frontiers | Research Topics

Management of agroecosystems for enhancement of soil microbial communities and soil natural fertility

Edited by
Patricia Dorr De Quadros, Nigel Victor Gale and Vanessa Sacramento Cerqueira

Published in
Frontiers in Soil Science

ISSN 1664-8714
ISBN 978-2-8325-2323-0
DOI 10.3389/978-2-8325-2323-0

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Management of agroecosystems for enhancement of soil microbial communities and soil natural fertility

Topic editors

Patricia Dorr De Quadros — University of Waterloo, Canada
Nigel Victor Gale — University of Toronto, Canada
Vanessa Sacramento Cerqueira — Federal University of Pelotas, Brazil

Citation

De Quadros, P. D., Gale, N. V., Cerqueira, V. S., eds. (2023). *Management of agroecosystems for enhancement of soil microbial communities and soil natural fertility*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-2323-0

Table of contents

frontiers in Soil Science

ORIGINAL RESEARCH
published: 02 December 2021
doi: 10.3389/fsoil.2021.765901

Microbial Signatures in Fertile Soils Under Long-Term N Management

María B. Villamil[1], Nakian Kim[1], Chance W. Riggins[1], María C. Zabaloy[2], Marco Allegrini[3] and Sandra L. Rodríguez-Zas[4]*

[1] Department of Crop Sciences, University of Illinois, Urbana, IL, United States, [2] Departamento de Agronomía, Centro de Recursos Naturales Renovables de la Zona Semiárida (CERZOS, Universidad Nacional del Sur - Consejo Nacional de Investigaciones Científicas y Técnicas), Universidad Nacional del Sur, Bahía Blanca, Argentina, [3] Laboratorio de Biodiversidad Vegetal y Microbiana, Instituto de Investigaciones en Ciencias Agrarias Rosario (IICAR-Consejo Nacional de Investigaciones Científicas y Técnicas), Universidad Nacional de Rosario, Zavalla, Argentina, [4] Department of Animal Sciences, University of Illinois, Urbana, IL, United States

Long-term reliance on inorganic N to maintain and increase crop yields in overly simplified cropping systems in the U.S. Midwest region has led to soil acidification, potentially damaging biological N_2 fixation and accelerating potential nitrification activities. Building on this published work, rRNA gene-based analysis via Illumina technology with QIIME 2.0 processing was used to characterize the changes in microbial communities associated with such responses. Amplicon sequence variants (ASVs) for each archaeal, bacterial, and fungal taxa were classified using the Ribosomal Database Project (RDP). Our goal was to identify bioindicators from microbes responsive to crop rotation and N fertilization rates following 34–35 years since the initiation of experiments. Research plots were established in 1981 with treatments of rotation [continuous corn (Zea mays L.) (CCC) and both the corn (Cs) and soybean (Glycine max L. Merr.) (Sc) phases of a corn-soybean rotation], and of N fertilization rates (0, 202, and 269 kg N/ha) arranged as a split-plot in a randomized complete block design with three replications. We identified a set of three archaea, and six fungal genera responding mainly to rotation; a set of three bacteria genera whose abundances were linked to N rates; and a set with the highest number of indicator genera from both bacteria (22) and fungal (12) taxa responded to N fertilizer additions only within the CCC system. Indicators associated with the N cycle were identified from each archaeal, bacterial, and fungal taxon, with a dominance of denitrifier- over nitrifier- groups. These were represented by a nitrifier archaeon *Nitrososphaera*, and *Woesearchaeota AR15*, an anaerobic denitrifier. These archaea were identified as part of the signature for CCC environments, decreasing in abundance with rotated management. The opposite response was recorded for the fungus *Plectosphaerella,* a potential N_2O producer, less abundant under continuous corn. N fertilization in CCC or CS systems decreased the abundance of the bacteria genera *Variovorax* and *Steroidobacter*, whereas *Gp22* and *Nitrosospira* only showed this response under CCC. In this latter system, N fertilization resulted in increased abundances of the bacterial denitrifiers *Gp1, Denitratisoma, Dokdonella,* and *Thermomonas*, along with the fungus Hypocrea, a known N_2O producer. The identified signatures could help

OPEN ACCESS

Edited by:
Patricia Dorr De Quadros, University of Waterloo, Canada

Reviewed by:
Durgesh Kumar Jaiswal, Savitribai Phule Pune University, India
Rodrigo Gouvea Taketani, Rothamsted Research, United Kingdom

****Correspondence:***
María B. Villamil
villamil@illinois.edu

Specialty section:
This article was submitted to Soil Biology, Ecosystems and Biodiversity, a section of the journal Frontiers in Soil Science

Received: *27 August 2021*
Accepted: *10 November 2021*
Published: *02 December 2021*

Citation:
Villamil MB, Kim N, Riggins CW, Zabaloy MC, Allegrini M and Rodríguez-Zas SL (2021) Microbial Signatures in Fertile Soils Under Long-Term N Management. Front. Soil Sci. 1:765901. doi: 10.3389/fsoil.2021.765901

future monitoring and comparison across cropping systems as we move toward more sustainable management practices. At the same time, this is needed primary information to understand the potential for managing the soil community composition to reduce nutrient losses to the environment.

Keywords: archaea, bacteria, fungi, nitrogen, maize, soybean, monoculture, rotation

INTRODUCTION

Due to the excessive rates and inefficiencies associated with N fertilizer use, agricultural soil management is a major source of N losses to the environment (1, 2). Although the consequences are global, the challenges and potential solutions are local because the main players in the biological N cycle are soil microorganisms. All major transformative steps of the N cycle, fixation, mineralization, nitrification, and denitrification are microbially mediated (3).

Previous studies aimed to characterize the distribution and diversity of N relevant microorganisms in agroecosystems have used cultivation independent techniques targeting genes encoding phylogenic genes and critical enzymes of the microbial N cycle: *nif*H (N_2 fixation), *amo*A (first step of nitrification), *nir*K and *nir*S (second step of denitrification), and *nos*Z (last step of denitrification) (1, 4–6) yet only a few long-term studies provided a complete picture of N-fertilization effects on the N transformation cycle within a single agroecosystem (7–9). New metagenomics tools allow for exploration of the changes in the composition of the soil microbiota that accompany the reported functional changes brought about by agricultural practices, thereby granting the identification of bioindicators of agricultural use (8, 10).

Although the soil microbiota is thought to be functionally redundant, the biodiversity loss commonly observed under intensive agricultural management, could seriously impair ecosystem functioning (11–13). Therefore, identifying bioindicators can provide primary information to monitor longitudinal responses to management practices, categorize core microbiota, and provide the raw material to further study microbial networks and their potential for manipulation to increase the sustainability of current operations (13, 14). To that end, the identification of bioindicators from well-replicated, long-term agricultural experiments are essential, as these systems represent a unique "steady" setting to characterize baselines and evidence changes in microbiota abundance and diversity in relation to management practices (15).

The Midwest region of the US is predominantly an area of corn and soybean production where corn crops are cultivated continuously or in short rotation with soybeans. Throughout the region, the rotated crops typically return higher yields compared to monocultures due to increased N availability, residue management, and improved yield stability (16, 17). Higher volumes of residue are left in the field following corn compared to soybeans due to higher yields. In turn, greater residue production and slower decomposing residues of corn compared to soybean crops lead to an accumulation of stable soil organic matter (SOM) in rotations that feature corn crops more frequently (18, 19). Soybean residues with characteristically low C:N ratio are quick to decompose and have been shown to increase the rates of nitrification and denitrification (20), while increasing the soil susceptibility to erosion (21). Nutrient requirements and removal rates also differ among crops: the removal of P and S per hectare is greater for corn, while K removal is greatest for soybean (22).

Although the N supply for soybean is carried out with biological N fixation, the crop must still obtain N from the soil and it is known to be a net N user (23). By altering the quantity and quality of residues, and of root exudates added to the soil, crop rotation influences the soil environment (24). Likewise, the differing requirement of N fertilizers among crops affects not only N cycling within the soil, but also several other soil properties, mainly pH (22, 25). In a meta-analysis by Ouyang et al. (25) crop rotation and soil pH influenced N cycling by changing the ammonia-oxidizing bacteria (AOB) and archaea (AOA) community dynamics as well as the community of denitrifiers in the soil. Fertilization accelerates each step of the microbial N cycle by loading the system with inorganic N (7, 25). Nitrification increases substantially, leading to greater losses of N to the environment through leaching and denitrification. Similarly, biological N fixation is reduced as the selective pressure for this function is relieved due to an abundance of easily accessible N in the system. Lower pH under continuous corn management has been reported to favor both the abundance and diversity of fungi (26). Another meta-analyses by Carey et al. (27) reported that additions of N increased both AOB and AOB abundances, with AOB showing increases an order of magnitude larger than AOA, correlated to increases in nitrification potential from agroecosystems.

Despite being economically successful, the widespread use of short rotations and continuous cropping systems in the Midwest region pose negative environmental consequences as these oversimplified intensive agricultural systems are driving global declines in biodiversity and soil health leading to reductions in agroecosystem functions and services (24, 28).

In a previous study, Huang et al. (7), reported that the long-term use of N fertilizers in continuous corn management, has led to acidification of the soils in the region. The reduction in soil pH was accompanied by increases in the abundance of fungal ITS and bacterial *amo*A counts, and parallel reductions in the abundance of *nif*H genes, potentially hampering biological N_2 fixation while accelerating potential nitrification activities (7). However, that previous work restricted the analyses to quantification of genes such as ITS (fungal), 16S rRNA (bacteria and archaea), and those involved in the microbial N cycle, that

are broad indicators of microbial community structure, while did not assess the signatures of long term management on the whole soil metagenome. Thus, because the main reported changes in these agroecosystems are strongly associated with the microbial cycling of N, we hypothesized that microbial groups responsive to crop rotation and N fertilization regimes, will be identified within each of the archaeal, bacterial, and fungal domains. Based on the results of our previous study, our goal was to identify bioindicators from those responsive microbes in a long-term experiment following 34–35 years since its initiation of experiments. We anticipate the identified signatures could help future monitoring and comparison across cropping systems and provide primary information to understand the potential for managing the soil community composition to reduce nutrient losses to the environment as we move toward more sustainable management practices.

MATERIALS AND METHODS

Experimental Site, Treatment Layout, and Field Management Practices

The long-term agronomic trial is located at the Northwestern Illinois Agricultural Research and Demonstration Center (40°55'50" N, 90°43'38" W), approximately 8 km northwest of Monmouth, Illinois. The research site was established in 1981 to study the effects of five rates of N fertilization (0, 67, 135, 202, and 269 kg N/ha) on corn crop yields in monoculture (CCC) or in a short rotation with soybeans, with each phase present every year (Cs, Corn phase within corn-soybean rotation; Sc, Soybean phase within corn-soybean rotation). The two systems together represent 11.2 million ha in Illinois alone (29). Soils belong to the Muscatune silt loam series (fine-silty, mixed, mesic Aquic Argiudoll), a series of dark and deep, prime agricultural soils, on nearly flat topography. Muscatune series is representative of the loess-derived soils of the glaciated region of Illinois, covering over 207,000 ha in the state (30). The mean annual precipitation at Monmouth is 978 mm with a mean annual temperature of 16°C (31).

Treatments were arranged as a split plot of rotation (Rot) and N fertilization rates (N rate) in a randomized complete block design with three replications. Rotation was assigned to the main plots (18 m long by 30 m wide) and N fertilization rates to the subplots (18 m long by 6 m wide). Only three of the original fertilization rates (N rate: 0, 202, and 269 kg N/ha) were used in the current study representing unfertilized controls, current average annual application of N in the region, and what can be considered over application of N for corn, respectively. No N fertilizer was added to the soybean crops.

Tillage occurred in late fall following harvest using a chisel plow 20–25 cm deep, and a field cultivator was used to prepare the seedbeds the next spring. Corn (DK 63–33 both years) and soybeans (Munson 8,364 in 2015; 8,366 in 2016) were planted in mid-April–May each year in 76 and 38 cm rows, respectively, using 75,000–85,000 seeds per ha for corn and 340,000–350,000 seeds per ha for soybeans. Corn was fertilized in the spring at or before planting with N in the form of urea (46% N) until 1996, and thereafter as incorporated urea ammonium nitrate solution (UAN 28%). For all treatments, the same P rate of 40 kg P per ha was applied every 2 years as diammonium phosphate, and additional K fertilizer and lime were occasionally added to the entire experimental area based on soil test results (last application occurred in 2011). Fertilizer and pest management decisions were based on best management practices for the site according to the Illinois Agronomy Handbook (32).

Soil Sampling, DNA Extraction, and Sequencing

Soil sampling occurred following the harvest of the cash crops in October 2015 and again in October 2016, after 34 and 35 years, respectively, since the experiment was set up inspired by renewed interest in soil health research. Each year, three composited soil subsamples to a depth of 10 cm were taken with an Eijelkamp grass plot sampler (Eijkelkamp Soil & Water, Morrisville, NC, USA) within each experimental unit. Each subsample consisted of about 10 plugs totaling about 500 g of soil, collected walking in a zig-zag pattern. Samples were preserved with ice packs in the field, and frozen to −20°C upon arrival at our lab facilities. A complete, multivariate examination of soil properties at the sampled depth (10 cm) was presented in Huang et al. (7), and a summary table of baseline properties is included as **Supplementary Table 1**. Baseline soil properties include soil pH (1:1 soil-water), soil organic matter determined by loss-on ignition (SOM, %), nitrate and ammonium [NO_3^- and NH_4^+, ln(ppm)], and available phosphorus [Pa, ln(ppm)], all by flow injection analysis with a Lachat Quick-Chem 8000 (LachatQuickchem Analyzer, Lachat Instruments, Loveland, CO, USA) (7).

Soil DNA was extracted using the PowerSoil® DNA isolation kits (MoBio Inc., Carlsbad, CA, USA), using 0.25 g of the composited soil samples, with the soils carefully homogenized before subsampling and following manufacturer's instructions. The extracted DNA quantity and quality were examined using a Nanodrop 100 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). An Illumina MiSeq compatible amplicon library containing individual barcodes for each sample was constructed using 25 μL volumes with 1 × buffer (GoTaq Flexi buffer; Promega Corp., Madison, WI, USA), 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM each primer (forward and reverse), 1.0 μL template DNA (pooled amplicons), and 1.0 unit of GoTaq polymerase. The primer sets used for amplification were 349F (GTGCASCAGKCGMGAAW) and 806R (GGACTACVSGGGTATCTAAT) for the archaeal 16S rRNA gene (33); 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACVSGGGTWTCTAAT) for the bacterial 16S rRNA gene (34), and 3F (GCATCGATGAAGAACGCAGC) and 4R (TCCTCCGCTTATTGATATGC) for the fungal ITS (internal transcribed spacer) region (35). The primers were designed as a 5′-PCR-specific + gene region + 3′-PCR-specific + 10 nt barcode, and the Fluidigm platform used two primer sets concurrently in the creation of the final DNA amplicon. A Qubit

TABLE 1 | Mean values, standard error of the mean (SEM), and probability values (*p*-values) associated with the ANOVA for the α-diversity parameters amplicon sequence variants (ASVs), Faith' phylogenetic diversity index (PD), Pielou Evenness Index (J'), and Shannon's Diversity Index (H') for bacteria, fungi, and archaea taxa, following 35 years of Rotation (Rot) and N fertilization (*N* rate)[†].

