N (V, VR and R + N) also showed an increase in Nm at 0–0.06 m ($p = 0.0237$). At 0.06–0.12 m depth all treatments had higher values of Cm in the third year ($p < 0.0001$) but no differences between treatments and time in Nm values were found.

Soil microbial biomass, dissolved organic matter, soil temperature, moisture and water filled pore space were the variables that most explained variations in soil organic matter fractions (Table 3). DOC was a regression variable for both Cp and Np models at 0–0.06 m depth, but at 0.06–0.12 m, microbial biomass and roots were better regression variables. The relationship between Cp and DOC at 0–0.06 m depth was significant for all treatments except for R ($p = 0.4208$) and control showed higher slope than VR ($p < 0.0001$), V($p = 0.0002$) and R + N ($p = 0.0018$) (Fig. 1(a)). Similar treatment response was observed for the Np and DOC relationship, with higher slope in control than in V ($p = 0.0085$), VR (0.0354) and R + N

Table 2
Particulate, mineral associated and total C (Cp, Cm and CT respectively) and N (Np, Nm and NT), microbial biomass C (MBC) and N (MBN), and dissolved organic C (DOC) and N (DON) for 0–0.06 m and 0.06–0.12 m depth. Treatments were control without cover crops (C), rye (R), rye + 40N (R + N), vetch (V) and vetch + rye (VR). ± *Values indicate standard errors. Different letters indicate significant difference between treatments and years, according to the p values indicated, for each depth layer separately.*

