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Cover crops prevent the deleterious effect of nitrogen fertilisation on bacterial diversity by maintaining the carbon content of ploughed soil

Julien Verzeaux [a,*], Abdelrahman Alahmad [a], Hazzar Habbib [a], Elodie Nivelle [a], David Roger [a], Jérôme Lacoux [a], Guillaume Decocq [a], Bertrand Hirel [b], Manuella Catterou [a], Fabien Spicher [a], Frédéric Dubois [a], Jérôme Duclercq [a], Thierry Tetu [a]

[a] Ecologie et Dynamique des Systèmes Anthropisés (EDYSAN, FRE 3498 CNRS UPJV), Laboratoire d'Agroécologie, Ecophysiologie et Biologie intégrative, Université de Picardie Jules Verne, 33 rue St Leu, 80039 Amiens Cedex, France

[b] Adaptation des Plantes à leur Environnement, Unité Mixte de Recherche 1318, Institut Jean-Pierre Bourgin, Institut National de la Recherche Agronomique, Centre de Versailles-Grignon, R.D. 10, F-78026 Versailles Cedex, France

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ABSTRACT

Synthetic nitrogen (N) fertilisers are widely used for enhancing agrosystem productivity and are thus thought to increase organic inputs from crop residues. However, many crop rotations have a low amount of organic residue returned to the soil since the whole aboveground crop biomass is harvested and exported. To compensate for such organic outputs and to improve soil quality, the introduction of winter cover crops in rotations has been suggested. A 4-year controlled field experiment was conducted to quantify the respective and combined effects of chemical N fertilisation and winter cover crops on plant productivity, organic carbon (C) and N inputs from crop residues and cover crops, changes in soil C and N concentrations, C:N ratio, soil mineral N, pH, soil moisture and soil bacterial biodiversity. A ploughing tillage system with low organic input was assessed, for which the main crops were spring wheat, green pea, forage maize, along with cover crops of different legume and non-legume species.

N fertilisation did not have an impact on the aboveground biomass except following forage maize. Cover crops increased the total amount of C and N inputs, irrespective of N fertilisation which had no significant effect. The soil N concentration decreased in all treatments, particularly when N fertilisers were applied under bare fallow conditions. The latter treatment also caused decreased soil C concentrations (slightly increased in the other treatments) and decreased bacterial biodiversity (no change in the other treatments). Bacteria from the Proteobacteria and Bacterioidetes phyla were highly correlated with soil from fertilised bare fallow conditions. While Verrucomicrobia was characteristic of non-fertilised bare fallow soils, Acidobacteria and Cyanobacteria were associated with the high C and N concentrations present in soils following cover crop treatments.

Taken together, these results demonstrate that in ploughing systems, under low organic restitution regimes, intensive N fertilisation decreases the diversity of the bacterial soil community and reduces soil C and N concentrations, but only in bare fallow conditions. There is a protective effect of winter cover crops against the deleterious effect of chemical N fertilisation on soil biodiversity and nutrient cycling, since they can maintain soil C and N concentrations. The use of winter cover crops containing legumes is thus a practice that is able to meet the criteria of a sustainable agriculture.

1. Introduction

Several studies have reported that there is a decline in soil organic carbon (C) content worldwide and especially in Europe (Capriel, 2013; Heikkinen et al., 2013). Nevertheless, most cultivated soils are still tilled using the moldboard ploughing technique (Higashi et al., 2014; Triplett and Dick, 2008), which is known to provoke deleterious effects on both soil carbon content and on soil living organisms (Kladivko, 2001; Jacobs et al., 2009; Leite et al., 2009; Nyamadzawo et al., 2009). An increase in the soil organic C pool can be obtained by the use of cover crops, which provide additional C through better energy conversion during crop rotation (Sainju et al., 2007; Smith et al., 2014). In addition, cover crops improve aboveground biodiversity (Balota et al., 2014; Calderon et al., 2016) and provide substantial amounts of N when they are composed of legume species (Kramberger et al., 2014). In conventional crop cultivation systems, the increase in crop productivity by mineral N fertilisers can lead to an increase in N and C inputs into the soil (Sainju et al., 2002, 2006;

* Corresponding author.
E-mail addresses: julien.verzeaux@u-picardie.fr (J. Verzeaux), jerome.duclercq@u-picardie.fr (J. Duclercq), thierry.tetu@u-picardie.fr (T. Tetu).

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Bakht et al., 2009; Mazzoncini et al., 2011), depending on the type of crop rotation. Intensive N fertilisation can also promote soil organic matter (SOM) mineralisation through modifications of soil microbe communities (Jenkinson et al., 1985; Kuzyakov et al., 2000; Kuzyakov, 2010; Majumder and Kuzyakov, 2010). However, the impact of fertilisers on soil microbial diversity is complex because it involves several factors such as the type and the amount of fertiliser applied, as well as the nature of the soil and the crop rotation (Lupwayi et al., 2012). Generally, N fertilisation induces a loss of microbial diversity and modification of the composition of the bacterial community (Ramirez et al., 2010; Coolon et al., 2013; Cederlund et al., 2014; Willekens et al., 2014; Zhao et al., 2014).

The impact of N fertilisation (Shen et al., 2010; Gomez and Garland, 2012; Zhao et al., 2014; Sun et al., 2015; Zhou et al., 2015; Zeng et al., 2016) and the use of winter cover crops (Carrera et al., 2007; Liu et al., 2007; Nair and Ngouajio, 2012) have not been studied previously in an integrated manner. In particular, soil C sequestration, soil N content and microbial diversity in ploughing-based agricultural systems have received little attention. We thus developed a field experiment to quantify the individual and synergistic effects of cover crops and chemical N fertilisation on the soil microbial community and soil C and N contents under tillage conditions. We found that over a short period of time, intensive N fertilisation reduced both soil C and N contents. We also observed that under high N fertilisation input, the diversity of the soil microbial community decreased. In addition, we found that the detrimental effect of intensive N fertilisation on both soil properties and soil microbial communities can be substantially reduced when using a cover cropping system.

2. Materials and methods

2.1. Site description and experimental design

The field experiment was conducted at the experimental site of La Woestyne, in North France (50°44′N, 2°22′E, 40 m a.s.l.). The average annual air temperature and total rainfall were 10.5 °C and 675 mm, respectively, with amounts of rainfall relatively homogeneous across seasons. Soil particle size composition was characterized by silt 66.8%, clay 21.2% and sand 12%. The concentrations of organic C, total N and SOM before the beginning of the experiment were 13.1 g kg^{-1}, 1.5 g kg^{-1} and 22.5 g kg^{-1} respectively (see Table 1 for more characteristics).