			ASVs	PD	J'	H'	
Taxa	**Treatment**	**Level**	**Mean**	**Mean**	**Mean**	**Mean**	
Archaea	Rot	CCC	37.74	7.63	0.93	4.78	
		Cs	38.35	7.92	0.94	4.83	
		Sc	38.98	7.97	0.93	4.81	
		SEM	13.04	0.26	0.02	0.62	
		p-value	0.8458	0.4296	0.7842	0.9320	
	N rate	0	39.04	7.73	0.93	4.80	
		202	37.00	7.72	0.93	4.75	
		269	39.04	8.07	0.94	4.87	
		SEM	13.08	0.32	0.02	0.62	
		p-value	0.7081	0.6721	0.8235	0.7551	
Bacteria	Rot	CCC	1,308	119.94	0.97	9.96	
		Cs	1,336	122.97	0.97	10.00	
		Sc	1,359	125.88	0.97	10.02	
		SEM	330.0	21.16	0.01	0.43	
		p-value	0.8828	0.6089	0.9529	0.8679	
	N rate	0	1,364	124.41	0.97	9.98	
		202	1,310	121.51	0.97	9.97	
		269	1,329	122.87	0.97	10.02	
		SEM	329.2	21.18	0.01	0.43	
		p-value	0.8474	0.8902	0.6234	0.9085	
Fungi	Rot	CCC	34.22	10.47	0.82	4.15	
		Cs	32.89	9.76	0.82	4.09	
		Sc	30.72	9.37	0.83	4.03	
		SEM	2.54	0.59	0.02	0.19	
		p-value	0.6368	0.4238	0.9615	0.7964	
	N rate	0	30.15	9.35	0.81	3.95	b
		202	32.11	9.75	0.83	4.09	ab
		269	35.57	10.50	0.83	4.22	a
		SEM	2.20	0.55	0.02	0.17	
		p-value	0.2316	0.2734	0.4354	0.0453	

[†]*CCC, continuous corn; Cs, corn phase of the corn-soybean rotation; Sc, soybean phase of the corn-soybean rotation; N rate: 0, 202, 269 Kg N/ha.*

Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify the resulting amplicon libraries, which were then computed on a bioanalyzer (Agilent, Santa Clara, CA, USA) to assess the profile of fragment lengths. The barcoded libraries were pooled in equimolar concentrations, diluted to 10 nM, and sequenced at the Roy Carver Biotechnology Center, Functional Genomics lab at the University of Illinois at Urbana-Champaign (Urbana, IL, USA) using paired-end sequencing on the Illumina MiSeq (Illumina, San Diego, CA, USA), resulting in reads 250 nt in length.

Bioinformatic Processing and Microbial Diversity Analyses

Quality control and processing of sequences were conducted with QIIME2 (36, 37). Demultiplexed sequences were trimmed using a Q score threshold of 30 (38), leading to retention of base pair positions between 5 and 250 for forward sequences of all three taxa, and reverse sequences between 5 and 221 for archaea, 5 and 231 for bacteria, and 5 and 223 for fungi. The plugin DADA2 (39) was used for denoising, the removal of chimeric and low-quality sequences, with the chimera-method consensus

TABLE 2 | Results of the principal component analysis of archaea genera showing the eigenvalues, the cumulative proportion of variability explained, and the eigenvectors for each of the three principal components (PC) extracted.

	PC1	PC2	PC3
Eigenvalue	2.22	1.31	1.10
Cum. proportion	0.37	0.59	0.77
Eigenvector†	**Component correlation scores**		
Methanomassiliicoccu	**−80**	35	−34
Nitrososphaera	−39	**55**	**50**
Woesearchaeota_IS_AR15	**65**	16	**54**
Woesearchaeota_IS_AR16	−30	**−88**	33
Woesearchaeota_IS_AR18	**74**	25	4
Woesearchaeota_IS_AR20	**62**	−10	**−60**

†*Complete classification is provided in* ***Supplementary Table 5****.*
Component correlation scores (eigenvectors) with loadings >|0.45| are bolded.

option, and the resulting sequences were clustered into amplicon sequence variants (ASVs).

The rarefaction curves plateaued at sampling depths of 500 archaeal sequences per sample 24,000 bacterial sequences per sample, and 4,000 fungal sequences per sample (**Supplementary Figure 1**). The α-diversity measures calculated were observed number of ASVs, Faith' phylogenetic diversity index (PD), Pielou's evenness parameter (J'), and Shannon's diversity index (H') (**Table 1**). To calculate β-diversity measures for each taxon in QIIME2, the ASV sequences were aligned using MAFFT (40) to build a maximum likelihood phylogenetic tree with fasttree and midpoint-root methods. The β-diversity measures for each microbial group were obtained based on weighted UniFrac distances (**Supplementary Tables 2–4**).

Amplicon sequence variants (ASVs) for each taxon were classified with the Ribosomal Database Project (RDP) naïve Bayesian classifier (41), or with the RDPTool package (42) using 16S rRNA training set 18 and Warcup Fungal ITS trainset 2. The classified ASVs for each taxon were then grouped by genus and those with low (<0.1 %) per-sample relative abundances, averaged across all samples, were filtered out to reduce the sparsity of the dataset (43) using package dplyr (44) in R (45). The resulting data sets were comprised of eight genera of archaea, 195 genera of bacteria, and 128 genera of fungi.

Statistical Analysis and Visualization

The data sets of genera with their ASV reads for archaea, bacteria and fungi were input into the JMP® predictor screening platform to provide an initial identification of the microbes responsive to treatment effects using a bootstrap forest partitioning method (10, 26, 46). This led to the selection of 6 archaea, 39 bacteria, and 45 fungi genera (**Supplementary Table 5**) each contributing at least one percent to the variability of the model algorithms for each set. Next, each data set was centered log-ratio (clr) transformed after replacing zero values with the zCompositions package (47) in R (45), as recommended for compositional data (48).

Principal Component Analyses (PCA), were then deployed as a data reduction technique on the top contributing genera for each taxon to further remove redundancy. Using the FACTOR procedure in SAS software version 9.4 (SAS Institute, Cary, NC) with priors = 1 (default), the ASV reads of each set of genera were summarized into new sets of uncorrelated composite variables, or Principal Components (PCs). PCs with eigenvalues ≥1 that also explained at least 5% of the variability in each data set, were retained and used as independent variables for further analysis. Genera with an important correlation with each PC (PC loading value > |0.45|) were considered responsive microbes and used as descriptors of such PC (**Tables 2–4**). The "List of Prokaryotic Names with Standing in Nomenclature" or LPSN (49), as well as primary research, were used to find reports on the characteristics of the responsive genera. The GLIMMIX procedure in SAS was deployed to fit linear mixed models to each of the α-diversity measures (**Table 1**), and the extracted PCs for each microbial group (**Table 5**). Rotation (Rot) and N fertilization (Nrate) were considered fixed effects whereas blocks, years, and their interactions with fixed effects were considered random terms in the analyses of variance that ensued. When appropriate, least-square means of the response variables were separated by treatment levels, using the lines option of the lsmeans statement, setting the probability of Type I error at α = 0.05. Probability values 0.05–0.10 are reported and deemed "marginally" significant; by providing complete information on all studied variables up to this threshold (0.1), we are enabling readers to prioritize the biological and statistical information and make their own decisions when considering our assertions.

The β-diversity measures were calculated with pairwise permutational analysis of variance [PERMANOVA, (50)] in QIIME2, a non-parametric multivariate approach to compare distances between treatment levels. The approach uses pseudo-F test statistics, rendering pseudo-F probability values (*p*-values) and q-values to correct for multiple hypothesis testing, both based on false discovery rate (FDR) (51).

Package ggplot2 (52) in R (45), was used to plot the statistically indicative relationship detected between PCs and treatments for

TABLE 3 | Results of the principal component analysis of bacteria genera showing the eigenvalues, the cumulative proportion of variability explained, and the eigenvectors for each of the four principal components (PC) extracted.

	PC1	PC2	PC3	PC4
Eigenvalue	13.22	3.02	2.89	2.67
Cum. proportion	0.34	0.42	0.49	0.56
Eigenvector†	**Component correlation scores**			
Actinoallomurus	**−72**	9	−23	−2
Algisphaera	**63**	13	3	−3
Allostreptomyces	**79**	0	6	26
Aquisphaera	−21	43	26	**47**
Arboricoccus	**49**	**46**	−23	−5
Arenimonas	−32	**48**	18	−11
Chujaibacter	**−74**	−35	−24	−8
Dasania	28	−35	32	23
Denitratisoma	**−76**	31	−21	−14
Devosia	−39	−38	38	−33
Dokdonella	**−74**	−4	1	−15
Ferribacterium	**61**	17	−21	−16
Geminisphaera	36	20	43	**45**
Gemmata	−18	43	−5	**46**
Gp1	**−81**	−7	−31	6
Gp22	**77**	5	−20	−5
Hyphomicrobium	25	**−60**	−8	4
Lacibacterium	−41	−30	24	**63**
Laribacter	−28	9	**53**	−1
Lentimicrobium	39	**−48**	18	33
Mesorhizobium	37	−19	39	−7
Micropepsis	**−80**	11	−1	37
Nitrosospira	**64**	26	−5	9
Ornatilinea	**72**	13	6	3
Parviterribacter	**82**	16	−2	−9
Pedomicrobium	**80**	26	−25	−12
Porphyrobacter	**−82**	−5	−19	−14
Pseudolabrys	**−54**	**45**	2	−1
Pyxidicoccus	9	**−48**	−22	31
Rhizobium	17	7	36	**−53**
Rhizomicrobium	**−76**	8	−17	12
Roseisolibacter	**−73**	35	−4	24
Steroidobacter	−20	30	**70**	15
Terrimonas	27	3	−1	**54**
Thermoanaerobaculum	**86**	−3	−13	15
Thermodesulfitimonas	70	12	−36	2
Thermomonas	**−69**	−8	−34	20
Thiobacter	41	4	−33	12
Variovorax	−21	6	**52**	−43

†*Complete classification is provided in* ***Supplementary Table 5****.*
Component correlation scores (eigenvectors) with loadings >|0.45| are bolded.

a given taxon. **Figures 1**–**6**, each has two panels, where one panel depicts the PC means and their standard errors (as whiskers) for a given treatment, and the second panel shows the contribution of each responsive microbe to the PC mean value for each treatment. This contribution is thus calculated as the mean PC value for a given treatment (from **Table 5**) multiplied by the loading of the specific genera within the PC (from **Tables 2**–**4**), named MxL in each plot.

TABLE 4 | Results of the principal component analysis of fungal genera showing the eigenvalues, the cumulative proportion of variability explained, and the eigenvectors for each of the five principal components (PC) extracted.

	PC1	PC2	PC3	PC4	PC5
Eigenvalue	4.31	3.47	3.32	3.23	3.06
Cum. proportion	0.12	0.21	0.28	0.34	0.39
Eigenvector†	**Component correlation scores**				
Aleuria	−24	20	18	−21	**69**
Alternaria	35	43	−12	10	**−49**
Candida	−8	**−49**	1	−8	12
Coemansia	−7	−7	0	−22	4
Cortinarius	−25	43	−41	**−46**	28
Corynascus	−1	8	9	**48**	9
Cryptococcus	−5	−35	−14	−5	−3
Davidiella	40	−3	13	−21	**−49**
Epicoccum	−29	−7	14	−5	−44
Eupenicillium	−39	−1	8	25	23
Exophiala	−21	−6	−11	**46**	−11
Fusarium	39	17	41	3	1
Gibberella	2	19	−12	39	−16
Glomus	−31	−9	**−49**	**−56**	2
Herpotrichia	−3	−12	−6	−6	12
Humicola	−11	−43	5	**50**	−31
Hypocrea	1	−41	1	**48**	12
Lasiosphaeris	−2	**68**	−8	0	16
Lectera	75	25	6	24	8
Lecythophora	−9	−43	−15	−13	−3
Metacordyceps	−44	−14	44	−42	−19
Morchella	**52**	−12	20	−19	−19
Mortierella	−20	−18	−17	−40	−21
Mycosphaerella	**59**	22	19	11	2
Nectria	−1	−25	35	−16	44
Neonectria	12	−3	1	0	**53**
Ochroconis	−21	**49**	**52**	−14	−9
Ophiostoma	−16	**56**	−5	−4	27
Pestalotiopsis	−38	−28	−9	25	−8
Phialocephala	−33	−15	5	33	−27
Phoma	10	1	−27	−22	**−46**
Plectosphaerella	44	18	**50**	−3	−19
Plectosporium	71	9	13	13	2
Pochonia	**47**	−3	−1	−3	4
Podospora	3	−25	−24	3	−15
Pseudodictyosporium	−35	26	−5	−27	5
Pseudogymnoascus	−20	**−56**	−2	6	3
Rhizophagus	−13	20	**−59**	−19	2
Rhizophydium	−36	−10	**−67**	−29	−4
Spizellomyces	−30	10	−26	−33	**50**
Stachybotrys	0	26	33	15	−7
Staphylotrichum	10	21	−22	−5	**49**
Talaromyces	−37	15	17	10	17
Tetracladium	−6	13	**64**	−28	6
Thielavia	−32	−7	7	**49**	3

†*Complete classification is provided in* ***Supplementary Table 5****.*
Component correlation scores (eigenvectors) with loadings >|0.45| are bolded.

TABLE 5 | Mean values, standard error of the mean (SEM), and probability values (*p*-values) associated with the analysis of variance (ANOVA) results for the effects of Rotation (Rot), N fertilization (Nrate), and their interaction on each group of principal components (PCs) calculated for archaea, bacteria, and fungi taxa datasets comprised of indicator ASVs.