	Treatment	Cp ($g\ m^{-2}$)		Np ($g\ m^{-2}$)		Cm ($g\ m^{-2}$)		Nm ($g\ m^{-2}$)		CT ($g\ m^{-2}$)		NT ($g\ m^{-2}$)		MBC ($g\ m^{-2}$)		MBN ($g\ m^{-2}$)		DOC ($g\ m^{-2}$)		DON ($g\ m^{-2}$)	
2010																					
0–0.06 m	C	399 ± 44	a	37 ± 2	a	1035 ± 44	cd	86 ± 4	cd	1434 ± 70	cd	123 ± 4	a	13 ± 1	d	0.9 ± 0.1	d	6 ± 0.7	c	0.5 ± 0.1	a
	R	391 ± 34	a	35 ± 2	a	1015 ± 34	cd	91 ± 4	bcd	1407 ± 54	cd	120 ± 4	a	19 ± 1	b	2 ± 0.1	a	9 ± 0.7	b	0.5 ± 0.1	a
	R + N	288 ± 29	a	31 ± 2	a	984 ± 29	d	82 ± 4	d	1272 ± 46	d	113 ± 4	b	16 ± 1	cd	1.4 ± 0.1	bc	9 ± 0.7	ab	0.7 ± 0.1	a
	V	341 ± 27	a	33 ± 2	a	898 ± 27	d	84 ± 4	d	1331 ± 43	d	116 ± 4	b	18 ± 1	bc	1.3 ± 0.1	c	10 ± 0.7	a	0.6 ± 0.1	a
	VR	360 ± 29	a	35 ± 2	a	1051 ± 29	cd	85 ± 4	cd	1411 ± 63	cd	120 ± 4	b	21 ± 1	ab	1.7 ± 0.1	ab	10 ± 0.7	ab	0.7 ± 0.1	a
0.06–0.12 m	C	355 ± 23	a	35 ± 2	a	1001 ± 44	b	97 ± 3	a	1356 ± 38	b	130 ± 4	a	8 ± 0.8	c	0.7 ± 0.1	f	9 ± 0.7	a	1 ± 0.2	a
	R	290 ± 28	a	29 ± 2	a	1014 ± 34	b	93 ± 3	a	1302 ± 44	b	121 ± 4	a	11 ± 0.8	ab	1.3 ± 0.1	cde	8 ± 0.7	a	0.6 ± 0.2	a
	R + N	338 ± 28	a	31 ± 2	a	1014 ± 29	b	91 ± 3	a	1352 ± 44	b	122 ± 4	a	12 ± 0.8	ab	1.3 ± 0.1	de	9 ± 0.7	a	0.8 ± 0.2	a
	V	335 ± 29	a	30 ± 2	a	1020 ± 27	b	89 ± 3	a	1355 ± 48	b	119 ± 4	b	13 ± 0.8	a	1.9 ± 0.1	a	7 ± 0.7	a	0.7 ± 0.2	a
	VR	300 ± 25	a	32 ± 2	a	997 ± 29	b	97 ± 3	a	1298 ± 41	b	129 ± 4	a	9 ± 0.8	bc	1.3 ± 0.1	cde	8 ± 0.7	a	0.9 ± 0.2	a
2012																					
0–0.06 m	C	288 ± 27	a	31 ± 2	a	1097 ± 27	cd	90 ± 3	cd	1385 ± 44	d	121 ± 4	a	18 ± 1	bc	1.4 ± 0.1	a	3 ± 0.4	d	0.3 ± 0.05	b
	R	336 ± 31	a	31 ± 2	a	1058 ± 31	cd	93 ± 3	bcd	1394 ± 49	d	124 ± 4	a	19 ± 1	b	1.9 ± 0.1	a	3 ± 0.4	d	0.3 ± 0.05	b
	R + N	351 ± 27	a	30 ± 2	a	1179 ± 27	b	100 ± 3	ab	1530 ± 44	bc	130 ± 4	a	20 ± 1	b	1.8 ± 0.1	bc	2 ± 0.4	d	0.2 ± 0.05	b
	V	377 ± 31	a	33 ± 2	a	1311 ± 31	a	103 ± 3	a	1687 ± 50	a	136 ± 4	a	21 ± 1	ab	1.8 ± 0.1	a	2 ± 0.4	d	0.3 ± 0.1	b
	VR	326 ± 25	a	31 ± 2	a	1297 ± 25	a	103 ± 3	a	1622 ± 41	ab	135 ± 4	a	24 ± 1	a	1.9 ± 0.1	a	2 ± 0.4	d	0.4 ± 0.1	b
0.06–0.12 m	C	280 ± 22	a	28 ± 2	a	1114 ± 27	a	90 ± 3	a	1394 ± 34	a	117 ± 4	b	16 ± 1	a	1.7 ± 0.1	ab	2 ± 0.3	b	0.3 ± 0.04	b
	R	303 ± 28	a	30 ± 2	a	1165 ± 31	a	93 ± 3	a	1475 ± 44	a	123 ± 4	a	17 ± 1	a	1.1 ± 0.1	e	2 ± 0.3	b	0.2 ± 0.04	b
	R + N	261 ± 28	a	27 ± 2	a	1113 ± 27	a	95 ± 3	a	1373 ± 44	a	122 ± 4	a	16 ± 1	a	1.6 ± 0.1	abc	2 ± 0.3	b	0.3 ± 0.04	b
	V	337 ± 24	a	32 ± 2	a	1193 ± 31	a	98 ± 3	a	1530 ± 38	a	130 ± 4	a	18 ± 1	a	1.3 ± 0.1	cde	2 ± 0.3	b	0.3 ± 0.04	b
	VR	293 ± 23	a	30 ± 2	a	1165 ± 25	a	97 ± 3	a	1457 ± 36	a	127 ± 4	a	18 ± 1	a	1.5 ± 0.1	bcd	2 ± 0.3	b	0.3 ± 0.04	b
0–0.06 m	Time	0.5406		0.0188		<0.0001		<0.0001		<0.0001		0.0001		<0.0001		0.0001		<0.0001		<0.0001	
	Treatment	0.3241		0.5712		<0.0001		0.4259		0.0006		0.4497		0.0004		<0.0001		0.0139		0.4014	
	$T \times T$	0.0824		0.6071		0.0002		0.0237		0.0015		0.0992		0.0079		0.0514		0.0011		0.5349	
0.06–0.12 m	Time	0.0857		0.1985		<0.0001		0.8324		0.0003		0.7809		<0.0001		0.0428		<0.0001		<0.0001	
	Treatment	0.3976		0.8881		0.2128		0.5727		0.1960		0.4301		<0.0001		0.0025		0.6497		0.5706	
	$T \times T$	0.1328		0.2370		0.5840		0.0915		0.2713		0.0894		0.0547		<0.0001		0.8400		0.6593	

Table 3
Multiple regression models for soil organic matter fractions at 0–0.06 and 0.06–0.12 m depth. Letter subscript "p" refers to particulate organic matter and "m" to organic matter associated with soil mineral particles. SE: standard error.