Prior to the start of the experiment in 2009, the field was managed using a chisel plough and rotary power system. A crop rotation method for which organic restitution was known for being low and weakly affected by N fertilisers was employed to highlight the effect of N fertiliser on biodiversity and soil C and N concentrations (Fig. 1). It consisted of spring wheat (*Triticum aestivum* L.) in 2010, followed by green peas (*Pisum sativum* L.) in 2011, maize (*Zea mays* L.) in 2012, and spring wheat in 2013. In order to study the effect of fertilisation and cover crops on biodiversity and chemical parameters, the experimental field was split into four treatments with three replicate plots for each: bare fallow without (BFN0) or with (BFNX) N fertilisation; winter cover crops without (CCN0) or with (CCNX) N fertilisation. BFN0 and CCN0 plots measured 7 × 8 m, while BFNX and CCNX plots measured 14 × 8 m. The cover crops consisted of a mixture of three legumes and three non-legume species, which were sown as follows: 400 seeds m^{-2} of Egyptian clover (*Trifolium alexandrinum* L.), 30 seeds m^{-2} of faba bean (*Vicia faba* L.), 50 seeds m^{-2} of vetch (*Vicia sativa* L.), 80 seeds m^{-2} of flax (*Linum usitatissimum* L.), 200 seeds m^{-2} of phacelia (*Phacelia tanacetifolia* Benth.), 60 seeds m^{-2} of oat (*Avena sativa* L.). Before the main crops were sown, cover crops were buried by a conventional moldboard plough to a depth of 30 cm. Spring wheat was sown at a row spacing of 12.5 cm using an AS 400 drill (Alpego, Italia) combined with a rotating harrow and crop residues were returned to the soil. Green pea was sown at a row spacing of 17 cm using a Turbosem drill (Herriau, France) combined with a rotating harrow. According to European regulations, pea did not receive N fertilisation in NX treatments. Crop residues of green pea were returned to the soil. Maize was sown at a row spacing of 75 cm with a Maxima drill (Kuhn, France) following a rotating harrow. Maize was grown for silage, meaning that all the aboveground residues were removed from the field. The dose of N fertiliser applied in the NX treatments was determined according to the N budget method for maize (108 kg N ha^{-1}) and wheat (160 kg N ha^{-1}). N fertilisation was applied in the form of urea for both maize and wheat and crop protection was ensured conventionally.

Table 1
Main characteristics of the 0–10 cm soil layer before the beginning of the experiment in 2009.

Parameters (units)	Mean ± SE
pH in H_2O	6.28 ± 0.160
CEC[a] (cmol + kg^{-1})	11.67 ± 0.272
P_2O_5[b] (g kg^{-1})	0.13 ± 0.008
Organic C (g kg^{-1})	13.08 ± 0.042
Total N (g kg^{-1})	1.48 ± 0.004
SOM (g kg^{-1})	22.50 ± 0.007
Soil C:N ratio	8.85 ± 0.105

SOM: soil organic matter.
SE: standard error of the mean.
[a] Cation-exchange capacity (Metson method).
[b] Available phosphorus (Olsen method).

2.2. Total biomass and C and N inputs from main crops and cover crop residues

During the experiment, a 3 × 1 linear meter row of the main crop was sampled each year at the time of harvest, in each plot. The part of the plant that is commonly harvested (i.e., grains) was separated from the rest of the aboveground biomass (i.e., crop residues incorporated into the soil). Samples were oven-dried at 65 °C for 3 days and subsequently weighed (± 0.1 g accuracy) to determine total aboveground biomass. Each sample was then ground into a powder prior to C and N analysis using an elemental analyser (Flash EA 1112 series, Thermo Electron, Germany).

For winter cover crops, 3 × 1 m^2 was sampled each year in each plot, just before ploughing. Samples were oven-dried at 65 °C for three days and subsequently weighed. The total aboveground biomass was determined and then ground into a powder prior to C and N analysis. C and N inputs for each treatment since the beginning of the experiment were measured as the sum of the amounts of C and N in the crop residues plus the C and N in cover crops since 2009.

2.3. Soil sampling and chemical analyses

In late March 2013, 1 month after the last ploughing, 11 months after the last N application and >1 year after the last cover crop incorporation, six 10 cm deep soil cores were randomly collected using a 2 cm diameter auger in each of the three replicate plots. Samples 10 cm deep were chosen since previous studies indicated that it was an appropriate depth for the assessment of the effects of N additions on microbial communities (Coolon et al., 2013; Zeng et al., 2016), even in ploughed soils (Sun et al., 2015). The six soil cores were composited together into a single sample. Soils were then sieved using a 2 mm mesh and divided into two parts: the first one was stored at 4 °C to await chemical analysis, and the second one was stored at −20 °C until DNA extraction.

The soil moisture concentration was determined by oven-drying at 105 °C. Soil pH was measured using a pH meter FE20-FiveEasy™ (Mettler Toledo, Switzerland) at a ratio of 1:5 (weight/volume) of soil to distilled water, following shaking for 45 min. Nitrate (NO_3^--N) and ammonium (NH_4^+-N) were extracted at a ratio of 20 g fresh soil to 100 mL 1 M KCl. After shaking for 1 h, the extracts were centrifuged for 10 min at 4000 rpm, and the supernatants were analysed by a continuous flow analytical system (San^{++} system, Skalar, Holland). Total