		Archaea				**Bacteria**					**Fungi**					
		PC1	**PC2**	**PC3**		**PC1**		**PC2**	**PC3**	**PC4**	**PC1**	**PC2**	**PC3**		**PC4**	**PC5**
Rot	CCC[†]	−0.03	0.03	0.42	a	−0.52	b	−0.14	0.09	0.22	−0.29	−0.32	−0.69	b	0.08	0.06
	Cs	0.02	0.02	−0.09	ab	0.15	ab	0.03	0.18	−0.28	0.33	0.01	0.14	ab	0.13	0.19
	Sc	0.01	−0.05	−0.33	b	0.37	a	0.11	−0.27	0.06	−0.05	0.31	0.55	a	−0.21	−0.25
	SEM	0.76	0.25	0.32		0.24		0.69	0.30	0.41	0.55	0.24	0.47		0.58	0.45
	p-value	0.9846	0.9663	0.0793		0.0003		0.7377	0.4168	0.6358	0.7057	0.1106	0.0253		0.6299	0.9167
Nrate	0	−0.10	−0.27	0.21		0.66		0.01	0.41	a 0.07	−0.11	0.53	a −0.19		−0.28	0.27
	202	0.06	0.24	−0.03		−0.22		0.00	−0.31	b 0.05	0.05	0.00	ab 0.07		0.02	−0.06
	269	0.04	0.03	−0.18		−0.44		−0.01	−0.09	ab −0.12	0.06	−0.53	b 0.12		0.26	−0.21
	SEM	0.76	0.27	0.46		0.30		0.70	0.30	0.44	0.41	0.26	0.47		0.59	0.46
	p-value	0.8440	0.5107	0.8478		0.1278		0.9992	0.0803	0.9407	0.8331	0.0307	0.6891		0.4943	0.5164
Rot × Nrate	CCC × 0	−0.26	−0.54	0.51		0.89	a	−0.19	0.06	0.42	−0.58	0.27	ab −0.51		−0.87	b 0.17
	CCC × 202	0.04	−0.15	0.21		−0.97	c	0.06	−0.10	0.01	−0.07	0.23	ab −1.22		0.48	a 0.06
	CCC × 269	0.13	0.77	0.54		−1.49	c	−0.29	0.31	0.24	−0.21	−1.44	c −0.34		0.62	a −0.06
	CS × 0	−0.23	−0.16	0.01		0.46	ab	0.21	0.61	−0.43	0.30	0.49	ab 0.04		−0.17	ab 0.83
	CS × 202	0.28	0.37	0.26		−0.09	b	0.22	−0.44	0.24	0.14	−0.45	bc 0.44		0.11	ab 0.26
	CS × 269	0.00	−0.16	−0.53		0.07	ab	−0.35	0.36	−0.64	0.56	−0.02	ab −0.06		0.47	ab −0.52
	Sc × 0	0.19	−0.11	0.10		0.62	ab	0.00	0.54	0.22	−0.04	0.84	a −0.10		0.20	ab −0.20
	SC × 202	−0.14	0.48	−0.56		0.40	ab	−0.28	−0.40	−0.09	0.08	0.21	ab 0.99		−0.53	ab −0.50
	SC × 269	−0.02	−0.52	−0.53		0.10	ab	0.62	−0.96	0.04	−0.18	−0.13	ab 0.76		−0.31	ab −0.04
	SEM	0.81	0.42	0.56		0.36		0.75	0.43	0.65	0.61	0.38	0.61		0.65	0.56
	p-value	0.7785	0.1231	0.5730		0.0012		0.2446	0.1635	0.8010	0.6040	0.0903	0.3476		0.0047	0.3378

[†]*CCC, continuous corn; Cs, corn phase of the corn-soybean rotation; Sc, soybean phase of the corn-soybean rotation; N rate: 0, 202, 269 Kg N/ha.*
For each taxon and within a given column, treatment mean values followed by the same lowercase letter were not statistically different (α = 0.05).

To link our findings with concurrently published research, Pearson's correlation coefficients obtained with the CORR procedure of SAS, were used to evaluate the relationships among the statistically indicative PCs extracted for each taxon in this study, with the baseline soil properties (**Supplementary Table 1**) determined in the study of Huang et al. (7). The baseline soil properties were here analyzed using the above-mentioned linear mixed models and the results included within **Supplementary Table 1**.

RESULTS

Overall Characterization of the Soil Microbiota

The archaeal taxa had 118,618 archaeal 16S rRNA region sequences clustered into 8 ASVs, the bacterial taxa had 14,136,085 16S V4 region sequences clustered into 1701 ASVs, whereas the fungal kingdom had 3,166,008 ITS region sequences clustered into 342 ASVs. The α-diversity measurements of ASV count, Faith' phylogenetic diversity index (PD), Pielou's evenness index (J'), and Shannon's diversity index (H'), for bacteria and archaea, revealed no statistical differences for effects of crop rotation and N rate (**Table 1**) or their interaction. On the other hand, the J' index for fungi showed a statistically significant effect of the Rot × Nrate interaction term ($p = 0.01$) with the highest value of J' determined for Cs-202 (0.85), the lowest J' determined for both CCC-202 (0.80) and Cs-0 (0.80), and the remaining combinations showing intermediate values between these two sets of responses. Fertilization rate had a statistically significant effect on fungal H' measurements ($p = 0.04$) where the diversity index under 269 kg N/ha was statistically higher compared to the unfertilized controls, with intermediate values for the 202 kg N/ha treatments (**Table 1**).

The β-diversity of the archaeal communities was not statistically different for any of the 36 pairwise comparisons of the Rot × Nrate treatment interactions according to the q-values obtained, correcting for multiple comparisons (**Supplementary Table 2**). The β-diversity of Bacteria differed significantly for 16 out of the 36 Rot × Nrate treatment interactions, driven largely by differences between the continuous corn system (CCC) and the Cs or SC phases of the corn-soybean rotations (**Supplementary Table 3**). The bacterial β-diversity of the CCC controls (CCC-0) also differed from the diversity measured at higher N rates (CCC-202 and CCC-269), with similar levels of β-diversity between them. Bacterial β-diversity under CCC-0 however, did not differ from that measured under the Cs controls (Cs-0) or at the highest N rate (Cs-269) for that phase. Likewise, the β-diversity under CCC-0 did not differ from that measured within soybean phases, at any N fertilization rate (Sc-0, Sc-202, Sc-269 kg N/ha). The β-diversity of the fungal community structure differed significantly only for the rotation treatment, and within those, β-diversity was statistically different under CCC compared to the Cs or Sc phases of the corn-soybean rotation (**Supplementary Table 4**).

The indicator ASVs extracted for the archaeal community (**Supplementary Table 5**) belong mainly to the phylum Thaumarchaeota (54%), followed by Euryarchaeota (27%), and in much lower percentages to the Woesearchaeota (2%), and Pacearchaeota (0.1%) phyla. Bacterial reads at the phylum level showed that Proteobacteria (44%) was the most abundant, followed by Planctomycetes (15%), Gemmatimonadetes (13%), Acidobacteria (10%), Bacteroidetes (7%), Chloroflexi (6%), Actinobacteria (4%), Firmicutes (1%), and Verrucomicrobia (1%) (**Supplementary Table 5**). The fungal community was dominated by the phylum Ascomycota (63%), followed by Zygomycota (19%), Chytridiomycota (9%), Glomeromycota (6%), and Basidiomycota (3%) (**Supplementary Table 5**).

Soil Microbiota Responses to Crop Rotation and N Fertilization

Archaea

The PCA on the 16S rRNA archaeal domain rendered a set of three uncorrelated PCs (PC1-PC3; **Table 2**), explaining a total of 77% of the variability contained within the top-contributing archaeal dataset. Archaeal indicators with a significant correlation with a given PC, loadings $\geq |0.45|$, were considered important contributors and used in the description of such PC. Thus, PC1 explained 37% of the dataset variability and contained negative loadings for *Methanomassiliicoccus*, and positive loadings from three *Woesearchaeota incerta sedis (IS): AR15, AR18,* and *AR20.* PC2 explained an additional 22% of the variability, representing a contrast between *Nitrososphaera* (positive loading) and *AR16* (negative loading). PC3 explained an additional 18% and showed a contrast between the abundance of *Nitrososphaera* and *AR15*, both with positive loadings, and the abundance of *AR20*, with negative loadings.

Results from linear mixed model ANOVAs assessing effects of crop rotation (Rot) and N fertilization rate (Nrate) on each of the PCs representing the archaeal community (**Table 5**) indicate no statistically significant effects for either PC1 or PC2, and a marginal yet statistically significant effect of rotation on PC3 ($p = 0.079$). A plot of PC3 mean values for each Rot level with its standard error bars is shown in **Figure 1A**, accompanying the means separation results included in **Table 5**. Means separation procedures on this relation showed CCC with the largest and Sc with the lowest treatment means, while the Cs showed intermediate values. Looking at the contributions from each indicator archaea to these mean separation results in **Figure 1B**, differences were driven by higher relative abundances of *Nitrososphaera* and *AR15* and lower relative abundance of *AR20* in CCC when compared to Sc.

Bacteria

The PCA on top bacterial indicators rendered a set of four uncorrelated PCs (PC1–PC4; **Table 3**) that together explained 56% of the variability of the data set. PC1 explained 34% of the variability and its eigenvector contained a contrast between two groups of microbes, those with positive loadings: *Algisphaera, Allostreptomyces, Arboricoccus, Ferribacterium, Gp22, Nitrosospira, Ornatilinea, Parviterribacter, Pedomicrobium, Thermoanaerobaculum,* and *Thermodesulfitimonas*; whereas the group with negative loadings included *Actinoallomurus, Chujaibacter, Denitratisoma, Dokdonella, Gp1, Micropepsis,*

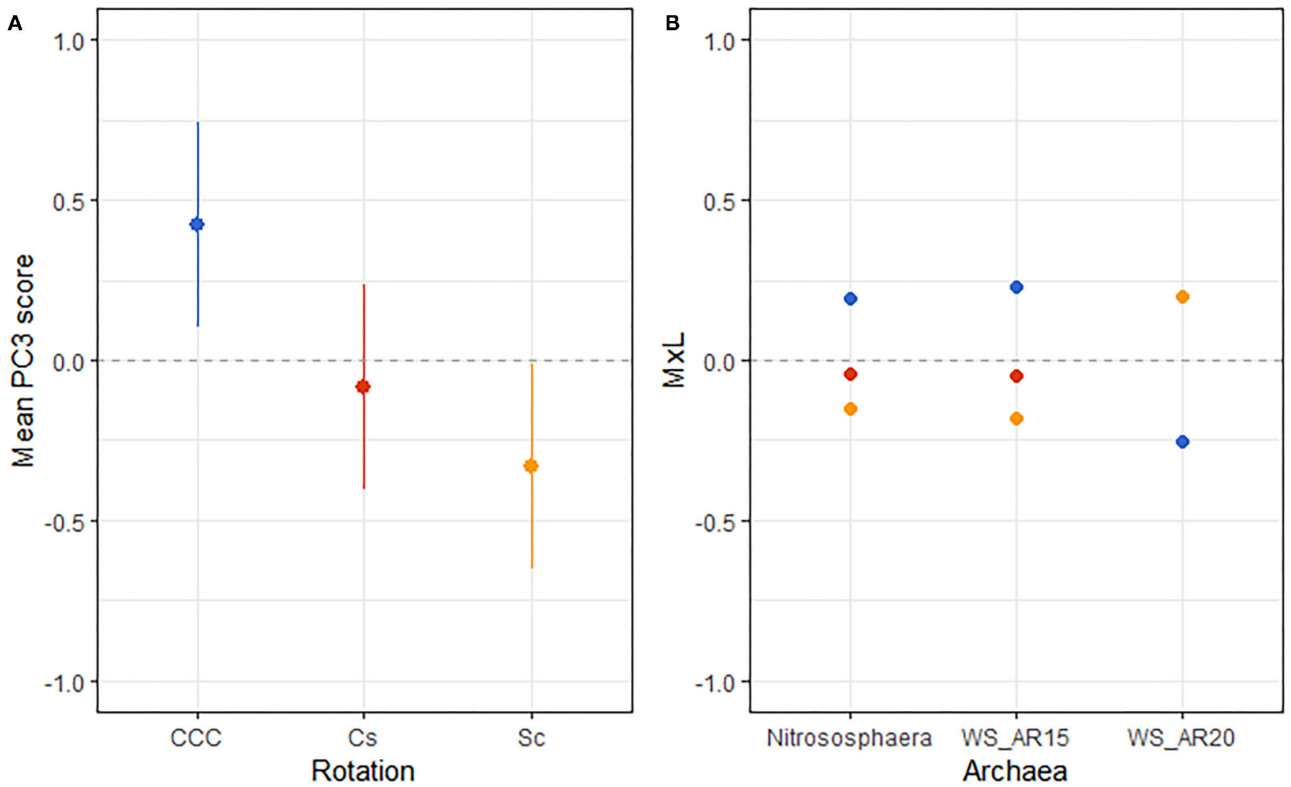


FIGURE 1 | (A) Mean value of the archaeal PC3 for each rotation treatment with their standard errors (as whiskers). **(B)** Contribution of each indicator archaeon to the PC mean value (MxL) for each rotation treatment: continuous corn (blue), corn phase of the corn-soybean rotation (red), and soybean phase of the corn-soybean rotation (orange).

Porphyrobacter, Pseudolabrys, Rhizomicrobium, Roseisolibacter, and *Thermomonas*. PC2 explained an additional 8% of the variability and its eigenvector contained a contrast between two groups of microbes, those with positive loadings: *Arboricoccus, Arenimonas,* and *Pseudolabrys*; whereas the group with negative loadings included *Hyphomicrobium, Lentimicrobium,* and *Pyxidicoccus*. PC3 explained an additional 7% and was represented by three genera all with positive loadings, *Laribacter, Steroidobacter,* and *Variovorax*. Lastly, PC4 contrasted the abundance of *Rhizobium* (negative loading) with that of a group of bacteria including *Aquisphaera, Geminisphaera, Gemmata, Lacibacterium,* and *Terrimonas*.

Linear mixed model ANOVAs assessing effects of Rot and Nrate and their interaction, on each of the PCs representing the bacterial community (**Table 5**), indicate statistically significant interaction effects (Rot × Nrate) on PC1 ($p = 0.0012$), and a marginal yet statistically significant main effect of Nrate on PC3 ($p = 0.0803$). No statistically significant effects were detected for PC2 or PC4 and thus, there will not be further discussed. A graph of the PC1 means for each Rotation and N rate level with their respective standard error bars is shown in **Figure 2A**, accompanying the means separation results included in **Table 5**. The contribution of each bacterial genera to these results is depicted in **Figure 2B**. Thus, it became evident that the differential pattern of the response observed for the rotations at successive N rates was mainly driven by a greater magnitude of the response of the microbial groups under CCC compared to the response to N observed for the Cs or Sc phases of the corn-soybean rotation. Compared to the CCC-0 controls, the group of indicator microbes with positive loadings within PC1, significantly decreased with the addition of N at either rate of 202 or 269 kg N/ha, and the opposite behavior was observed for those indicators with negative loadings (**Figure 2B**). This marked response to Nrate was however not observed within each of the Cs or Sc rotation phases, which showed intermediate values to the CCC-0 on one end, and the Cs-202 on the other, yet were all statistically different from the strong response to N under CCC. A graph of the PC3 means for each N rate level with their respective standard error bars is shown in **Figure 3A**, which accompanies the means separation results included in **Table 5**. The contribution of each bacterial genera to these results is depicted in **Figure 3B**. The three bacteria genera statistically decreased in abundance at 202 kg N/ha yet their relative abundance at the maximum Nrate showed values intermediate between those registered at 0 and 202 kg N/ha.