y	x	Estimated value	SE	p value	R^2
Cp at 0–0.06 m (g m^{-2})	Constant	253.7	29.8	0.0007	0.29
	DOC (g m^{-2})	22.9	1.9	0.0175	
	WFPS (%)	3.3	10.8	0.0443	
	MBN (g m^{-2})	−34.5	0.5	0.0660	
Cp at 0.06–0.12 m (g m^{-2})	Constant	321.7	19.0	<0.0001	0.17
	Moisture at 0–0.20 m (mm)	−1.7	0.3	<0.0001	
	MBC (g m^{-2})	4.2	0.9	<0.0001	
Np at 0–0.06 m (g m^{-2})	Constant	28.5	0.8	<0.0001	0.25
	DOC (g m^{-2})	1.02	0.2	<0.0001	
Np at 0.06–0.12 cm (g m^{-2})	Constant	25.2	2.7	<0.0001	0.20
	Moisture at 0–0.20 m (mm)	−0.26	0.1	<0.0001	
	MBC (g m^{-2})	0.35	0.1	0.0190	
	N-nitrate at 0–0.20 m (g m^{-2})	3.9	1.1	0.0007	
	WFPS (%)	0.26	0.1	0.0015	
	Roots (g MS m^{-2})	−0.02	0.0	0.0332	
Cm at 0–0.06 m (g m^{-2})	Constant	558.3	67.8	<0.0001	0.43
	Moisture at 0–0.20 m (mm)	9.0	0.9	<0.0001	
	N-nitrate at 0–0.20 m (g m^{-2})	132.2	23.6	<0.0001	
	MBC (g m^{-2})	5.5	2.4	0.0249	
Cm at 0.06–0.12 m (g m^{-2})	Constant	1226.9	96.7	<0.0001	0.14
	Soil temperature	−7.2	2.6	0.0058	
	MBC (g m^{-2})	4.7	2.5	0.0588	
Nm at 0–0.06 m (g m^{-2})	Constant	59.2	4.5	<0.0001	0.32
	Moisture at 0–0.20 m (mm)	0.66	0.1	<0.0001	
	N-nitrate at 0–0.20 m (g m^{-2})	8.3	1.9	<0.0001	
Nm at 0.06–0.12 cm (g m^{-2})	Constant	85.0	4.1	<0.0001	0.03
	N-nitrate at 0–0.20 m (g m^{-2})	4.5	1.9	0.0163	
	WFPS (%)	0.14	0.1	0.0549	

treatments ($p = 0.0287$) (Figure b). MBN also explained Cp, but resulted in a negative relationship for V ($p = 0.0050$) and VR treatments ($p = 0.0016$) (Fig. 1(c)). At 0.06–0.12 m depth, linear regressions between Cp and MBC were significant for R + N ($p = 0.0234$), V ($p = 0.0434$) and VR ($p = 0.0290$) treatments (Fig. 1(d)). These treatments also showed a positive relationship between Np and MBC with similar slope (Fig. 1(e)). Multiple regression models for Cm also included MBC as a variable in both depths (Table 3). Linear regressions confirmed a positive relationship between MBC and Cm as shown in the multiple regression models ($p = 0.0003$ and $p < 0.0001$ for 0–0.06 and 0.06–0.12 m respectively) (Fig. 2(a), (b)). Differences between treatments were found only at 0.06–0.12 m with higher slopes ($p = 0.09$) in V and VR compared to control (Fig. 2(c)).

Net mineralization rates were obtained from the difference between gross mineralization and MBN in the upper 0.12 m depth. Negative values of this parameter indicate net N immobilization. The values varied between −0.01 and 0.04 g $m^{-2}d^{-1}$, equivalent to −0.9 to 5.7% of total soil N. Differences between treatments were observed in both years ($p < 0.0001$) with a generalized increase towards the third year ($p < 0.0001$). The first year, V showed the highest mineralization rate of all treatments and R + N was the only treatment with negative values, and in the third year, VR showed higher rates (Fig. 3(a)). We found a linear positive relationship between net N mineralization rates and Cm in the first 0.12 m depth (Fig. 3(b)).

3.3. Changes in microbial community structure

Quantitative PCR analysis revealed that cover crop inclusion altered bacterial and fungal relative abundances and this response varied through time at 0–0.06 m ($p < 0.0001$) and 0.06–0.12 m depth ($p = 0.0002$) (Table 4). The first year of the experiment R showed higher fungal abundance than the control treatment while bacteria were more abundant in V and VR treatments at 0–0.06 m depth. However, at 0.06–0.12 m, V also showed more fungal abundance than VR with no differences between other treatments. At the end of the experiment, all treatments showed a significant increase in bacteria abundance in both depths. Likewise, in V and VR treatments fungi to bacteria ratio also suggested bacteria dominance (F:B < 1) during the three years at 0–0.06 m and in VR at 0.06–0.12 m (Fig. 4). Control and cover crop treatments with rye alone or fertilized (R and R + N) with an initial community dominated by fungi (F:B > 1) evidenced a significant decrease in this ratio the third year in both depths (p values of 0.0089 at 0–0.06 m and $p = 0.0037$ at 0.06–0.12 m). Moreover, fungal to bacterial ratio showed a negative relationship with net N mineralization rate (Fig. 5).