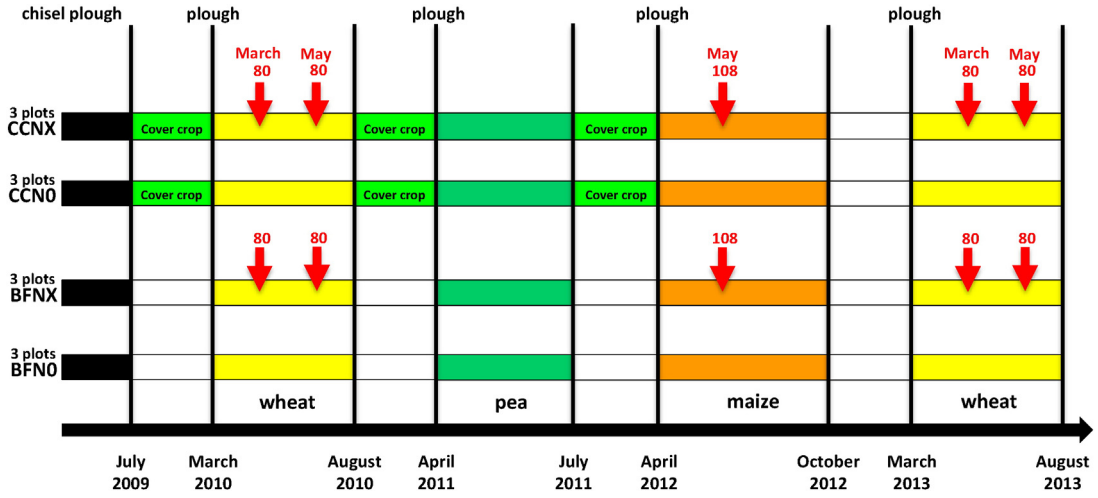

Fig. 1. Chronological representation of crop rotation, ploughing and N fertilisation over the 4-year experiment. CC: winter cover crop treatment with (NX) and without (N0) nitrogen fertilisation. BF: bare fallow treatment with (NX) and without (N0) nitrogen fertilisation; Red arrows indicate nitrogen fertilisation (with month of the year and amount in kg N ha^{-1}). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

soil C and total soil N concentrations were determined using the same elemental analyser as for biomass after drying at 35 °C for 48 h and ball milled using a grinder MM 400 (Retsch, Germany). Since the soil was free of carbonate, the soil organic C was assumed to be equal to the total C. Soil C and N analyses were carried out on samples collected from each of the 12 plots before the beginning of the experiment in 2009 and with the same method than in 2013. Changes in soil C and N concentrations were calculated as the difference between the soil C and N concentrations in March 2013 and the soil C and N concentrations in 2009.

2.4. Soil bacterial community analysis

2.4.1. Soil DNA extraction and PCR amplification

Prior to the pyrosequencing of 16S rRNA gene sequences, DNA was extracted and purified from 0.3 g of defrosted soil using the Fast DNA ™ Spin Kit for Soil kit (MP Biomedicals, USA) and the NucleoSpin® gDNA Clean-up XS kit (Macherey-Nagel, Germany), respectively. The total DNA concentration in each sample was fluorometrically quantified with the AccuBlue™ High Sensitivity dsDNA Quantitation Kit (Biotium, USA) using a monochromator based multimode microplate reader (Tecan Infinite® M1000 PRO, Tecan Systems, USA). From the purified DNA, the 16S V3 and V4 region was PCR amplified with the Bakt_341F (5′-CCTACGGGNGGCWGCAG-3′) and Bakt_805R (5′-GACTACHVGGGTATCTAATCC-3′) primers (Herlemann et al., 2011). The forward and reverse primers were designed containing overhang sequences compatible with Illumina Nextera XT index (forward primer overhang: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG, reverse primer overhang: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG). For each sample, 5 ng of DNA was used for a PCR of 25 μL, conducted under the following conditions: 95 °C for 3 min, 25 cycles of 30 s at 95 °C, 55 °C for 30 s and 72 °C for 30 s, followed by 5 min at 72 °C.

2.4.2. Purification and sequencing

Each synthesised amplicon was purified using the AMPure XP beads kit (Beckman Coulter, USA) and subsequently quantified with the AccuBlue™ High Sensitivity dsDNA Quantitation Kit. Illumina Nextera XT Index sequencing adapters were integrated into the amplicons by PCR (95 °C for 3 min; 8 cycles of 30 s at 95 °C, 55 °C for 30 s and 72 °C for 30 s, followed by 5 min at 72 °C for 5 min). The final libraries were purified once again with the AMPure XP beads before quantification with the AccuBlue™ High Sensitivity dsDNA Quantitation Kit. To validate the library, 1 μL of a 1:50 dilution of the final library was used on a Bioanalyzer DNA 1000 chip using a Bioanalyzer 2100 (Agilent Technologies, USA) to verify the library size and to check for the presence of primer dimer contamination. Purified libraries were pooled at equal molarity, denatured, diluted to 4pM, spiked with a premade PhiX control library at 5% (PhiX control v2, Illumina, USA) and loaded into a MiSeq v2 Reagent Kit (500 Cycles PE, Illumina, USA) to be sequenced in a MiSeq system (Illumina, USA). Illumina sequence reads in the FastQ format were uploaded into the Galaxy instance (v.2.3.0) of the Genotoul bioinformatics platform (http://sigenae-workbench.toulouse.inra.fr) to be used in the FROGS (Find Rapidly OTU with Galaxy Solution) pipeline (Auer et al., 2015). FLASH (Fast Length Adjustment of SHort reads, Magoc and Salzberg, 2011) was used to merge paired-end reads with a maximum of 10% mismatch in the overlapped region. A filter was set to select the contig sequence with a length between 340 and 450 nucleotides. During the FROGS pre-process, the sequences with ambiguous bases (N) and which did not contain good primers were removed. Cutadapt (Martin, 2011) was used to search and trim the primer sequences with <10% of differences. Clustering of the sequences was done using the SWARM algorithm (v2.1.5, Mahé et al., 2014). A first denoising step was carried out to build very fine clusters with minimal differences (d = 1) and a second one was completed with an aggregation distance of 3. The resulting representative seed for each cluster or OTU (Operational Taxonomic Units) was subjected to chimera detection and removal using the VSEARCH algorithm (v.1.9, https://github.com/torognes/vsearch). Taxonomic classification of each OTU was performed using RDPClassifier and BLAST tools against the non-redundant small subunit database from SILVA (v123). To identify differences in soil bacterial richness and diversity, Chao 1 (Chao, 1984) and ACE (abundance-based coverage estimators), as well as Shannon–Wiener evenness, Pielou equitability and Simpson diversity indices were computed using the *vegan* package (Oksanen et al., 2015) in *R* software v. 3.1.2 (R Development Core Team, http://www.R-project.org).

2.5. Statistical analysis

All statistical analyses were performed using *R* v. 3.1.2. Mean values are given with their standard error. Total aboveground biomass, soil C and N concentrations and bacterial diversity indices were compared among treatments using a non-parametric Kruskal–Wallis one-way analysis of variance (*H*-value) followed by a Dunn's *post hoc* test whenever significant. Correlations between environmental variables (aboveground biomass, C and N inputs, soil C and N) on the one hand and bacterial diversity indices and relative abundances of the most abundant phyla were computed using Pearson's product–moment correlation coefficient (Hmisc package). A non-metric multidimensional

Table 2
Total aboveground biomass of main crops (Mg ha^{-1} dry matter; mean ± standard error of the three plots per treatment).