Fungi

The PCA on top fungal indicators rendered a set of five uncorrelated PCs (PC1–PC5; **Table 4**) that together explained 39% of the variability of the data set. PC1 explained 12%

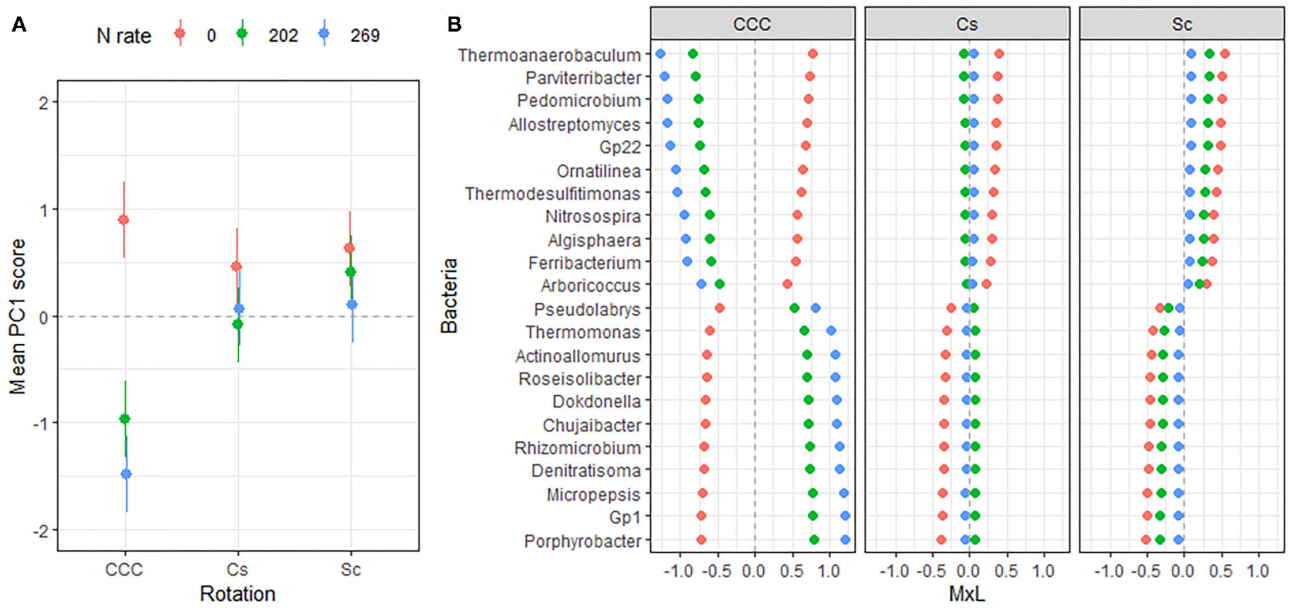


FIGURE 2 | (A) Mean value of the bacterial PC1 scores for each rotation at each level of N fertilization. Standard errors are indicated as whiskers. **(B)** Contribution of each indicator genera to the PC mean value (MxL) for each rotation at each level N fertilization: CCC, continuous corn; Cs, corn phase of the corn-soybean rotation; Sc, soybean phase of the corn-soybean rotation.

of the variability and its eigenvector was dominated by three genera with positive loadings: *Morchella, Mycosphaerella*, and *Pochonia*. PC2 explained an additional 9% of the variability and its eigenvector contained a contrast between two groups of microbes, with positive loadings: *Lasiosphaeris, Ochroconis,* and *Ophiostoma,* and *Candida* and *Pseudogymnoascus* with negative loadings. PC3 explained an additional 7% presenting a contrast of two microbial groups: *Ochroconis, Plectosphaerella,* and *Tetracladium* with positive loadings, and *Glomus* and *Rhizophagus*, with negative loadings. PC4 explained an additional 6% contrasting the abundance of *Corynascus, Exophiala, Humicola, Hypocrea,* and *Thielavia*, with that of *Cortinarius* and *Glomus* (both with negative loading). Lastly, PC5 explained the remaining 6% of the variability in the fungal dataset and contained a contrast between *Aleuria, Neonectria, Spizellomyces,* and *Staphylotrichum* and the group of indicators with negative loadings comprised of *Alternaria, Davidiella,* and *Phoma*.

Linear mixed model ANOVAs assessing effects of Rot and Nrate and their interaction, on each of the PCs representing the fungal community (**Table 5**), indicate statistically significant interaction effects (Rot × Nrate) on PC2 ($p = 0.0903$), and PC4 ($p = 0.0047$), and a statistically significant main effect of Rot on PC3 ($p = 0.0025$). No statistically significant effects were detected for PC1 or PC5 and thus, there will not be further discussed. A graph of the fungal PC2 means for each Rotation and N rate level with their respective standard error bars is shown in **Figure 4A**, the means separation results included in **Table 5**. The contribution of each fungal genera to these results is depicted in **Figure 4B**. Again, the differential pattern of the response observed for the rotations at contrasting N rates (0 vs. 269 kg N/ha), was mainly driven by a greater magnitude of the response of the fungal groups under CCC. Thus, compared to the CCC-0 and CCC-202, the group of fungal indicator genera with positive loadings within PC2 significantly decreased in abundance at 269 kg N/ha whereas those with negative loadings increased with more N in the system (**Figure 4B**). Thought the highest PC2 means were registered for the Sc-0 controls, no differences in the response to Nrate was observed within Sc or the Cc rotation phases, which showed intermediate values to the Sc-0 and the Cs-202. With the exemption of Cs-202, they were all statistically different than the strong response to the maximum N rate under CCC (**Figure 4A**). PC3 means for each Rot level with their respective standard error bars are shown in **Figure 5A**, which accompanies the means separation results included in **Table 5**. The contribution of each fungal indicator genera to these results is depicted in **Figure 5B**. Thus, PC3 means showed a statistically significant increase within the Sc compared to the CCC, with the Cs showing intermediate values. The fungal indicators with positive loadings increased in abundance with Sc, while those with negative loadings increased with CCC. Last, **Figure 6A** shows PC4 treatment means for each Rot × Nrate level with their respective standard error bars, alongside the contribution of each fungal indicator genera to the PC4 means separation results (**Figure 6B, Table 5**). Once again, the interaction is driven by a strong response of the PC4 microbial groups to the addition of N under CCC, not observed under either phase of the rotated crops (**Figure 6A**). Thus, compared to the fertilized systems, the CCC-0 system is characterized by a higher relative abundance of *Glomus* and *Cortinarius* and lower abundances of *Corynascus, Exophiala, Humicola, Hypocrea*, and *Thelavia*. However, once N is added

at either 202 or 269 kg N/ha, the group dominance is reverted. Fungal indicators within Cs and Sc at any given N rate, all showed relative abundances intermediate to those registered under CCC (**Figure 6B**).

Relations Between Soil Microbiota and Soil Properties

Linear mixed model ANOVAs assessing effects of Rot and Nrate and their interaction, on each of soil parameters determined for the site at the time of sampling (**Supplementary Table 1**), indicate statistically significant interaction effects (Rot × Nrate) on pH ($p = 0.0021$), and NO_3^- ($p = 0.0285$), and a statistically significant main effect of Nrate on Pa ($p = 0.0066$). At 0 Nrate, pH was similar across rotations; the interaction effect on pH becomes evident once N is added within the CCC system, a response that is not observed within the Sc phase of the corn-soybean rotation, and that is only partially evident within the Cs phase. Within the latter, the reduction in pH occurs when N is added at the lowest rate, Cs-202, yet the pH measured at higher Nrate was intermediate between that of the control Cs-0, and the pH at Cs-202. No significant differences associated with the treatments were detected for SOM and NH_4^+. The lowest levels of NO_3^- were measured within the CCC-0 and the Cs-0 controls, and the highest within CCC- 269 kg N/ha, the remaining combinations showing intermediate values between these responses. Regardless of the rotation, however, Pa showed a statistically significant decrease with the addition of N.

Table 6 shows the matrix of Pearson' correlation coefficients among pH, SOM, NO_3^-, NH_4^+, and Pa and the PCs responsive to treatments for each taxon, Archaea PC3 (APC3), Bacteria PC1 and PC3 (BPC1 and BPC3), Fungi PC2, PC3, and PC4 (FPC2, FPC3, FPC4). Nineteen of the 25 statistically significant correlation coefficients found in the analyses (bolded, **Table 6**) fell within the "weak" (|0.2–0.4|) association range, whereas 3/25 correlations were classified as "moderate" (|0.4–0.6|) range, and only 2/25 and 1/25 correlations were within the "strong" (|0.6–0.8|) and very strong (>|0.8|) association ranges, respectively. Thus, APC3 was weakly and positively associated with BPC3 values ($r = 0.25$, $p = 0.0706$), and to Pa ($r = 0.27$, $p = 0.0464$) and weakly and negatively associated with NH_4^+ ($r = -0.26$, $p = 0.0563$). Bacterial PC1 was positively associated with FPC2 scores ($r = 0.68$, $p < 0.0001$), soil pH ($r = 0.92$, $p < 0.0001$), and Pa ($r = 0.43$, $p = 0.0012$), relations that fell within the strong, very strong, and moderate association ranges, respectively. At the same time, BPC1 was found to be weakly and negatively associated to FPC4 ($r = -0.37$, $p = 0.0054$), SOM ($r = -0.30$, $p = 0.0298$), and NO_3^- ($r = -0.30$, $p = 0.0253$). Bacterial PC3 had a weak and negative association with FPC3 ($r = -0.25$, $p = 0.0675$), and a weak positive association with Pa ($r = 0.23$, $p = 0.0932$). Fungal PC2 was strongly and positively associated with soil pH ($r = 0.69$, $p < 0.0001$), and weakly associated with Pa ($r = 0.27$, $p = 0.0458$), while showing a negative weak association to NO_3^- ($r = -0.25$, $p = 0.0690$). Fungal PC3 displayed weak and negative associations to both NO_3^- (r =-0.25, $p = 0.0662$), and Pa ($r = -0.33$, $p = 0.0134$). Fungal PC4 was moderately and negatively associated with pH ($r = -0.42$, $p = 0.0015$), and positively associated with SOM ($r = 0.51$, $p < 0.0001$) while showing a weak negative relation to Pa ($r = -0.25$, $p = 0.0706$). Among the soil properties examined, pH was weakly and negatively associated to SOM ($r = -0.36$, $p = 0.0074$), and NO_3^- ($r = -0.25$, $p = 0.0642$), and positively related to Pa ($r = 0.37$, $p = 0.0060$). Likewise, NO_3^- was found to be weakly and negatively associated with SOM ($r = -0.40$, $p = 0.0031$), NH_4^+ ($r = -0.31$, $p = 0.0213$), and Pa ($r = -0.25$, $p = 0.0721$).

DISCUSSION

Overall, results from this study indicate that both agronomic practices of crop rotation and N fertilization rates leave a significant imprint on the soil microbial groups following over three decades of management. Continuously cropped corn had distinct microbial taxa, while annually rotated communities were similar in both crop phases. These findings agree with those of Chamberlain et al. (53) in southern Wisconsin, Ashworth et al. (54) in Tennessee, and Behnke et al. (26) in Illinois, who studied the long-term impacts of rotating corn and soybean compared to their monocultures on bulk soil microbial communities. The general lack of differences between the corn and soybean phases of the corn-soybean rotation supports a legacy rotational effect rather than an immediate yearly impact of each crop of the rotation on the structure of microbial communities, as observed in other studies (26, 53).

Because of the annual addition of fertilizers within the continuous corn system compared to the every-other-year application in the rotated ones, when interactions between crop rotation and N fertilization were present, the responses were closely associated with the changes in soil pH and total inorganic N within the continuously cropped corn systems compared to the rotated ones. As it was observed for the crop rotation, alternating corn and soybean phases seemed to ameliorate the response of the soil microbiota to N fertilization detected in the continuous corn system. This ameliorated response could be further supported by similar levels of SOM and Pa measured across monocropped and rotated corn crops despite obvious differences in amount and quality of residue returned, consistent with previous studies in soils of the region (26, 53, 55). Across rotations, however, N fertilization had a significant impact on increasing NO_3^- in the soil while reducing pH and available P. The latter is attributed to the enhanced P uptake by crops in rotation, that yielded 10% more grain on average than monocropping (56), as well as to the potential damage to microbial acid phosphatase activities with N-fertilization (57).

The following subsections Archaeal indicators, Bacterial indicators, and Fungal indicators provide an overview of the archaeal, bacterial, and fungal indicators identified and their relations with soil properties. Lastly, subsection Final remarks and future directions delineates future directions and integrates these findings with previously published information and relevant literature pertaining to crop rotation and N fertilization in agroecosystems.