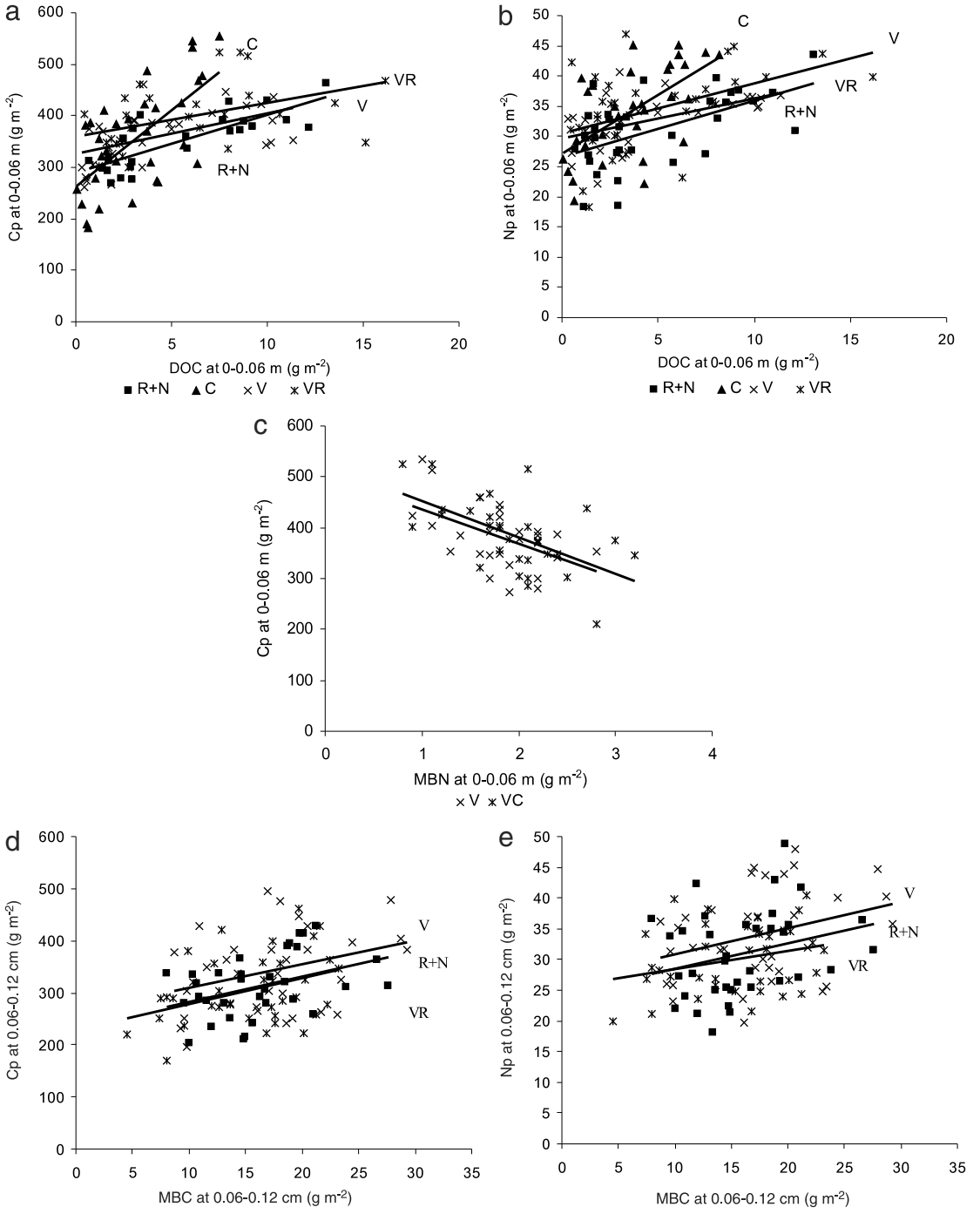

Fig. 1. Linear regressions between particulate organic matter (Cp and Np) and dissolved organic carbon (DOC), microbial biomass (MBC and MBN) at 0–0.06 m (a; b; c) and 0.06–0.12 m (d; e) depths.

3.4. Changes in soil fatty acids methyl ester profiles and Shannon–Weaver index

Three years under no-till with cover crops showed differences in relative abundance of Gram positive bacteria compared to control without cover crops. At 0–0.06 m depth all cover crop treatments had higher values of Gram positive bacteria except for the fertilized treatment (R + N) while at 0.06–0.12 m, higher values were observed in R and V. Dissolved organic nitrogen explained in part the observed variations in Gram positive abundance ($R^2 = 0.68$; $y = 39.8x - 0.9$; $p = 0.0009$). Fungal to bacterial ratio indicated bacteria dominance at both depths and only revealed treatment differences between VR with lower values compared to C, R and R + N at 0–0.06 m depth. Shannon–Weaver Index was highest in V, indicating more