	H	BFN0	BFNX	CCN0	CCNX
2010 Wheat	2.59 (NS)	9.67 ± 1.14	11.57 ± 0.64	10.53 ± 1.52	12.02 ± 0.53
2011 Pea	1.34 (NS)	8.35 ± 0.81	8.22 ± 0.67	8.40 ± 0.08	8.33 ± 0.21
2012 Maize	1.46 (NS)	24.97 ± 1.09	24.55 ± 1.58	25.19 ± 1.90	28.80 ± 2.90
2013 Wheat	8.44 (*)	6.71 ± 0.90b	14.45 ± 1.85a	7.84 ± 1.28b	15.32 ± 0.52a

H: value of the Kruskal–Wallis test with its significance in brackets (*$P < 0.05$). Letters give the result of a Dunn's *post hoc* test. BF: bare fallow; CC: winter cover crop; N0: no fertiliser; NX: chemical N fertilisation. NS: non-significant.

scaling (NMDS) of the plot × phyla (expressed as relative abundances) matrix was employed to visualize the differences in the composition of the bacterial phylum among treatments, in addition to the Bray–Curtis distance and the *vegan* package in R. To see how these compositional differences were related to environmental variables, the significant impacting variables were subsequently fitted onto the NMDS ordination using the 'envfit' function in *vegan*.

3. Results

3.1. Plant and soil parameters

Overall, N fertilisation and winter cover crops had no significant effect on aboveground biomass, except for spring wheat in 2013, when the biomass was doubled following the application of N ($P < 0.05$) and was nearly 1 Mg ha^{-1} greater for CC than for BF treatment, irrespective of N fertilisation (Table 2).

The two treatments including cover crops had significantly higher organic N and C inputs than the two BF treatments (Fig. 2). Overall, the total N input over the 4-year period was approximately 0.55 Mg ha^{-1} from CCNX, 0.50 Mg ha^{-1} from CCN0, 0.25 Mg ha^{-1} from BFNX and 0.20 Mg ha^{-1} from BFN0. C inputs followed the same trend as N inputs, with significantly greater values in CCN0 and CCNX than in BFN0 and BFNX treatments (8 to 9 Mg ha^{-1} and 5 to 6 Mg ha^{-1}, respectively; $P < 0.05$).

Under bare fallow conditions, (BFNX) N fertilisation decreased soil C and N concentrations, of 0.35 g kg^{-1} (against ca. 0.1 g kg^{-1} for the other treatments) and 1.5 g kg^{-1} (against an increase of 0.2 to 1.8 g kg^{-1} in the other treatments), respectively (Fig. 3). Winter cover crops tended to increase the soil C concentration, an effect that was particularly important in the absence of N fertilisation (+ 2 g kg^{-1} in CCN0). Soil moisture varied from 194 to 207 g kg^{-1}, pH from 6.81 to 6.95, NH_4^+-N from 0.1 to 4.5 mg kg^{-1} and NO_3^--N from 3.54 to 8.45 mg kg^{-1}, but there was no trend between treatments, nor a statistically significant comparison (data not shown).

3.2. Soil bacterial community structure

The sequencing of the bacterial 16S rRNA gene amplicons resulted in 5,943,622 sequences with an average length of 394 bp. >90% of the bacterial sequences were classified into four "major" phyla, namely, Actinobacteria, Chloroflexi, Firmicutes and Proteobacteria. The remaining OTUs were classified within 17 "minor" phyla, of which Acidobacteria, Bacteroidetes, Cyanobacteria, Planctomycetes and Verrucomicrobia represented from 1 to 3% of all bacterial sequences. Although they shared the same phyla, the soil subjected to the four treatments differed by the relative abundances of these phyla, especially in BF treatments (Fig. 4). Compared to BFN0, BFNX showed a clear shift in the relative abundance of Proteobacteria (from 20% to 60%), Actinobacteria (from 40% to 30%), Chloroflexi (from 20% to 5%), Firmicutes (from 8% to 3%), Planctomycetes (from 3% to 1%), Cyanobacteria (from 1.5% to 0.2%) and Verrucomicrobia (from 0.75% to 0.25%). By contrast, soil that had received the two CC treatments exhibited very similar composition.

In addition, N fertilisation had no effect on the bacterial taxonomic richness of the soil but significantly reduced the bacterial diversity, evenness and equitability in BFNX, although no differences were determined between the three other treatments (Table 3).

3.3. Relationships between environmental factors and soil bacterial diversity

The soil C and N concentrations both positively correlated with all the diversity metrics (Fig. 5). Conversely, none of these metrics correlated with either C and N inputs, plant aboveground biomass or other soil parameters. Consistently, soil C and N concentrations correlated

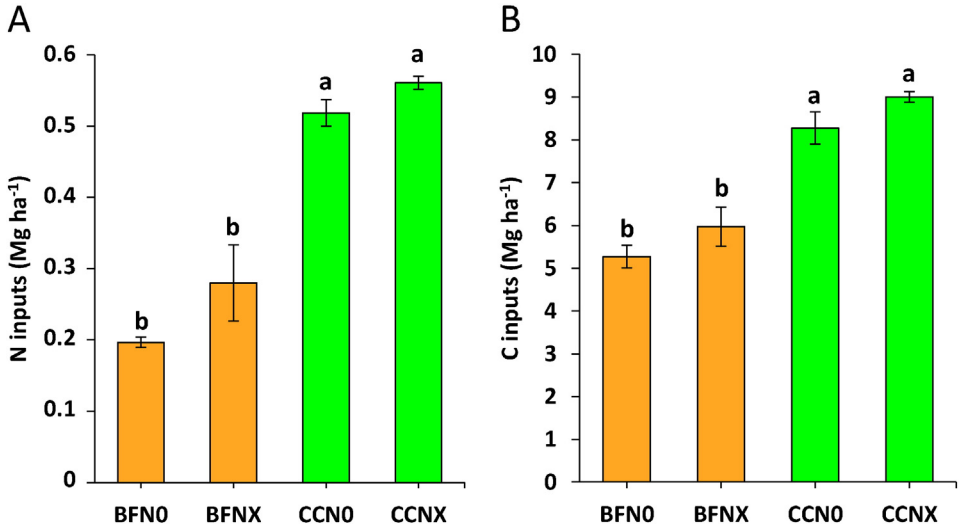

Fig. 2. Bar plots representing the total organic inputs of nitrogen (A) and carbon (B) through main crop and cover crop residues returned to the soil. Letters indicate differences among treatments according to a Dunn's *post hoc* test following a significant Kruskal–Wallis test ($P < 0.05$). BFN0: bare fallow without nitrogen fertilisation. BFNX: bare fallow with nitrogen fertilisation. CCN0: winter cover crop without nitrogen fertilisation. CCNX: winter cover crop with nitrogen fertilisation.