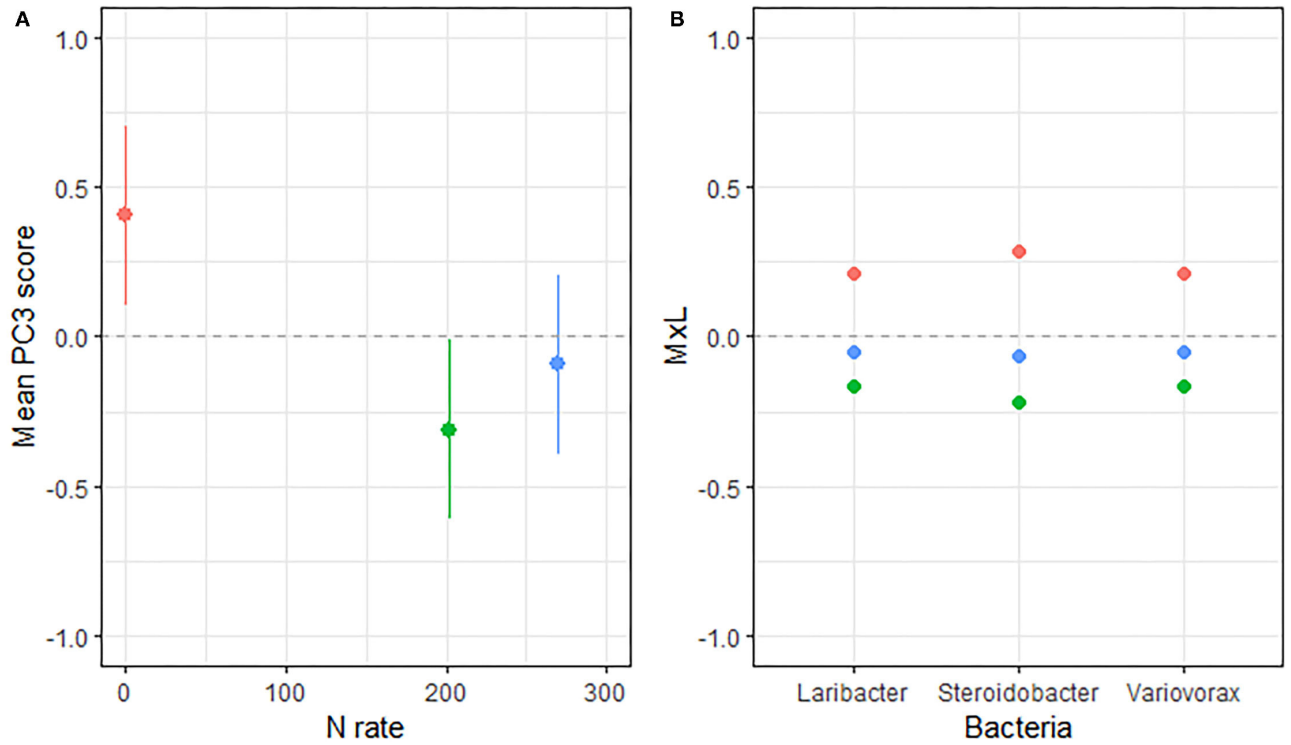


FIGURE 3 | (A) Mean value of the bacterial PC3 scores for each level of N fertilization with their standard errors (as whiskers). **(B)** Contribution of each indicator genera to the PC mean value (MxL) for each level of N fertilization: 0 kg N/ha (pale red), 202 kg N/ha (pale green), and 269 kg N/ha (pale blue).

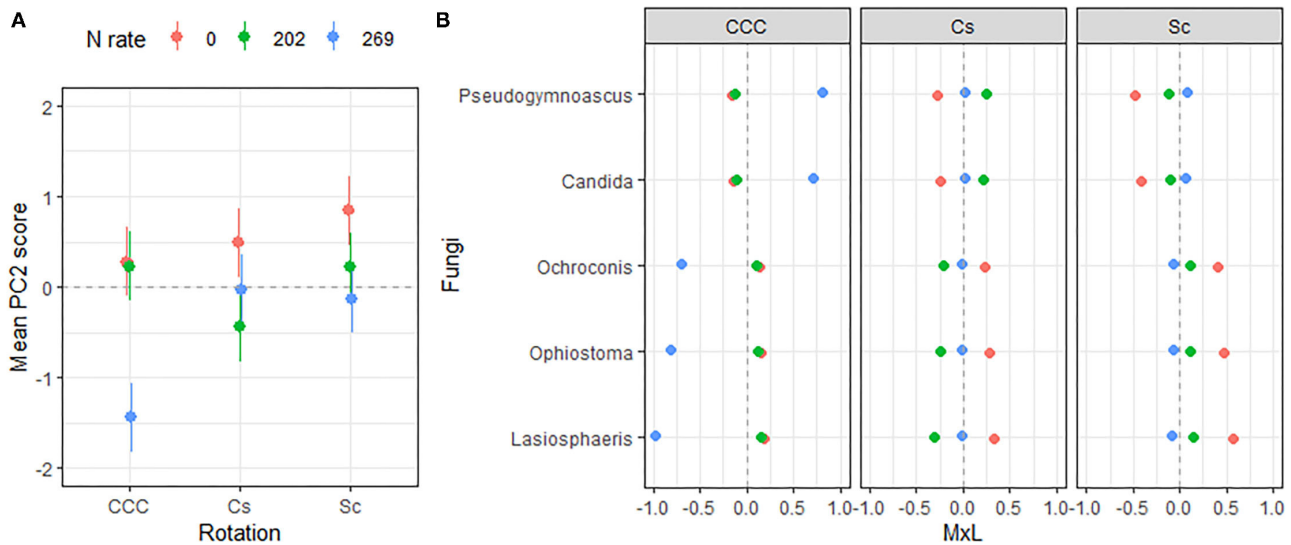


FIGURE 4 | (A) Mean value of the bacterial PC2 scores for each rotation at each level of N fertilization. Standard errors are indicated as whiskers. **(B)** Contribution of each indicator genera to the PC mean value (MxL) for each rotation (panels) at each level of N fertilization: CCC, continuous corn; Cs, corn phase of the corn-soybean rotation; Sc, soybean phase of the corn-soybean rotation.

Archaeal Indicators

Archaeal indicators found for the rotation factor in this study clearly distinguished between the continuous corn and the rotated corn and soybean phases. Higher abundances of *Nitrososphaer*a and the *Woesearchaeota IS AR15* along with the reduced abundance of the *Woesearchaeota IS AR20* were the

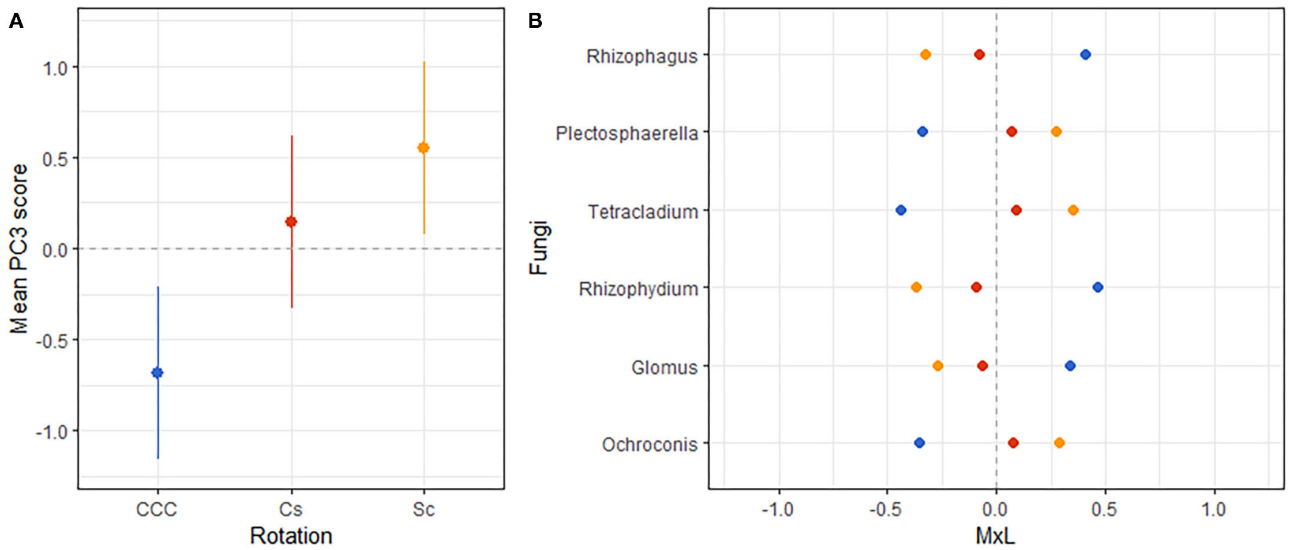


FIGURE 5 | (A) Mean value of the fungal PC3 for each rotation treatment with their standard errors (as whiskers). **(B)** Contribution of each indicator fungi to the PC mean value (MxL) for each rotation treatment: continuous corn (blue), corn phase of the corn-soybean rotation (red), and soybean phase of the corn-soybean rotation (orange).

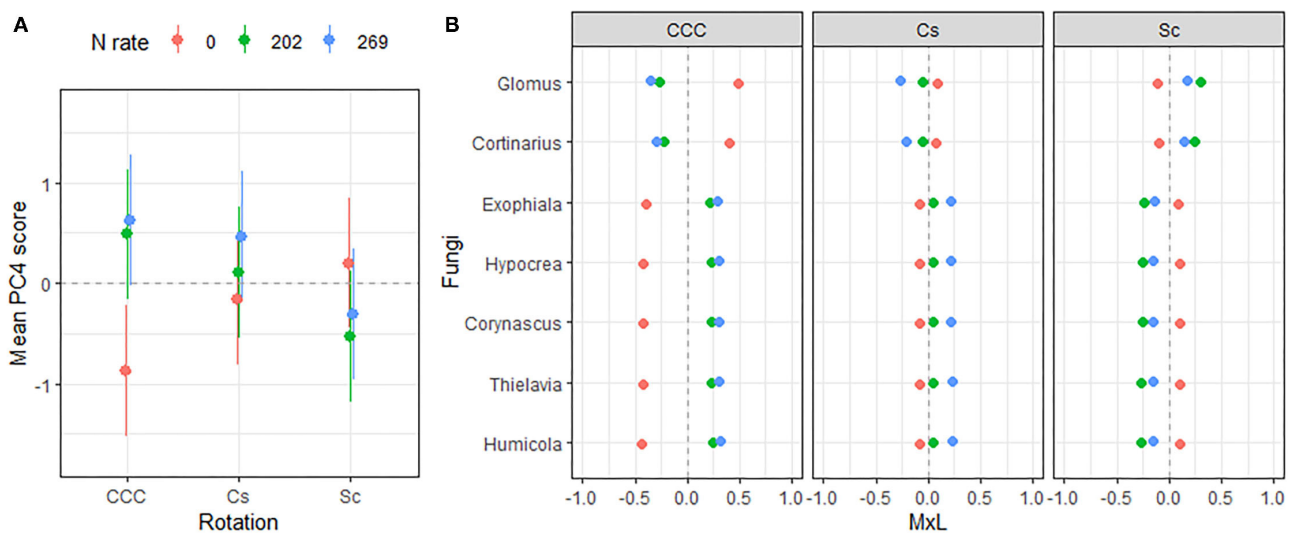


FIGURE 6 | (A) Mean value of the fungal PC4 scores for each rotation at each level of N fertilization. Standard errors are indicated as whiskers. **(B)** Contribution of each indicator genera to the PC mean value (MxL) for each rotation (panels) at each level of N fertilization: CCC, continuous corn; Cs, corn phase of the corn-soybean rotation; Sc, soybean phase of the corn-soybean rotation.

signature of the continuous corn management when compared to the rotated systems (**Figure 1**).

Nitrososphaera is an aerobic, neutrophilic, ammonia-oxidizing archaea (AOA), predominantly found in terrestrial ecosystems (58). Due to its high abundance in agricultural soils and strong positive correlation of *Nitrososphaera* with agricultural management, in particular with soil pH and ammonium levels, Zhalnina et al. (59) proposed this genus as a signature microorganism for agricultural land use. Likewise, Kim et al. (10) looking for microbial indicators from corn-soybean rotations with and without cover crops, identified *Nitrososphaera* as the only archaeal indicator, prospering under the oligotrophic conditions of the corn-soybean rotations without cover crops. The higher abundance of this genus in the continuous corn management relative to the rotated system could be explained by differences in the quality of organic matter supplied to the soil (including straw and root exudates). Labile organic matter favors the growth of the soil AOA community (60, 61) and thus,

TABLE 6 | Matrix of Pearson' correlation coefficients among microbial indicator groups for each taxa responsive to treatments (Archaea PC3, Bacteria PC1 and PC3, Fungi PC2, PC3, and PC4) and soil properties of pH, soil organic matter (SOM), nitrate and ammonium (NO_3^-, NH_4^+), and available P (Pa).

	APC3	BPC1	BPC3	FPC2	FPC3	FPC4	pH	SOM	NO_3^-	NH_4^+	Pa
APC3[†]	1										
BPC1	0	1									
BPC3	**0.25**	0	1								
FPC2	0.15	**0.68**	0	1							
FPC3	−0.15	0.14	**−0.25**	0	1						
FPC4	−0.07	**−0.37**	0.19	0	0	1					
pH	−0.01	**0.92**	0.03	**0.69**	0.11	**−0.42**	1				
SOM	0.11	**−0.30**	0.15	−0.07	0.15	**0.51**	**−0.36**	1			
NO_3^-	−0.01	**−0.30**	0.03	**−0.25**	**−0.25**	−0.19	**−0.25**	**−0.40**	1		
NH_4^+	**−0.26**	0.10	−0.09	0.17	−0.08	−0.03	0.06	0.07	**0.31**	1	
Pa	**0.27**	**0.43**	**0.23**	**0.27**	**−0.33**	**−0.25**	**0.37**	−0.02	**−0.25**	−0.07	1

[†]*APC3, Archaea PC3; BPC1, BPC3, Bacteria PC1 and PC3, respectively; FPC2, FPC3, FPC4, Fungi PC2, PC3, and PC4, respectively.*
Bolded correlation coefficients indicate statistical significance at α = 0.10.

a higher supply of this labile SOM in CCC could be a potential explanation for the observed results.

Woesearchaeota represents a newly established member of the superphylum DPANN (Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanoarchaeota, and Nanohaloarchaea) that are predicted to have fermentation-based lifestyles and/or are symbionts (62). Both ecological distribution patterns and metabolic predictions conducted by Liu et al. (63, 64) support a key role of woesearchaeotal lineages of anoxic environments in the cycling of carbon, nitrogen, and sulfur. Because *nir*K and *nosZ* genes are detected in Woesearchaeota, they may play a role in the nitrogen cycle under anoxic conditions, typical of inner aggregates, such as denitrification from nitrite to N_2 (63, 64). Representative sequences from the *AR15* genome were only previously identified in samples from hypersaline anoxic environments (63), while the metabolic reconstruction of the *AR20* genome indicate the ability to play a role in sulfur cycling (62, 64). The negative correlation with soil NH_4^+ detected for these archaeal indicators as a group (APC3), and the different behavior of AR20 could be hinting to an ammonium sensitivity of this archaeon compared to AR15 and *Nitrososphaera*. With their potential importance in soil N cycling, more efforts should be dedicated to culturing and studying archaeal metabolism and ecology.

Bacterial Indicators

Indicators of N Fertilization Within Rotations

A distinct bacterial signature for the continuous corn systems was found in response to N additions. Two groups, each comprised of 11 genera, behaved oppositely when N fertilizer was applied within the continuous corn systems, regardless of the rate supplied (**Figure 2**). Compared to their abundances within the unfertilized controls, the abundance of *Thermoanaerobaculum, Parviterribacter, Pedomicrobium, Allostreptomyces,* Acidobacteria subgroup 22 (*Gp22*), *Ornatilinea, Thermodesulfitimonas, Nitrosospira, Algisphaera, Ferribacterium*, and *Arboricoccus*, decreased when N was added to the system. On the other hand, the abundance of *Pseudolabrys, Thermomonas, Actinoallomurus, Roseisolibacter, Dokdonella, Chujaibacter, Rhizomicrobium, Denitratisoma, Micropepsis,* Acidobacteria subgroup 1 (*Gp1*), and *Porphyrobacter* increased with N fertilization.