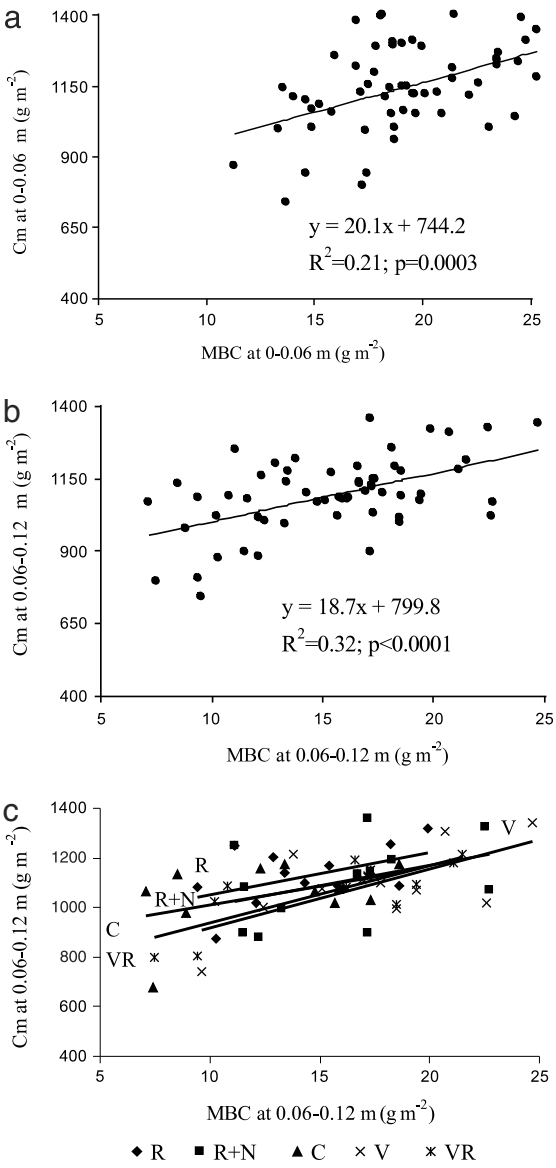

Fig. 2. Relationships between soil organic carbon associated with mineral particles (Cm), and soil microbial biomass carbon (MBC) at 0–0.06 (a) and 0.06–0.12 m (b; c) depths. Treatments were control without cover crops (C), rye (R), rye + 40N (R + N), vetch (V) and vetch + rye (VR).

biodiversity than control ($p = 0.0542$) at 0–0.06 m, and lowest in R + N with differences against V ($p = 0.019$) and VR ($p = 0.1$) at this depth. No differences in diversity were found at 0.06–0.12 m depth.

4. Discussion

In simplified systems such as agroecosystems based on crop monocultures, soil biota can suffer a reduction in its size and in its diversity. Both would reduce soil biological activity and the associated ecosystem functions. The recovery of these parameters through soil management have been postulated, and comparative studies of different management system showed the benefits of cover crops for MBC, but the time span involved for the restoration of soil health has not yet been studied. Our results showed a rapid response of soil microbial biomass size and its community composition to cover crop inclusion due to residue inputs and an active rhizosphere throughout the year (Djigal et al., 2012; Lehman et al., 2012).

Cropping sequences with low quality residues (rye alone or with urea, and sorghum monoculture) showed an apparent microbial succession throughout the three-year experiment of no-till, with a community initially dominated by fungi that changed to a community dominated by bacteria. Interestingly, successional changes observed in crop sequences with vetch alone or in consociation with rye (high residue quality) led to an early establishment of an apparent stable community dominated by bacteria. Under laboratory conditions similar successional changes had been observed (Baumann et al., 2009).

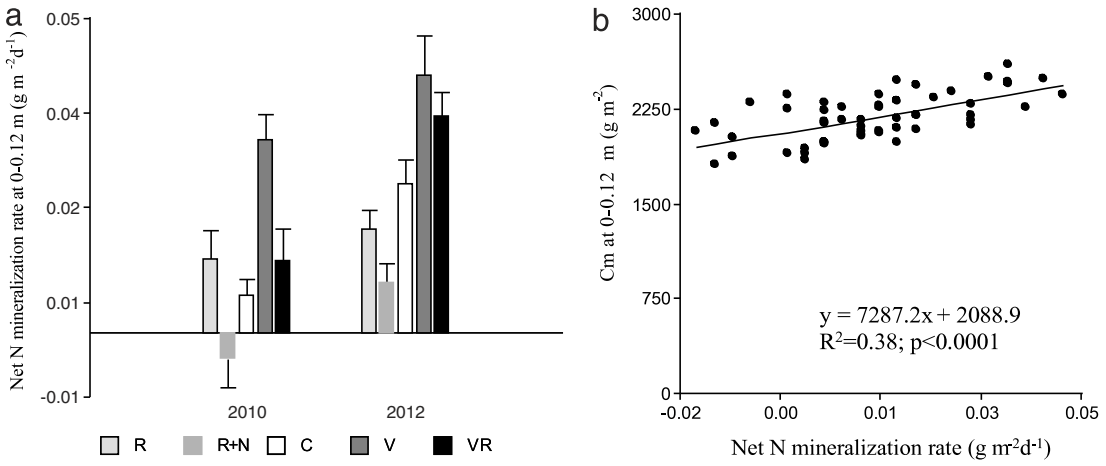

Fig. 3. Net N mineralization rates at 0.12 m the first and third year under no-till with and without cover crops (a) and its relationship with mineral associated carbon (Cm) (b). Treatments were control without cover crops (C), rye (R), rye + 40N (R + N), vetch (V) and vetch + rye (VR). *Bars indicate standard errors.*