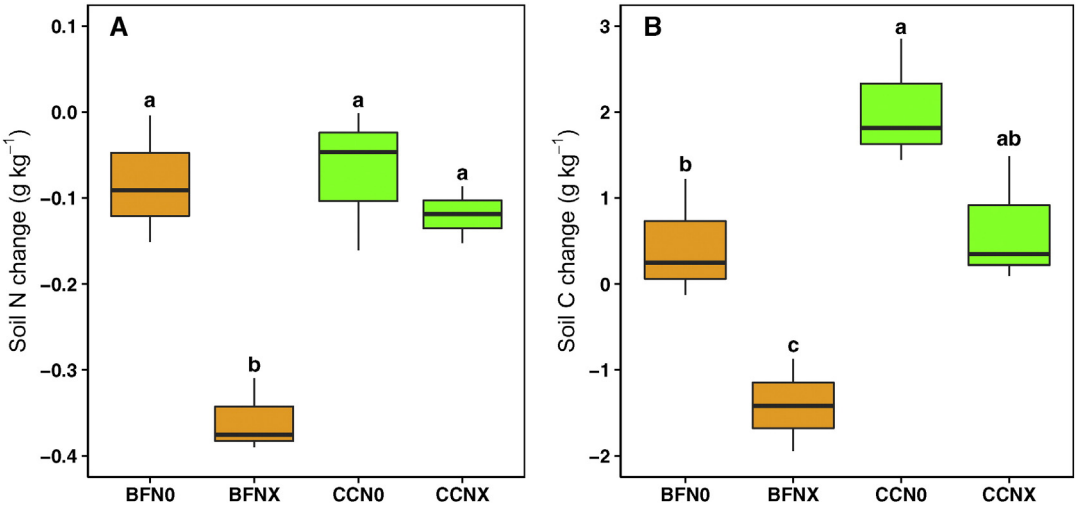

Fig. 3. Box plots representing changes in soil nitrogen (A) and carbon (B) concentrations over the 4-year experiment. Letters indicate differences among treatments according to a Dunn's *post hoc* test following a significant Kruskal–Wallis test ($P < 0.05$). BFN0: bare fallow without nitrogen fertilisation. BFNX: bare fallow with nitrogen fertilisation. CCN0: winter cover crop without nitrogen fertilisation. CCNX: winter cover crop with nitrogen fertilisation.

positively with the relative abundance of all the phyla with a relative abundance of >1%, except Proteobacteria (negative correlation), Cyanobacteria (no correlation) and Bacteroidetes (non-significant and negative correlation with C and N, respectively). However, there was a positive correlation between organic N input and Acidobacteria, organic C input and Cyanobacteria, NO_3^--N and Firmicutes/Acidobacteria, NH_4^+-N and Acidobacteria (Fig. 6). The NMDS revealed that the soils that had received the CCN0 and CCNX treatments contained close bacterial communities. By contrast, BFN0 and BFNX soils were strongly separated. The first axis clearly divided BFNX treated soil plots from the others, due to a greater relative abundance of Proteobacteria and Bacteroidetes phyla. Remarkably, the compositional dissimilarity was much more evident for BFNX soils than for the other treatments, suggesting a greater environmental heterogeneity. The second axis mainly separated CC from BFN0 plots and correlated positively with soil C and N concentrations ($P < 0.05$). CC plots were characterized by an increased relative abundance of Cyanobacteria and Acidobacteria, while BFN0 plots were characterized by Actinobacteria and Verrucomicrobia. The phylum