The mirrored segregation between the groups of genera is likely driven by soil pH and nutrient levels, supported by the strong positive correlation measured between bacterial BPC1 (**Table 6**) and soil pH ($r = 0.92$), the moderate association with available P (Pa, $r = 0.43$), and the negative weak correlations detected between the bacterial groups with SOM and NO_3^- (**Table 6**). Changes in soil properties associated with N fertilization are noticeable within continuous corn rotations (**Supplementary Table 1**), i.e., addition of N caused reductions in pH and available P, while increasing total inorganic N. Within this system, there seems to be a trend to increased SOM with increased fertilization, yet no statistically significant effect was detected. Thus, while the relative abundances of the first group of genera decreased in response to reductions in soil pH and available P, and parallel increases in N with fertilization, the

second group thrived under those same conditions. These results agree with other reports from long-term agricultural systems in the region (26, 53, 54), who found that soil pH, SOM, and nutrient levels, are the main soil factors modeling the responses of the microbiota to crop rotation and tillage practices. Relevant literature at the level of phyla generally supports these findings as well, although specifics remain unknown for many genera (65–69).

Both Acidobacteria in this group (*Thermoanaerobaculum,* and *Gp22*) of indicators that responded negatively to N fertilization, have previously shown a positive correlation to pH (67, 70). Most other acidobacteria subgroups, like the dominant *Gp1*, which showed a negative correlation with soil pH, have a preference for acidic growth conditions that characterizes the phylum (71). Acidobacteria has been proposed as a keystone taxon (72), associated with the decomposition of SOM in soils and playing a major role in denitrification processes, thus central in carbon and N cycling (71).

The two actinobacteria in this group were *Allostreptomyces,* originally isolated from the endorhizosphere of *Psammosilene tunicoides* in southeast China (73), and *Parviterribacter* (74), isolated from a dark loam grassland soil. The endophyte might confer protection to the plant from pathogens and environmental stresses, as well as promoting plant growth (69). *Ornatilinea* responded negatively to fertilization and it was the only indicator genus identified from the phylum Chloroflexi. *Ornatilinea* is the first representative of the family Anaerolineacea, characterized as strictly anaerobic chemoautotrophs, capable of growth on cellulose as the sole source of energy and carbon whose niche may be favored under unfertilized treatment poorer in C and N (75).

Thermodesulfitimonas is also the only identified indicator from the phylum Firmicutes in our study; it is a thermophilic, anaerobic, chemolithoautotrophic bacterium, isolated from a terrestrial hot spring (76). Their lithoautotrophic metabolism may explain their positive responses to comparatively nutrient poor unfertilized continuous corn system. Another lone representative of its phylum, *Algisphaera* (Planctomycetes) belongs to the group of indicators that decreased in abundance with the addition of N in the systems. The phylum Planctomycetes constitutes a significant proportion of aquatic and terrestrial environments, although most of them are uncultivated and the known ecophysiological types of planctomycetes are quite limited, as is the case with *Algisphaera*, a strictly aerobic bacterium isolated from algae (77). However, without much knowledge about this genus, it is difficult to explain their response to N rate treatment.

Four members of the Proteobacteria phylum, belong to the group of indicators with a negative relation to fertilization: *Pedomicrobium, Arboricoccus* (both Alphaproteobacteria), and *Nitrosospira* and *Ferribacterium* (both Betaproteobacteria). Though phylogenetically related, members of this phylum are physiologically, morphologically, and ecologically extremely diverse; they include various pathogens of humans, animals, and plants, as well as several taxa that play key roles in the carbon, sulfur, and nitrogen cycles (65). *Arboricoccus* is a facultative aerobic bacterium isolated from the endophytic microbiome of pine trees (78), grown in acidic soil environments. *Nitrosospira* includes the most relevant ammonia oxidizing bacteria (AOB) in soil environments and an increase in diversity and abundance of this taxa have been reported under long-term N applications (60, 79). The negative response observed in our study is likely associated to the uniformity of ammonium levels across treatments, indicating that the gradual pH decreases induced by the fertilizer was the main controller of this AOB abundance. Our results agree with Pereira e Silva et al. (80) who reported that fluctuations in ammonia oxidizing communities in agricultural soils are mainly influenced by pH and soil type.

The group of 11 indicator genera with a positive response to N fertilization, included one member of each of the Acidobacteria, Actinobacteria, and Gemmatimmonadetes phyla, genera Acidobacteria subgroup 1 (*Gp1*), *Actinoallomurus*, and *Roseisolibacter*, respectively. The group also included eight Proteobacteria, four Alphaproteobacteria (*Micropepsis, Rhizomicrobium, Pseudolabrys, and Porphyribacter*), one Betaproteobacteria (*Denitratisoma*), and three Gammaproteobacteria (*Dokdonella, Chujaibacter,* and *Thermomonas*).

Acidobacteria is one of the nine dominant phyla in soil with critical roles as decomposers and denitrifiers, and as previously stated, the response of *Gp1* to fertilization is typical of most members of the phylum, which prefer acidic conditions (70). The Actinomycetes are well-established as prolific producers of a wide range of bioactive secondary metabolites, among them, *Actinoallomurus* is one of the newly discovered genera, known as an endophyte of rice (*Oryza sativa*), and *Acacia spp*. Like other endophytes, they might confer plants resistance to stress and pathogenic invasions, aid in the solubilization and availability of phosphates, and facilitate the solubilization of iron via the production of siderophores (69).

Representatives of the phylum Gemmatimonadetes have been detected in a wide range of soil environments, where they seem to be well-adapted to the low moisture/low oxygen conditions of inner aggregates (81). *Roseosolibacter*, an aerobic bacterium, was recently isolated from an agricultural floodplain (82). Within the Alphaproteobacteria, genera *Micropepsis* and *Rhizomicrobium* are chemoheterotrophic bacteria widespread in soils, strict or facultative anaerobes capable of fermenting a wide range of carbon sources in neutral or mildly acid soils, which make them well-adapted to the soil environment under heavily fertilized continuous corn (83). *Pseudolabrys,* described by Kampfer et al. (84), were isolated from acidic soils whereas *Porphyrobacter*, an aerobic bacterium, is often associated with aquatic environments (85, 86). The Betaproteobacteria in this second group of microbial indicators is represented by *Denitratisoma*, a denitrifying bacterium isolated from activated sludge of a wastewater treatment plant able to perform anaerobic oxidation of estradiol to CO_2 using nitrate as the electron acceptor, thus reduced to a mixture of N_2O and N_2 (87).

The last three genera of this indicator group with a positive response to N fertilization, were all members of the Gammaproteobacteria phylum. *Dokdonella* genera are strictly aerobic rods isolated from soil; a few species can perform the aerobic reduction of nitrate (88). *Chujaibacter* is an aerobic rod isolated from soil shown to be unresponsive to land management

in a metagenomic survey of Polish soils (89). However, this genus was recently identified in agricultural soils as belonging to a cluster of microbes favoring the low pH and high SOM environments found under continuous corn systems, which agrees with our results (26). *Thermomonas* is an aerobic genus isolated from a variety of habitats, i.e., soils, hot springs, slurry, and biofilms (90). A few species of this genus are proven denitrifiers isolated from denitrification reactors (91).

Indicators of N Fertilization Across Rotations

Three Proteobacteria genera decreased in abundance when N was added to the systems (**Figure 3**) and the response was consistent for continuous and rotated corn phases. Though the abundances of these genera seem to follow the overall decrease in soil pH and available P across rotations (**Supplementary Table 1**), they did not show a statistically significant correlation with soil pH, and they were only weakly and positively correlated to Pa (**Table 6**, BPC3). Among them, the genera *Variovorax* is an aerobic or facultative anaerobic group of denitrifying bacteria isolated from soils (92). Lastly, the genera *Steroidobacter* (closely related to *Denitratisoma* mentioned in the previous group of indicators) are rod-shaped denitrifying Gammaproteobacteria that degrade steroids (oestradiol and testosterone) as the sole source of carbon and energy with nitrate as the electron acceptor (93).

Fungal Indicators

Indicators of N Fertilization Within Rotations

Rotation and N fertilization, alone and in combination, all favored distinct fungal groups that suggest an important role of fungi within these simplified agroecosystems typical of production agriculture. Within continuous corn, maximum N rate increased the abundances of *Pseudogymnoascus* and *Candida*, while decreasing those of *Ochroconis*, *Ophiostoma*, and *Lasiosphaeris* (**Figure 4**). The abundances of all these genera within the continuous corn systems did not differ between unfertilized control and intermediate N rate (202 kg N/ha). Interestingly, the highest PC2 mean score is found under the unfertilized controls of the soybean phase of the corn-soybean rotation, although not statistically different from the microbial abundances found under N fertilization (**Table 5**). Therefore, the abundances of these five fungal indicators contrasted the most between unfertilized soybean phase of the corn-soybean rotation and continuous corn with highest N rate (**Figure 4B**). The fungal abundances within the corn phase of the corn-soybean rotation reflect again the suggested legacy effect of the rotation, with abundances and responses intermediate between those observed under continuous corn and the soybean phase of the rotation.

As it was the case for the bacterial indicators of N fertilization and rotation, the segregation between the groups of fungal genera are likely driven by soil pH and nutrient levels, supported by the strong positive correlation measured between fungal FPC2 (**Table 6**) and soil pH, the weak association with available P, and the negative weak correlation detected with NO_3^-. Changes in soil properties associated with N fertilization are most noticeable within continuous corn rotations, yet soil pH is similar among unfertilized controls while available P decreases with N additions regardless of the rotation (**Supplementary Table 1**). On the other hand, the NO_3^- measured within the soybean phases tends to decrease as N is added yet they are all statistically similar to NO_3^- measured under the intermediate N rate application within continuous corn. Thus, while the relative abundances of *Pseudogymnoascus* and *Candida* increased in response to the strongest reductions in soil pH and available P and increases at the maximum N rate fertilization within the continuous corn system, *Ochroconis, Ophiostoma*, and *Lasiosphaeris* abundances suffered under those same conditions.

All these indicator genera belong to the Ascomycota phylum, dominant in agricultural soils consistent with other studies (10, 26, 94–96). *Pseudogymnoascus* species are cellulolytic saprotrophs and usually grow in low temperatures (97). Ubiquitous in soil, species of this genus are known to form ericoid mycorrhizae with the roots of alpine Ericales and other perennial hosts, helping these plants adapt to low-nutrient environments (98, 99). The genera *Ochroconis, Ophiostoma*, and *Lasiosphaeris* are all known saprobes on wood and plant litter. *Ochroconis* species are usually found in moist environments poor in nutrients (100), while *Lasiosphaeris* are typically isolated from decaying wood yet their role in nutrient and energy cycling remains poorly understood (101).

Another group of fungi shown in **Figure 6**, created a distinct microbial signature for the fertilized continuous corn; this time discriminating the unfertilized controls from both N systems, regardless of N rate. Thus, we detected that *Glomus* and *Cortinarius* relative abundances were greater within the unfertilized controls and decreased in the presence of N at any rate. On the other hand, the abundances of *Exophiala, Hypocrea, Corynascus, Thielavia*, and *Humicola* increased with N fertilization. The genus *Glomus* comprise arbuscular mycorrhizal fungi (AMF) that forms obligate symbioses with plant roots, where it obtains carbon from the host plant in exchange for water and nutrients, mainly P (98). *Cortinarius* is an ectomycorrhizal mycobiont (102), suspected to be the largest genus of order Agaricales, containing over 2,000 widespread species.

All the genera that increased in abundance with N fertilization belong to the Ascomycota phylum, a response consistent with their dominance in agricultural soils. *Exhophiala* is a mesophilic black yeast and member of the dark septate endophytes found commonly in soil environments, colonizing maize roots (103), and sorghum plants (68). In soils of the U.S. Midwest region, *Exhophiala* has been found to parasite eggs and adult females of the soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe (Phylum Nematoda), the most important pest affecting soybean production worldwide (104, 105). *Hypochrea* species, also known as *Trichoderma* (106), are widespread in agricultural fields providing important benefits to cultivated plants such as plant growth promotion, yet Trichoderma species have also been reported to be active producers of N_2O in agroecosystems (107). The three genera representative of the Chaetomiacea family of the Sordariales order, *Corynascus, Thielavia*, and *Humicola*, are saprobes commonly found in agricultural soils (108, 109).

Indicators of Crop Rotations

A clear fungal signature emerged for the rotation factor (**Figure 5**) where the abundances of *Rhizophagus, Rhizophydium,*

and *Glomus* increased within the continuous corn systems compared to rotated corn-soybean systems. Genera *Rhyzophagus* and *Glomus* belong to the Glomeromycota phylum and include known AMF that trade P, water, and other nutrients with the photosynthates (C) of their plant hosts. *Rhyzophydium* was the only genus represented in this study from the phylum Chytridiomycota, which is abundant in soils as decomposers of recalcitrant materials such as cellulose, chitin, and keratin, and parasitize plants and other fungi (110). *Rhyzophydium*, is common in nutrient-deficient soils, where they may have a role in nutrient recycling (110). The detrimental effect of increased soil nutrient levels on these potentially beneficial genera in our study is supported by their weak and negative association with available P and with NO_3^- (FPC3, **Table 6**). On the other hand, these soil conditions favored the increased abundances of the Ascomycota genera *Plectosphaerella, Tetrachladium*, and *Ochroconis* within the rotated corn-soybean systems. *Plectosphaerella* (an anamorph of *Fusarium* spp) is well-known as a pathogen of several plant species of agricultural and horticultural importance worldwide (111). *Fusarium* spp have also been reported to be potent N_2O producers in agricultural soils (107), and as its anamorph, *Plectosphaerella* potentially possess this capacity.

Final Remarks and Future Directions

A previous report from this research site explored shifts in the microbiota and the main steps of the microbial N cycle, using targeted gene abundances as proxies, with soil properties (7). Results indicate that long-term N fertilization under continuous corn management acidified the topsoil leading to a parallel increase in the abundance of fungal counts (ITS), potentially damaging biological N_2 fixation and accelerating potential nitrification activities, as was evidenced by the reduced abundance of *nif*H genes, and increased abundance of bacterial *amo*A genes (7). Likewise, Benhke et al. (8), in a long-term study comparing tillage options within monocropped and rotated corn and soybean systems, found that the acidification measured within continuous corn systems following 20 years of management, resulted in increased abundance of fungal ITS and bacterial *amo*A counts (AOB), and parallel reductions in archaeal *amo*A copy numbers (AOA), regardless of tillage. The authors posed that the continuous corn system, with its increased and yearly need for N fertilization, intensifies the microbial N cycle, enhancing bacterial nitrification and exacerbating acidification, thus creating an environment primed for N leaching and increased N_2O emissions (8).