Table 4
Relative abundances of bacteria and fungi under no-till with or without cover crops at the beginning (2010) and the end (2012) of the experience. Treatments were control without cover crops (C), rye (R), rye + 40N (R + N), vetch (V) and vetch + rye (VR). *Letters indicate significant differences between treatments and years and ± value indicates standard error.*

Depth	Treatment	Bacteria		Fungi	
2010					
0–0.06 m	C	0.35 ± 0.04	f	0.65 ± 0.04	b
	R	0.17 ± 0.04	g	0.83 ± 0.04	a
	R + N	0.35 ± 0.04	f	0.65 ± 0.04	b
	V	0.61 ± 0.04	de	0.39 ± 0.04	cd
	VR	0.59 ± 0.04	de	0.41 ± 0.04	cd
0.06–0.12 m	C	0.35 ± 0.04	d	0.65 ± 0.04	a
	R	0.36 ± 0.04	d	0.64 ± 0.04	a
	R + N	0.30 ± 0.04	d	0.70 ± 0.04	a
	V	0.32 ± 0.04	d	0.68 ± 0.04	a
	VR	0.61 ± 0.04	c	0.39 ± 0.04	b
2012					
0–0.06 m	C	0.89 ± 0.04	ab	0.11 ± 0.04	fg
	R	0.69 ± 0.04	cd	0.31 ± 0.04	de
	R + N	0.57 ± 0.04	e	0.43 ± 0.04	c
	V	0.78 ± 0.04	bc	0.22 ± 0.04	ef
	VR	0.91 ± 0.04	a	0.09 ± 0.04	g
0.06–0.12 m	C	0.91 ± 0.04	a	0.09 ± 0.04	d
	R	0.78 ± 0.04	b	0.22 ± 0.04	c
	R + N	0.89 ± 0.04	a	0.11 ± 0.04	d
	V	0.81 ± 0.04	ab	0.19 ± 0.04	cd
	VR	0.85 ± 0.04	ab	0.15 ± 0.04	cd
0–0.06 m	Time	<0.0001		<0.0001	
	Treatment	<0.0001		<0.0001	
	$T \times T$	<0.0001		<0.0001	
0.06–0.12 m	Time	<0.0001		<0.0001	
	Treatment	0.0004		0.0003	
	$T \times T$	0.0002		0.0002	

However, we are not aware of any study that showed temporal variations of microbial community composition in response to no-till and cover crop practices under field conditions.

Our results demonstrated that the continuity of residue input could drive soil community composition to a bacterial dominance independent of residue quality. Several previous studies postulated that fungal population would be favored by the presence of low quality residues (Bossuyt et al., 2001; Frey et al., 1999; Holland and Coleman, 1987; Kramer et al., 2012; Six et al., 2006; Thiet et al., 2006). However, our results induce to question whether fungi dominance in older no-till systems is really an indicator of insufficient residue supply to soil biota or that they are dominating simply because fungi are more resistant to adverse conditions. Fungi were important components of soil microbial community at 0.06–0.12 m

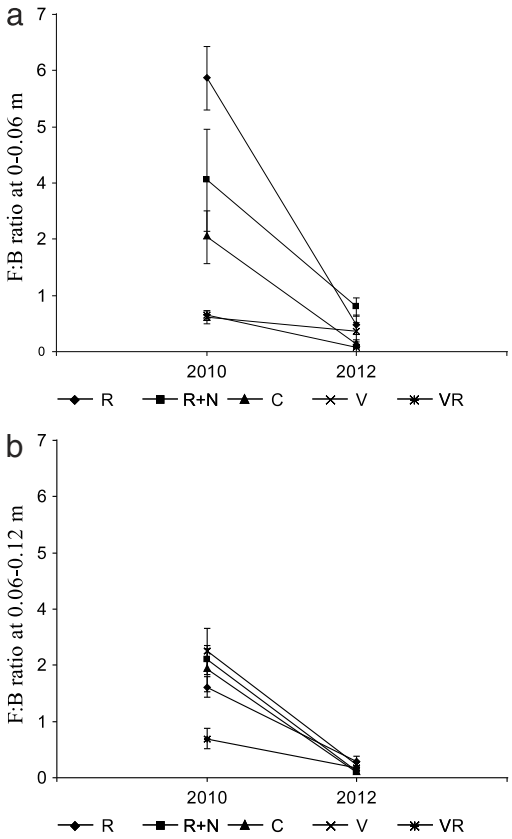

Fig. 4. Change of fungal to bacterial ratio from first to last year of the experiment at 0–0.06 m (a) and 0.06–0.12 m (b) depths. Treatments were control without cover crops (C), rye (R), rye + 40N (R + N), vetch (V) and vetch + rye (VR). *Bars indicate standard errors.*