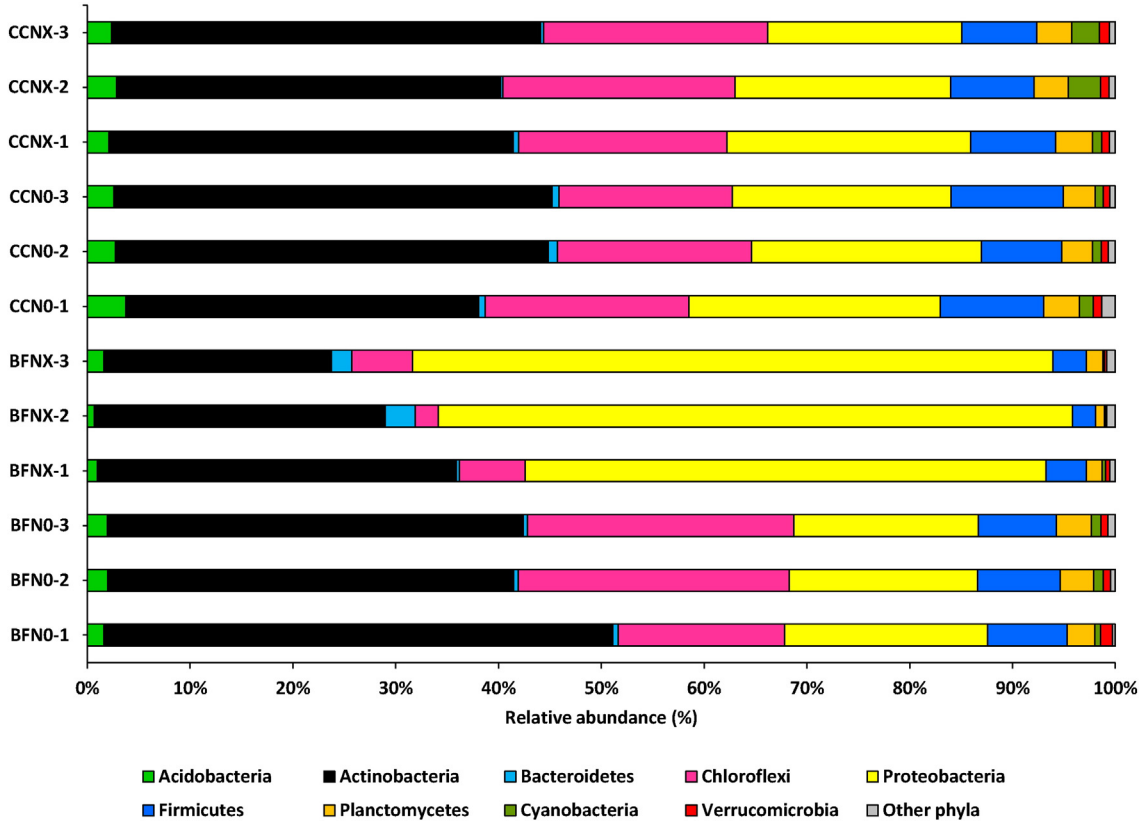

Fig. 4. Relative abundance of the most abundant bacterial phyla in the soil following the different experimental treatments. Sample numbers indicate the different replicates within each treatment. Phylogenetic groups accounting for <1% of all classified sequences are artificially grouped under "other phyla". BFN0: bare fallow without nitrogen fertilisation; BFNX: bare fallow with nitrogen fertilisation; CCN0: winter cover crop without nitrogen fertilisation; CCNX: winter cover crop with nitrogen fertilisation.

Table 3
Indices accounting for soil bacterial diversity (mean ± standard error).

	H	BFN0	BFNX	CCN0	CCNX
OTUs	6.69 (NS)	808.67 ± 67.92	601.33 ± 34.93	822.66 ± 6.57	831.00 ± 49.43
Chao 1	6.44 (NS)	966.23 ± 56.35	732.04 ± 34.70	988.29 ± 14.14	968.06 ± 29.59
ACE	7.81 (NS)	962.92 ± 49.86	734.23 ± 22.21	978.27 ± 13.02	975.70 ± 24.53
Shannon	9.36 (*)	4.62 ± 0.002a	3.32 ± 0.13b	4.81 ± 0.09a	4.77 ± 0.03a
Pielou	8,13 (*)	0.69 ± 0.008a	0.52 ± 0.02b	0.72 ± 0.01a	0.71 ± 0.003a
Simpson	8.74 (*)	0.97 ± 0.0007a	0.82 ± 0.02b	0.98 ± 0.002a	0.98 ± 0.001a

H: value of the Kruskal–Wallis test with its significance in brackets (*$P < 0.05$). Letters give the result of a Dunn's *post hoc* test. BF: bare fallow; CC: winter cover crop; N0: no fertiliser; NX: chemical N fertilisation. NS: non-significant. OTUs: Operational Taxonomic Units. ACE: abundance-based coverage estimator.

composition was not significantly affected by N fertilisation in the CC treated soils and tended to resemble the BFN0 treatment (Fig. 7).

4. Discussion

In this study, the individual and combined effects of cover crops and chemical N fertilisation on the bacterial community and the C and N content of the soil have been monitored following ploughing. Intensive N fertilisation decreased the diversity of the bacterial community of the soil and reduced the concentration of C and N under bare fallow conditions only. There is thus a protective effect of winter cover crops against the deleterious effect of chemical N fertilisation on both soil biodiversity and nutrient accumulation.

4.1. Impact of cover crop and N fertilisation on crop productivity

After 4 years of experimentation using a complete crop rotation system, neither winter cover crops nor N fertilisation had any significant effect on the total aboveground biomass production of the main cultivated crop (Table 2). However in 2013, wheat biomass production increased with N fertilisation, when no cover crop was grown, due to the late harvesting of maize. For both maize and wheat, the low impact of N