The observed increase in ITS abundances under continuous corn management, and the higher diversity found with increased N fertilization (**Table 1**), show that typical corn crops create a favorable environment for the proliferation of fungal populations. Not only the acidic conditions associated with fertilization but also the large volume of residues and root exudates returned to the soil by corn crops, seem to guarantee their ecological success (94–96, 112). Huang et al. (7) attributed the strong positive correlations detected between ITS counts and *nir*K and *nos*Z counts in our previous study, to the overall acidification of the systems preferred equally by fungi and main denitrifier groups. Yet the observed relations could also suggest an important contribution of fungal denitrification within these agroecosystems, recognized as an important biological process for N_2O emissions (107). Ninety percent of the fungi reported producing N_2O belong to the phylum Ascomycota, and in our data set, they are represented by species of *Trichoderma* and potentially, *Plectosphaerella*, as the *Fusarium* anamorph (107, 113). However, it should be acknowledged that detection of these genera in the soil metagenome does not necessarily mean that they are active members of the microbiome at the time of sampling. Future studies should explore the signatures of long-term nitrogen fertilization in CCC focusing on the soil meta-transcriptome.

In contrast, N fertilization and rotation have no measurable effect on the abundance (7) and alpha diversity (**Table 1**) of either archaeal or bacterial groups in this study. Although much less researched than bacteria, members of the archaea domain show homologous genes that allow them to perform different reductive pathways of the N-cycle, including both assimilatory processes, such as nitrate assimilation and N_2 fixation, and dissimilatory reactions, such as nitrate respiration and denitrification (114). Here, we identified *Nitrososphaera*, an AOA nitrifier archaeon, and *Woesearchaeota AR15*, an anaerobic denitrifier, both as markers for continuous corn environments, decreasing in abundance with rotated management. Seven out of the eight bacteria indicators associated with the N cycle (*Variovorax, Steroidobacter, Acidobacteria Gp22, and Gp1, Denitratisoma, Dokdonella,* and *Thermomonas*), are known denitrifiers, and only one is known as an AOB (*Nitrosospira*).

Interestingly, in our previous study by Huang et al. (7), no significant impacts of fertilization rates or rotations were detected on the estimated abundance of denitrifiers using the *nir*S, *nir*K, and *nos*Z gene counts in the soil as proxies. In the present study, the indicator genera identified as potential denitrifiers were evenly represented in both unfertilized and high N rate treatments, in agreement with the results reported by Huang et al. (7). Nonetheless, sensitivity of some of these denitrifying indicators to N rate was only detected under continuous corn system. Therefore, we could speculate that the pool of indicators identified in this study that are suspected as denitrifiers might be responsible for typical increase in N_2O emission recorded under continuous corn in fertilized conditions (56). However, both meta-transcriptomic as well as functional studies of potential nitrification and denitrification rates yet to be performed for these systems, could shed light on the role of these microbes in the CCC system.

This study identified potential bioindicators that could represent the important soil microbial processes under N fertilization and crop rotation. These processes included decomposition, plant-microbe symbiosis, nitrification, and denitrification. The behaviors of the indicators were largely consistent with the responses of soil properties and functional microbial genes reported in the literature. Moreover, some of the indicator genera are closely associated with important soil properties, namely SOM, NO_3^-, available P, and especially soil pH, thereby suggesting these microbes as potential markers of microbially driven changes in these soil properties.

Overall, we identified a set of three archaea, and six fungal genera responding mainly to rotation; a set of three bacteria

genera whose abundances were linked to N rates; and the set with the highest number of indicator genera from both bacteria (22) and fungal (12) taxa responded to N fertilizer additions within the CCC system. We then focused our discussion on those indicators with a known or a suspected role in the N cycle. Thus, a nitrifier archaeon *Nitrososphaera*, and *Woesearchaeota AR15*, an anaerobic denitrifier, were identified as part of the signature for continuous corn environments, decreasing in abundance with rotated management while the opposite response was recorded for the fungus *Plectosphaerella*, less abundant under continuous corn. Regarding fertilizer addition, bacteria of the genera *Variovorax* and *Steroidobacter* decreased in abundance once N was added to the rotations, whereas *Gp22* and *Nitrosospira* only showed this response under continuous corn. Within the latter system as well, the addition of N fertilizer resulted in increased abundances of the bacterial and fungal denitrifiers *Gp1, Denitratisoma, Dokdonella, Thermomonas*, and *Hypocrea*.

To fully appreciate the agricultural significance and environmental impacts of these bioindicators, we urge researchers to further explore these groups of genera from our results, especially the uncultured and ecologically obscure microbes. At the same time, additional research should identify indicator microbes in other systems and regions to expand the index of bioindicators to help in further monitoring strategies of soil health restoration programs, as we move toward more sustainable management practices worldwide.

DATA AVAILABILITY STATEMENT

The data have been deposited with links to BioProject accession number PRJNA771382 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/).

AUTHOR CONTRIBUTIONS

MV and MZ: conceptualization. NK, MA, CR, MZ, MV, and SR-Z: methodology. MV, SR-Z, and NK: formal analysis. MV, SR-Z, and CR: resources. MV, MZ, CR, and NK: data curation. MV: visualization, writing—original draft preparation, supervision, project administration, and funding acquisition. NK, MA, and MZ: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research was funded by awards ILLU-802-978 and AG 2018-67019-27807, both from the United States Department of Agriculture, USDA-NIFA.

ACKNOWLEDGMENTS

We acknowledge Drs. Alvaro Hernandez and Mark Band from the Roy Carver Biotechnology Center for Functional Genomics lab at the University of Illinois at Urbana-Champaign for their assistance in creating the amplicon libraries. We are thankful to Dr. Greg Steckel, and Mr. Marty Johnson for their contribution in managing the experimental plots, and to Dr. Gevan Behnke for his assistance with soil sampling and overall lab management.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsoil.2021.765901/full#supplementary-material

REFERENCES

1. Bender SF, Wagg C, Van Der Heijden MGA. An underground revolution: biodiversity and soil ecological engineering for agricultural sustainability. *Trends Ecol Evol.* (2016) 31:440–52. doi: 10.1016/j.tree.2016.02.016
2. Stevens CJ. Nitrogen in the environment. *Science.* (2019) 363:578. doi: 10.1126/science.aav8215
3. Hirsch PR, Mauchline TH. The importance of the microbial N cycle in soil for crop plant nutrition. *Adv Appl Microbiol.* (2015) 93:45–71. doi: 10.1016/bs.aambs.2015.09.001
4. De Vries M, Schöler A, Ertl J, Xu Z, Schloter M. Metagenomic analyses reveal no differences in genes involved in cellulose degradation under different tillage treatments. *Fems Microbiol Ecol.* (2015) 91:fiv069. doi: 10.1093/femsec/fiv069
5. Hink L, Nicol GW, Prosser JI. Archaea produce lower yields of N2O than bacteria during aerobic ammonia oxidation in soil. *Environ Microbiol.* (2017) 19:4829–37. doi: 10.1111/1462-2920.13282
6. Hink L, Gubry-Rangin C, Nicol GW, Prosser JI. The consequences of niche and physiological differentiation of archaeal and bacterial ammonia oxidisers for nitrous oxide emissions. *ISME J.* (2018) 12:1084–93. doi: 10.1038/s41396-017-0025-5
7. Huang L, Riggins CW, Rodriguez-Zas S, Zabaloy MC, Villamil MB. Long-term N fertilization imbalances potential N acquisition and transformations by soil microbes. *Sci Total Environ.* (2019) 691:562–71. doi: 10.1016/j.scitotenv.2019.07.154
8. Behnke GD, Zabaloy MC, Riggins C, Rodriguez-Zas S, Huang L, Villamil M. Acidification in corn monocultures favor fungi, ammonia oxidizing bacteria, nirK-denitrifier groups. *Sci Total Environ.* (2020) 720:137514. doi: 10.1016/j.scitotenv.2020.137514
9. Kim N, Riggins CW, Rodriguez-Zas S, Zabaloy MC, Villamil MB. Long-term residue removal under tillage decreases amoA-nitrifiers and stimulates nirS- denitrifier groups in the soil. *Appl Soil Ecol.* (2021) 157:103730. doi: 10.1016/j.apsoil.2020.103730
10. Kim N, Zabaloy MC, Riggins CW, Rodríguez-Zas S, Villamil MB. Microbial shifts following five years of cover cropping and tillage practices in fertile agroecosystems. *Microorganisms.* (2020) 8:1773. doi: 10.3390/microorganisms8111773
11. Philippot L, Spor A, Hénault C, Bru D, Bizouard F, Jones CM, et al. Loss in microbial diversity affects nitrogen cycling in soil. *ISME J.* (2013) 7:1609–19. doi: 10.1038/ismej.2013.34
12. Dudley N, Alexander S. Agriculture and biodiversity: a review. *Biodiversity.* (2017) 18:45–9. doi: 10.1080/14888386.2017.1351892
13. Xun W, Liu Y, Li W, Ren Y, Xiong W, Xu Z, et al. Specialized metabolic functions of keystone taxa sustain soil microbiome stability. *Microbiome.* (2021) 9:35. doi: 10.1186/s40168-020-00985-9
14. Berg G, Rybakova D, Fischer D, Cernava T, Vergès MC, Charles T, et al. Microbiome definition re-visited: old concepts and new challenges. *Microbiome.* (2020) 8:103. doi: 10.1186/s40168-020-00875-0
15. Ding JL, Jiang X, Ma MC, Zhou BK, Guan DW, Zhao BS, et al. Effect of 35 years inorganic fertilizer and manure amendment on structure of bacterial