in the vetch–sorghum sequence, probably related to arbuscular mycorrhizal fungi associations (Galvez and Douds, 1995), suggesting that at this depth rhizosphere microorganisms and their interactions with plants are more important than residue quality at least in the short term. Apparently, the methodology used to quantify fungal to bacterial ratio could lead to contradictory results (see review by Strickland and Rousk, 2010). In that sense, our study demonstrated that the DNA-based measurement (real-time PCR quantification) was a more robust method than fatty acid measurements to detect differences between treatments, but both methods demonstrated bacterial dominance after three years of no-till with or without cover crops. Similarly, Ferrari et al. (2015) and González-Chávez et al. (2010) also found a higher predominance of bacteria under no-till using the same methods although these authors did not compare both methods in the same study.

The higher availability of soluble N compounds in crop sequences that included vetch partly explained the increase in Gram-positive bacteria in the soil ($R^2 = 0.58$), and this was associated to higher Np and MBC. Recent studies reported similar results regarding the relative abundance of Gram-positive bacteria in no-till systems with cover crops (Mbuthia et al., 2015), whereas others found that cover crops in rotation with tomato decreased the proportion of Gram-positive bacteria in soil, and that vetch actually favored Gram-negative bacteria (Buyer et al., 2010). The response of the Gram-positive bacteria to cover crops might be explained by the difference in spatial location of Gram positive and Gram negative bacteria according to Hattori (1988). This author found that the former colonize more in large pores and the outer area of aggregates while Gram-negative bacteria were more abundant in the inner area of aggregates and therefore would be more protected against predators (protozoa) and changes in the soil environment.

The vetch treatment had the highest Shannon–Weaver diversity index, while the fertilized treatment had the lowest index (Table 5). This detrimental effect of synthetic fertilizers affected Gram + bacteria to a higher degree than other functional groups. Some recent studies already reported diminished microbial diversity as an effect of fertilizer application (Allison et al., 2007; He et al., 2013; Jangid et al., 2008; Sradnick et al., 2013).

Increases in the relative abundance of fungi in the soil microbial community have been associated with reduced rates of nutrient cycling and increased soil C and N sequestration (Bardgett et al., 2005; de Vries et al., 2006; Högberg, 2007). However, our results indicated that the vetch–sorghum rotation with high net N mineralization rates and a more diverse microbial community dominated by bacteria sequestered more C and N in stable soil organic matter fractions at the end of the 3-year experiment than no-till sorghum alone or with rye cover crops which had lower N turnover rates. Recent studies point to the existence of an intimate relationship between microbial activity and C sequestration (Bradford et al., 2013;