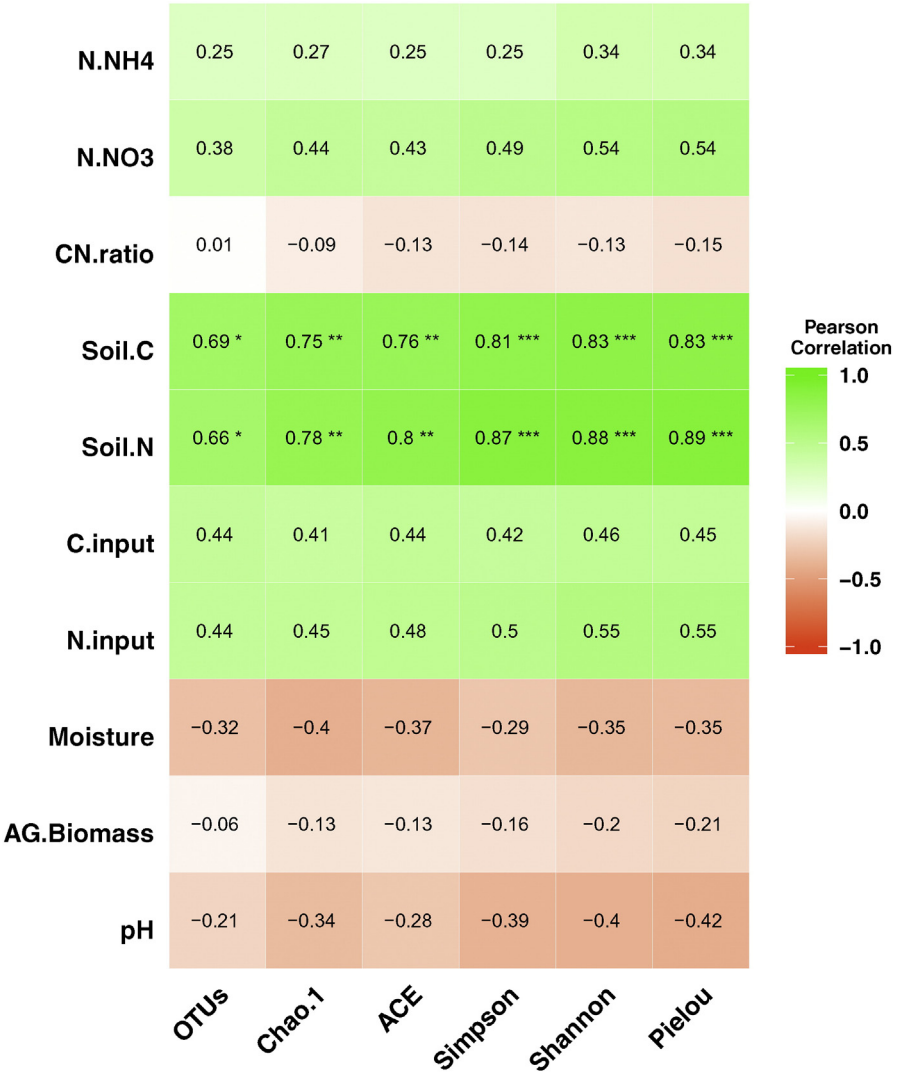

Fig. 5. Pearson correlations between diversity indices and environmental variables. Values of Pearson correlations are coloured by green as positive and orange as negative. Numbers in the squares are correlation coefficients and (*) represent significance level (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$). OTUs: operational taxonomic units. ACE: abundance-based coverage estimator. AG Biomass: aboveground biomass. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fertilisation on the aboveground biomass can be explained by an increase in N use efficiency, which was approximately two times higher under low N (N0) compared to high N (NX) fertiliser input (Sylvester-Bradley and Kindred, 2009). It is known that the crop cultivated previously (e.g. alfalfa) strongly influences maize yield (Peterson and Varvel, 1989), because the legume provides enough N (Asghari and Hanson, 1984). However for maize, a high level of N fertilisation is required to maintain an acceptable grain yield on the second and third year of cultivation (Stanger and Lauer, 2008). In this study, it is likely that green pea, which provides N to maize for one year, is not able to provide enough N to wheat on the following year.

4.2. Changes in C and N inputs and contents in the soil

In addition, following the CC treatment, the accumulation of C and N in the soil through crop residues and winter cover crops incorporation was higher than after the BF treatment, irrespective of the level of N fertilisation (Fig. 2). Interestingly, the soil C and N content changes were positive in the BFN0 treatment, while that of the BFNX treatment were strongly negative (Fig. 3). Since the changes in the soil C and N contents are expressed as a concentration, as has been the case in a number of recent reports (Shen et al., 2010; Geisseler and Scow, 2014; Tian et al., 2015; Zhou et al., 2015), they represent the availability of C and N for microbial processes rather than the actual stocks. The remarkable increase of C concentration observed in CCN0 (Fig. 3) can be explained by root biomass and rhizo-deposition, which were not included in the C and N input measurements shown in Fig. 2. It is likely that an increase of mineralisation under NX conditions was compensated by the organic matter originating from the winter cover crops in the CC treatments, thus stabilising the soil C and N contents over 4 years. It is known that intensive N fertilisation decreases (Khan et al., 2007; Russell et al., 2009; Zhong et al., 2015), or does not modify (Wilts et al., 2004; Higashi et al., 2014) the soil C content. In certain cases, intensive N fertilisation slows down the rate at which the soil organic C concentration is reduced (Ladha et al., 2011), which could also depend upon the amount of crop residues returned to the soil (Lou et al., 2011). For example, over 15 years in a no-till system, N fertilisation combined with the use of cover crops maintained or even increased soil N and C concentrations (Mazzoncini et al., 2011). Moreover, N fertilisation has been shown to stimulate the decomposition of soil residues (Conde et al., 2005), an effect that is likely to persist over long-term periods (Majumder and Kuzyakov, 2010). Such an effect, called the N-induced priming effect, (Jenkinson et al., 1985; Kuzyakov et al., 2000) accelerates organic matter mineralisation when the soil is enriched with N fertilisers. In the CCNX treatment, it is likely that the N-Induced priming effect was counterbalanced by the cover crop, which was able to provide enough new C for heterotrophic soil microorganisms thus preserving the older SOM from decomposition. In line with this hypothesis, a meta-analysis showed that C originating from the cover crops increased the sequestration capacity of soil C (Poeplau and Don, 2015). Therefore, this study has confirmed that in comparison to bare fallow conditions, winter legume cover crops can play a protective role against C and N losses from the topsoil in annual ploughing systems.

4.3. Soil bacterial community composition and diversity

It has been proposed that maintaining soil C and N stocks is the main factor influencing soil bacterial communities (Sul et al., 2013; Yuan et al., 2013). In this study, the considerable reductions in soil C and N