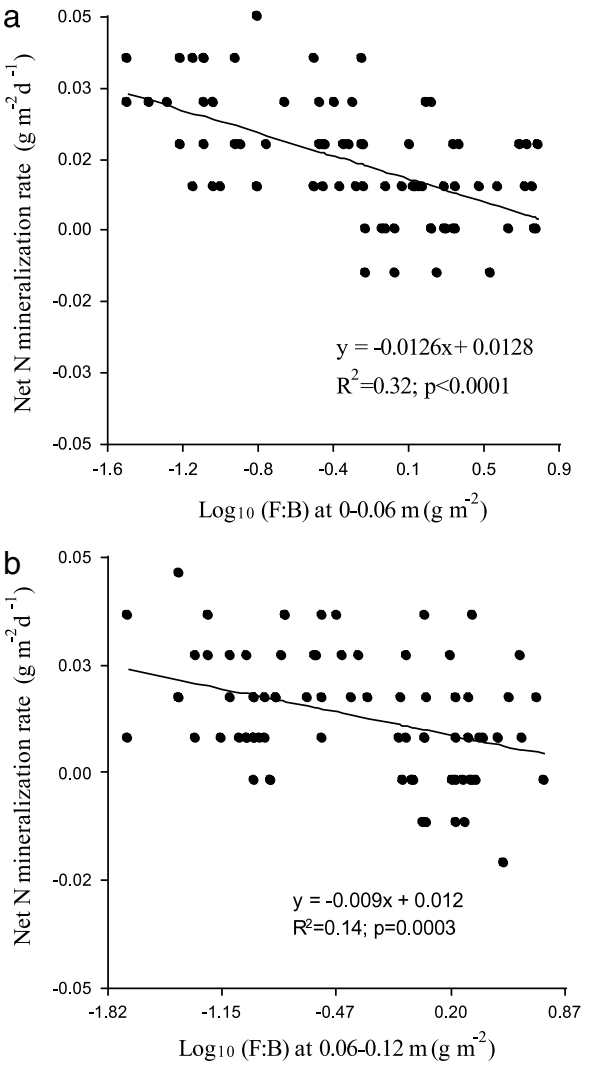

Fig. 5. Relationship between net N mineralization rate at 0–0.12 m depth and Log_{10} of fungal to bacterial ratio at 0–0.06 m (a) and 0.06–0.12 m (b) depth.

Table 5
Effects of three years under no-till with and without cover crop on relative abundance of different fatty acid biomarkers (% of total). Gram+ (G+) and Gram− (G−) bacteria, total bacteria, total fungi, fungal to bacterial ratio (F:B), and Shannon–Weaver Index (ShaW) at 0–0.06 and 0.06–0.12 m. Treatments were control without cover crops (C), rye (R), rye + 40N (R + N), vetch (V) and vetch + rye (VR). CI is the confidence interval at 95%. *Letters indicate differences between treatments for each soil depth* ($p < 0.05$).

Treatment	G+		G−		Bacteria		Fungi		F:B ratio		ShaW	CI
0–0.06 m												
C	7.8 ± 2.3	bc	18.1 ± 4	a	25.9 ± 5.1	ab	2.3 ± 0.6	a	0.09 ± 0.01	a	1.58	±0.16
R	15.0 ± 2.3	a	16.8 ± 4	a	33.1 ± 5.1	a	2.6 ± 0.6	a	0.08 ± 0.01	a	1.67	±0.39
R + N	6.5 ± 2.3	c	10.9 ± 4	a	18.8 ± 5.1	b	1.7 ± 0.6	a	0.08 ± 0.01	a	1.47	±0.14
V	13.0 ± 2.3	ab	19.2 ± 4	a	36.6 ± 5.1	a	2.1 ± 0.6	a	0.06 ± 0.01	ab	1.89	±0.16
VR	19.1 ± 2.3	a	19.1 ± 4	a	38.4 ± 5.1	a	1.3 ± 0.6	a	0.03 ± 0.01	b	1.74	±0.21
0.06–0.12 m												
C	7.2 ± 2.0	bc	17.9 ± 2.9	a	25.4 ± 4.4	a	3.0 ± 0.7	a	0.12 ± 0.02	a	1.63	±0.19
R	18.7 ± 2.0	a	17.8 ± 2.9	a	36.8 ± 4.4	a	3.4 ± 0.7	a	0.09 ± 0.02	a	1.75	±0.20
R + N	8.7 ± 2.0	bc	15.3 ± 2.9	a	26.9 ± 4.4	a	1.9 ± 0.7	a	0.07 ± 0.02	a	1.58	±0.23
V	11.2 ± 2.0	ab	19.1 ± 2.9	a	34.8 ± 4.4	a	3.6 ± 0.7	a	0.11 ± 0.02	a	1.84	±0.11
VR	5.3 ± 2.0	c	20.2 ± 2.9	a	26.2 ± 4.4	a	2.8 ± 0.7	a	0.11 ± 0.02	a	1.53	±0.14

Kong et al., 2011), which was confirmed by the positive relationship between soil microbial biomass and mineral associated organic matter in our data.

Interestingly, sequences with legume were more efficient in C stabilization associated with mineral particles (higher slope) evidencing the important role of nitrogen in the soil organic matter build-up. A recent study (Kallenbach et al., 2015) demonstrated that transformation of plant C into microbial biomass could be an effective strategy for building SOC in agricultural soils. A possible pathway of this transformation might be the use of DOC and Cp by the microbial biomass, as shown by the linear relationships between DOC or MBC and Cp found for our data (Fig. 1). These results reaffirm the importance of enhanced soil biodiversity for maintaining the crucial soil ecosystem functioning and services such as pointed out by Creamer et al. (2015) in a recent study across the most important biomes of Europe. It appears from our results that the supply of high amounts of N-rich residues such as provided by grass–legume cover crops could fulfill this objective.

5. Conclusions

Our results indicated that it is feasible to manage agricultural systems that have higher soil microbial diversity, better nutrient cycling, and more C and N sequestration by increasing the aboveground diversity, maintaining an active rhizosphere throughout the year and by including a biological nitrogen source (legume crop). The inclusion of grass–legume cover crops in intensive cash cropping- or animal fodder production systems could be a way of reversing the degradation of soils and ecosystem services that is currently associated to this kind of land-use.

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