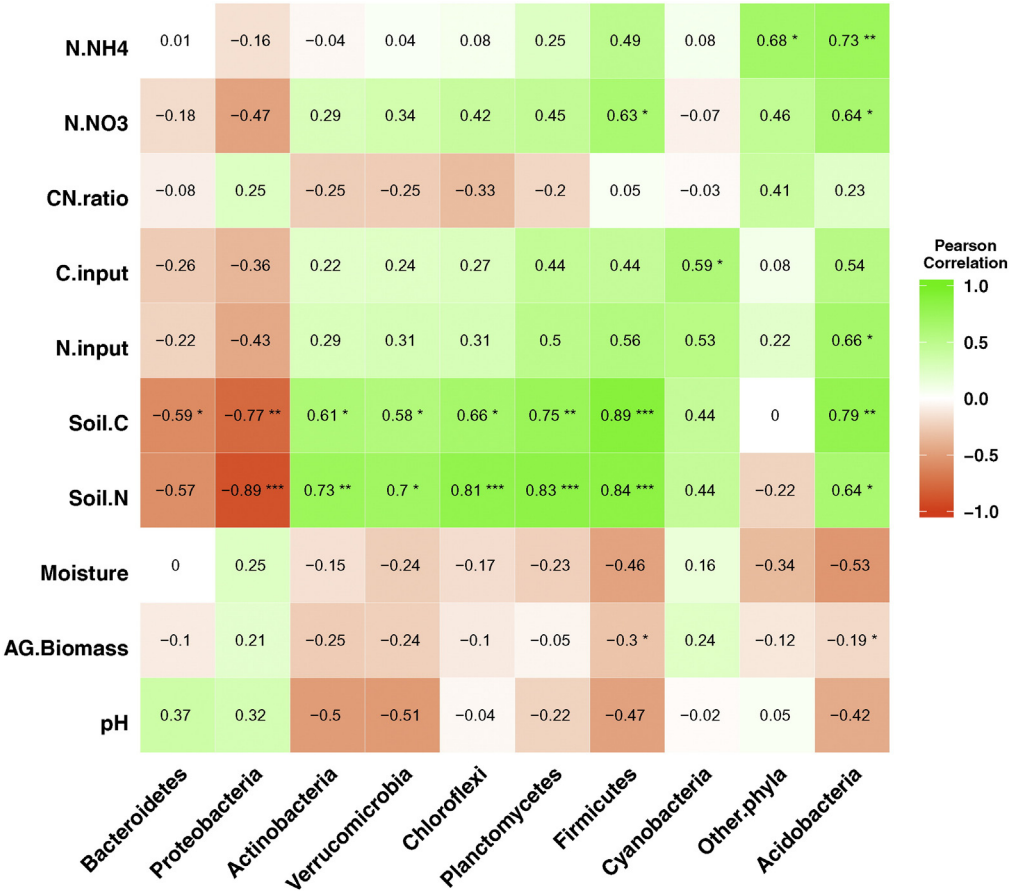

Fig. 6. Pearson correlations between relative abundances of the most abundant phyla and environmental variables. Values of Pearson correlations are coloured by green as positive and orange as negative. Numbers into the squares are correlation coefficients and (*) represent significance level (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$). AG Biomass: Aboveground biomass. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentrations in BFNX were clearly associated with a very low bacterial diversity, in agreement with a number of other studies showing that (1) N enrichment reduces soil bacterial diversity (e.g. Coolon et al., 2013; Geisseler and Scow, 2014; Zhou et al., 2015; Zeng et al., 2016); (2) bacterial diversity is positively correlated with soil N and C concentration (Sul et al., 2013). These results also indicate that the cover crop is able to counteract the negative impact of N fertilisation on soil bacterial diversity. Similarly, Sun et al. (2015) observed a decrease in soil bacterial diversity in N fertilised soils, which did not occur when manure was applied.

In this experimental system, the composition of soil bacteria exhibited important differences between the four different treatments. Moreover, the relative abundances of the dominant phyla were strongly correlated with soil C and N concentrations (except for Bacteroidetes and Cyanobacteria). In agreement with Pascault et al. (2013), fresh organic matter returned to the soil in the CC treatments stimulated oligotrophic phyla (i.e., slow growing bacteria that can use more recalcitrant C sources, such as Acidobacteria and Verrucomicrobia). In addition, some specialised copiotrophic bacteria were present (i.e., fast-growing bacteria such as Actinobacteria and Firmicutes, decomposers of complex C-compounds; Ramirez et al., 2012). The stimulating effect of CCs was maintained even under N fertilisation conditions. By contrast, under bare fallow conditions, there were considerable differences in the bacterial composition of the N0 and NX treated soils, characterised by an enrichment in more copiotrophic phyla (i.e., fast-growing bacteria such as Proteobacteria and Bacteroidetes requiring labile C sources) at the expense of oligotrophic phyla. A notable exception was the copiotrophic Actinobacteria, which were less abundant in BFNX in comparison with the three other treatments. Actinobacteria are important decomposers that play a major role in carbon cycling and nutrient transformation (Jenkins et al., 2009, Fierer et al., 2007; Ventura et al., 2007). It has been shown that their relative abundance is positively correlated with total soil N (Tian et al., 2015; Nacke et al., 2011), and as such we observed that it is lower in the BFNX treatment. This is somehow a paradox that N supply in bare fallow conditions leads to a depletion in the soil C and N contents, thus promoting a more oligotrophic environment compared to the other treatments.

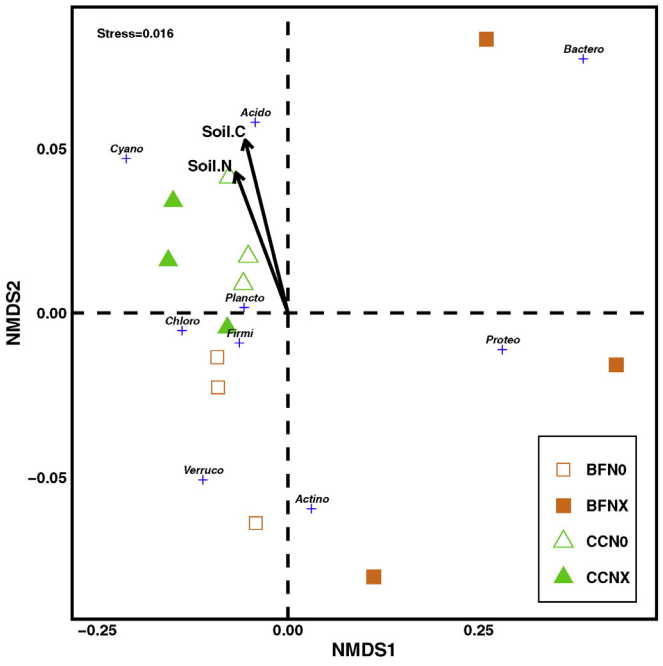

Fig. 7. NMDS diagram defined by the first two axes showing the ordination of the 12 plots (4 treatments × 3 replicates) and 9 bacterial phyla. Only environmental variables significantly fitting onto NMDS axes are shown ($P < 0.05$). *Firmi*: Firmicutes; *Cyano*: Cyanobacteria; *Actino*: Actinobacteria; *Verruco*: Verrucomicrobia; *Bactero*: Bacteroidetes; *Chloro*: Chloroflexi; *Proteo*: Proteobacteria; *Acido*: Acidobacteria; *Plancto*: Planctomycetes. BFN0: bare fallow without nitrogen fertilisation; BFNX: bare fallow with nitrogen fertilisation; CCN0: winter cover crop without nitrogen fertilisation; CCNX: winter cover crop with nitrogen fertilisation.

According to the copiotrophic–oligotrophic hypothesis, N-induced shifts in the microbial community composition should theoretically yield to cognate shifts in the functional and metabolic potentials of the soil microbial community. Such shifts should be able to modify the C decomposition rate (Ramirez et al., 2012) notably for the most recalcitrant compounds (Craine et al., 2007).

5. Conclusion

In this study, a 4-year crop rotation, which included wheat, green pea and maize was conducted in a ploughing system with a relatively low return of organic residues to the soil. Taking together, the results show that under bare fallow conditions, chemical N fertilisers did not increase N and C inputs into crop residues and cover crops. By contrast, chemical N fertilisers strongly depleted the soil C and N concentrations, while reducing bacterial diversity. Importantly, the incorporation of winter cover crops containing legumes between the two main crops, by increasing organic C and N inputs, suppressed the negative impact of N fertilisation, leading to (1) stable soil C and N concentrations and (2) stable bacterial community composition and diversity, while maintaining yields. The use of such practice should be encouraged within the framework of agricultural sustainability.

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