and archaeal communities in black soil of northeast China. *Appl Soil Ecol.* (2016) 105:187–195. doi: 10.1016/j.apsoil.2016.04.010
16. Gentry LF, Ruffo ML, Below FE. Identifying factors controlling the continuous corn yield penalty. *Agron J.* (2013) 105:295–303. doi: 10.2134/agronj2012.0246
17. Daigh ALM, Dick WA, Helmers MJ, Lal R, Lauer JG, Nafziger E, et al. Yields and yield stability of no-till and chisel-plow fields in the Midwestern US Corn Belt. *Field Crops Res.* (2018) 218:243–53. doi: 10.1016/j.fcr.2017.04.002
18. Kladivko EJ. Residue effects on soil physical properties. In: Unger PW, editor. *Managing Agricultural Residues*. Boca Raton, FL: Lewis Publishers (1994). p. 123–41.
19. Zuber SM, Behnke GD, Nafziger ED, Villamil MB. Crop rotation and tillage effects on soil physical and chemical properties in Illinois. *Agron J.* (2015) 107:971–8. doi: 10.2134/agronj14.0465
20. Baggs EM, Stevenson M, Pihlatie M, Regar A, Cook H, Cadisch G. Nitrous oxide emissions following application of residues and fertiliser under zero and conventional tillage. *Plant Soil.* (2003) 254:361–70. doi: 10.1023/A:1025593121839
21. Martens DA. Management and crop residue influence soil aggregate stability. *J Environ Qual.* (2000) 29:723–7. doi: 10.2134/jeq2000.00472425002900030006x
22. Zuber SM, Behnke GD, Nafziger ED, Villamil MB. Multivariate assessment of soil quality indicators for crop rotation and tillage in Illinois. *Soil Tillage Res.* (2017) 174:147–55. doi: 10.1016/j.still.2017.07.007
23. Salvagiotti F, Specht JE, Cassman KG, Walters DT, Weiss A, Dobermann A. Growth and nitrogen fixation in high-yielding soybean: impact of nitrogen fertilization. *Agron J.* (2009) 101:958–70. doi: 10.2134/agronj2008.0173x
24. McDaniel MD, Grandy AS, Tiemann LK, Weintraub MN. Crop rotation complexity regulates the decomposition of high and low quality residues. *Soil Biol Biochem.* (2014) 78:243–54. doi: 10.1016/j.soilbio.2014.07.027
25. Ouyang Y, Evans SE, Friesen ML, Tiemann LK. Effect of nitrogen fertilization on the abundance of nitrogen cycling genes in agricultural soils: a meta-analysis of field studies. *Soil Biol Biochem.* (2018) 127:71–8. doi: 10.1016/j.soilbio.2018.08.024
26. Behnke GD, Kim N, Zabaloy MC, Riggins CW, Rodriguez-Zas S, Villamil MB. Soil microbial indicators within rotations and tillage systems. *Microorganisms*. (2021) 9:1244. doi: 10.3390/microorganisms9061244
27. Carey CJ, Dove NC, Beman JM, Hart SC, Aronson EL. Meta-analysis reveals ammonia-oxidizing bacteria respond more strongly to nitrogen addition than ammonia-oxidizing archaea. *Soil Biology & Biochemistry*. (2016) 99:158–66. doi: 10.1016/j.soilbio.2016.05.014
28. Petersen B, Snapp S. What is sustainable intensification? Views from experts. *Land Use Policy*. (2015) 46:1–10. doi: 10.1016/j.landusepol.2015.02.002
29. USDA, NASS. *Quick Stats, Illinois County Data, Crops*. (2017). Available online at: http://quickstats.nass.usda.gov/ (accessed July 27, 2021).
30. Soil Survey Staff. *Official Soil Series Descriptions.* (2019). Available online at: https://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/home/?cid=nrcs142p2_053587 (accessed September 18, 2019).
31. Illinois Climate Network, Survey ISW, Editor. *Illinois State Water Survey*. Champaign, IL. (2019). Available online at: http://www.sws.uiuc.edu/warm/ (accessed August 1, 2019).
32. Fernandez FG, Hoeft RG. Managing soil pH and crop nutrients. In: Nafziger E, editor. *Illinois Agronomy Handbook, Crop Science Extension and Outreach*. Urbana, IL: University of Illinois (2009) 91–112.
33. Colman DR, Thomas R, Maas KR, Takacs-Vesbach CD. Detection and analysis of elusive members of a novel and diverse archaeal community within a thermal spring streamer consortium. *Extremophiles.* (2015) 19:307–13. doi: 10.1007/s00792-014-0715-0
34. Fierer N, Jackson JA, Vilgalys R, Jackson RB. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl Environ Microb.* (2005) 71:4117–20. doi: 10.1128/AEM.71.7.4117-4120.2005
35. Crawford JW, Deacon L, Grinev D, Harris JA, Ritz K, Singh BK, et al. Microbial diversity affects self-organization of the soil-microbe system with consequences for function. *J R Soc Interface.* (2012) 9:1302–10. doi: 10.1098/rsif.2011.0679
36. Hall M, Beiko RG. 16S rRNA Gene Analysis with QIIME2. *Methods Mol Biol.* (2018) 1849:113–29. doi: 10.1007/978-1-4939-8728-3_8
37. Bolyen E, Rideout J, Dillon M, Bokulich N, Abnet C, Al-Ghalith G, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol.* (2019) 37:852–7. doi: 10.1038/s41587-019-0209-9
38. Li X, Nair A, Wang S, Wang L. Quality control of RNA-seq experiments. In: Picardi E, editor. *RNA Bioinformatics*. New York, NY: Springer (2015). p. 137–46. doi: 10.1007/978-1-4939-2291-8_8
39. Callahan BJ, Mcmurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High resolution sample inference from amplicon data. *bioRxiv*. (2015) 13:024034. doi: 10.1101/024034
40. Rozewicki J, Li S, Amada KM, Standley DM, Katoh K. MAFFT-DASH: integrated protein sequence and structural alignment. *Nucleic Acids Res.* (2019) 47:W5–10. doi: 10.1093/nar/gkz342
41. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol.* (2007) 73:5261–7. doi: 10.1128/AEM.00062-07
42. Cole JR, Wang Q, Fish JA, Chai B, Mcgarrell DM, Sun Y, et al. Ribosomal database project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* (2014) 42:D633–42. doi: 10.1093/nar/gkt1244
43. Gloor GB, Reid G. Compositional analysis: a valid approach to analyze microbiome high-throughput sequencing data. *Can J Microbiol.* (2016) 62:692–703. doi: 10.1139/cjm-2015-0821
44. Wickham H, Francois R, Henry L, Muller K. *dplyr: A Grammar of Data Manipulation*. R package version 1.0.5 (2021).
45. R Core Team. *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing (2021). Available online at: https://www.R-project.org/
46. Sas Institute Inc. *JMP 14 Predictive and Specialized Modeling*. Cary, NC: SAS Institute (2018).
47. Palarea-Albaladejo J, Martín-Fernández JA. zCompositions — R package for multivariate imputation of left-censored data under a compositional approach. *Chemometr Intell Lab.* (2015) 143:85–96. doi: 10.1016/j.chemolab.2015.02.019
48. Gloor GB, Macklaim JM, Pawlowsky-Glahn V, Egozcue JJ. Microbiome datasets are compositional: and this is not optional. *Front Microbiol*. (2017) 8:2224. doi: 10.3389/fmicb.2017.02224
49. Parte AC. LPSN – list of prokaryotic names with standing in nomenclature (bacterio.net), 20 years on. *Int J Syst Evol Micr*. (2018) 68:1825–9. doi: 10.1099/ijsem.0.002786
50. Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecology*. (2001) 26:32–46. doi: 10.1111/j.1442-9993.2001.01070.pp.x
51. Storey JD. The positive false discovery rate: a Bayesian interpretation and the q -value. *Ann. Statist.* (2003) 31:2013–35. doi: 10.1214/aos/1074290335
52. Wickham H. *Ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag (2016). doi: 10.1007/978-3-319-24277-4
53. Chamberlain LA, Bolton ML, Cox MS, Suen G, Conley SP, An,é, et al.-M. Crop rotation, but not cover crops, influenced soil bacterial community composition in a corn-soybean system in southern Wisconsin. *Appl Soil Ecol.* (2020) 154:103603. doi: 10.1016/j.apsoil.2020.103603
54. Ashworth AJ, Debruyn JM, Allen FL, Radosevich M, Owens PR. Microbial community structure is affected by cropping sequences and poultry litter under long-term no-tillage. *Soil Biol Biochem.* (2017) 114:210–9. doi: 10.1016/j.soilbio.2017.07.019
55. Hoss M, Behnke GD, Davis AS, Nafziger ED, Villamil MB. Short corn rotations do not improve soil quality, compared with corn monocultures. *Agron J.* (2018) 110:1274–88. doi: 10.2134/agronj2017.11.0633
56. Behnke GD, Pittelkow CM, Nafziger ED, Villamil MB. Exploring the relationships between greenhouse gas emissions, yields, and soil properties in cropping systems. *Agric Basel.* (2018) 8:62. doi: 10.3390/agriculture8050062
57. Yang K, Zhu JJ, Gu JC, Yu LZ, Wang ZQ. Changes in soil phosphorus fractions after 9 years of continuous nitrogen addition in a larix gmelinii plantation. *Ann For Sci.* (2015) 72:435–42. doi: 10.1007/s13595-014-0444-7
58. Kerou M, Schleper C. *Nitrososphaera*. Hoboken, NJ: John Wiley & Sons, Inc (2016). doi: 10.1002/9781118960608.gbm01294

59. Zhalnina K, De Quadros P, Gano K, Davis-Richardson A, Fagen J, Brown C, et al. Ca nitrososphaera and bradyrhizobium are inversely correlated and related to agricultural practices in long-term field experiments. *Front Microbiol.* (2013) 4:104. doi: 10.3389/fmicb.2013.00104
60. Ai C, Liang G, Sun J, Wang X, He P, Zhou W. Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil. *Soil Biol Biochem.* (2013) 57:30–42. doi: 10.1016/j.soilbio.2012.08.003
61. Wessén E, Hallin S, Philippot L. Differential responses of bacterial and archaeal groups at high taxonomical ranks to soil management. *Soil Biol Biochem.* (2010) 42:1759–65. doi: 10.1016/j.soilbio.2010.06.013
62. Castelle CJ, Wrighton KC, Thomas BC, Hug LA, Brown CT, Wilkins MJ, et al. Genomic expansion of domain archaea highlights roles for organisms from new phyla in anaerobic carbon cycling. *Curr Biol.* (2015) 25:690–701. doi: 10.1016/j.cub.2015.01.014
63. Liu X, Li M, Castelle CJ, Probst AJ, Zhou Z, Pan J, et al. Insights into the ecology, evolution, and metabolism of the widespread woesearchaeotal lineages. *Microbiome.* (2018) 6:102. doi: 10.1186/s40168-018-0488-2
64. Liu X, Wang Y, Gu JD. Ecological distribution and potential roles of Woesearchaeota in anaerobic biogeochemical cycling unveiled by genomic analysis. *Comput Struct Biotec.* (2021) 19:794–800. doi: 10.1016/j.csbj.2021.01.013
65. Kersters K, De Vos P, Gillis M, Swings J. Proteobacteria. In: *Encyclopedia of Life Sciences (eLS)*. John Wiley & Sons, Ltd. (2006). doi: 10.1038/npg.els.0004312
66. Navarrete AA, Venturini AM, Meyer KM, Klein AM, Tiedje JM, Bohannan BJM, et al. Differential response of acidobacteria subgroups to forest-to-pasture conversion and their biogeographic patterns in the western brazilian amazon. *Front Microbiol.* (2015) 6:1443. doi: 10.3389/fmicb.2015.01443
67. Kielak AM, Barreto CC, Kowalchuk GA, Van Veen JA, Kuramae EE. The ecology of acidobacteria: moving beyond genes and genomes. *Front Microbiol.* (2016) 7:744. doi: 10.3389/fmicb.2016.00744
68. Zhang Q, Gong M, Yuan J, Hou Y, Zhang H, Wang Y, et al. Dark septate endophyte improves drought tolerance in sorghum. *Int J Agric Biol.* (2017) 19:53–60. doi: 10.17957/IJAB/15.0241
69. Singh R, Dubey AK. Diversity and applications of endophytic actinobacteria of plants in special and other ecological niches. *Front Microbiol.* (2018) 9:1767. doi: 10.3389/fmicb.2018.01767
70. Ivanova AA, Zhelezova AD, Chernov TI, Dedysh SN. Linking ecology and systematics of acidobacteria: distinct habitat preferences of the acidobacteriia and blastocatellia in tundra soils. *PLoS ONE.* (2020) 15:e0230157. doi: 10.1371/journal.pone.0230157
71. Kalam S, Basu A, Ahmad I, Sayyed RZ, El-Enshasy HA, Dailin DJ, et al. Recent understanding of soil acidobacteria and their ecological significance: a critical review. *Front Microbiol.* (2020) 11:580024. doi: 10.3389/fmicb.2020.580024
72. Banerjee S, Schlaeppi K, Van Der Heijden MGA. Keystone taxa as drivers of microbiome structure and functioning. *Nat Rev Microbiol.* (2018) 16:567–76. doi: 10.1038/s41579-018-0024-1
73. Huang MJ, Rao MPN, Salam N, Xiao M, Huang HQ, Li WJ. *Allostreptomyces psammosilenae* gen. nov., sp. nov., an endophytic actinobacterium isolated from the roots of *Psammosilene tunicoides* and emended description of the family *Streptomycetaceae* [Waksman and Henrici (1943)AL] emend. Rainey et al. (2017) 1997: emend. Kim et al. 2003, emend. Zhi et al. 2009. *Int J Syst Evol Microbiol.* (2017) 67:288–93. doi: 10.1099/ijsem.0.001617
74. Foesel BU, Geppert A, Rohde M, Overmann J. Parviterribacter kavangonensis gen. nov., sp. nov. and *Parviterribacter multiflagellatus* sp. nov., novel members of *Parviterribacteraceae* fam. nov. within the order *Solirubrobacterales*, and emended descriptions of the classes *Thermoleophilia* and *Rubrobacteria* and their orders and families. *Int J Syst Evol Micr.* (2016) 66:652–65. doi: 10.1099/ijsem.0.000770
75. Podosokorskaya OA, Bonch-Osmolovskaya EA, Novikov AA, Kolganova TV, Kublanov IV. Ornatilinea apprima gen. nov., sp. nov., a cellulolytic representative of the class Anaerolineae. *Int J Syst Evol Micr.* (2013) 63:86–92. doi: 10.1099/ijs.0.041012-0
76. Slobodkina GB, Baslerov RV, Novikov AA, Bonch-Osmolovskaya EA, Slobodkin AI. Thermodesulfitimonas autotrophica gen. nov., sp. nov., a thermophilic, obligate sulfite-reducing bacterium isolated from a terrestrial hot spring. *Int J Syst Evol Micr.* (2017) 67:301–5. doi: 10.1099/ijsem.0.001619
77. Yoon J, Jang JH, Kasai H. Algisphaera agarilytica gen. nov., sp. nov., a novel representative of the class Phycisphaerae within the phylum Planctomycetes isolated from a marine alga. *Antonie van Leeuwenhoek.* (2014) 105:317–24. doi: 10.1007/s10482-013-0076-1
78. Proença DN, Whitman WB, Varghese N, Shapiro N, Woyke T, Kyrpides NC, et al. Arboriscoccus pini gen. nov., sp. nov., an endophyte from a pine tree of the class Alphaproteobacteria, emended description of Geminicoccus roseus, and proposal of Geminicoccaceae fam. nov. *Syst Appl Microbiol.* (2018) 41:94–100. doi: 10.1016/j.syapm.2017.11.006
79. Xue C, Zhang X, Zhu C, Zhao J, Zhu P, Peng C, et al. Quantitative and compositional responses of ammonia-oxidizing archaea and bacteria to long-term field fertilization. *Sci Rep.* (2016) 6:28981. doi: 10.1038/srep28981
80. Pereira E Silva MC, Poly F, Guillaumaud N, Van Elsas JD, Falcão Salles J. Fluctuations in ammonia oxidizing communities across agricultural soils are driven by soil structure and pH. *Front Microbiol.* (2012) 3:77. doi: 10.3389/fmicb.2012.00077
81. DeBruyn JM, Nixon LT, Fawaz MN, Johnson AM, Radosevich M. Global biogeography and quantitative seasonal dynamics of gemmatimonadetes in soil. *Appl Environ Microbiol.* (2011) 77:6295–300. doi: 10.1128/AEM.05005-11
82. Pascual J, Foesel BU, Geppert A, Huber KJ, Boedeker C, Luckner M, et al. Roseisolibacter agri gen. nov., sp. nov., a novel slow-growing member of the under-represented phylum Gemmatimonadetes. *Int J Syst Evol Micr.* (2018) 68:1028–36. doi: 10.1099/ijsem.0.002619
83. Bräuer S, Harbison A, Ueki A. Micropepsales. In: Trujillo ME, Dedysh S, Devos P, Hedlund B, Kämpfer P, Rainey FA, Whitman WB, editors. *Bergey's Manual of Systematics of Archaea and Bacteria*. John Wiley & Sons, Inc., in association with Bergey's Manual Trust (2018). doi: 10.1002/9781118960608.obm00146
84. Kämpfer P, Young C-C, Arun AB, Shen F-T, Jäckel U, Rosselló-Mora R, et al. Pseudolabrys taiwanensis gen. nov., sp. nov., an alphaproteobacterium isolated from soil. *Int J Syst Evol Micr.* (2006) 56:2469–2472. doi: 10.1099/ijs.0.64124-0
85. Imhoff JF, Hiraishi A. Aerobic bacteria containing bacteriochlorophyll and belonging to the alphaproteobacteria. In: Trujillo ME, Dedysh S, Devos P, Hedlund B, Kämpfer P, Rainey FA, Whitman WB, editors. *Bergey's Manual of Systematics of Archaea and Bacteria* John Wiley & Sons, Inc., in association with Bergey's Manual Trust (2015) 1–7. doi: 10.1002/9781118960608.bm00001
86. Hördt A, López MG, Meier-Kolthoff JP, Schleuning M, Weinhold LM, Tindall BJ, et al. Analysis of 1,000+ type-strain genomes substantially improves taxonomic classification of alphaproteobacteria. *Front Microbiol.* (2020) 11:468. doi: 10.3389/fmicb.2020.00468
87. Fahrbach M, Kuever J, Meinke R, Kämpfer P, Hollender J. Denitratisoma oestradiolicum gen. nov., sp. nov., a 17beta-oestradiol-degrading, denitrifying betaproteobacterium. *Int J Syst Evol Microbiol.* (2006) 56:1547–52. doi: 10.1099/ijs.0.63672-0
88. Yoo SH, Weon HY, Anandham R, Kim BY, Hong SB, Jeon YA, et al. Dokdonella soli sp. nov., a gammaproteobacterium isolated from soil. *Int J Syst Evol Micr.* (2009) 59:1965–8. doi: 10.1099/ijs.0.005348-0
89. Wolińska A, Kuzniar A, Zielenkiewicz U, Banach A, Błaszczyk M. Indicators of arable soils fatigue – bacterial families and genera: a metagenomic approach. *Ecol Indicat.* (2018) 93:490–500. doi: 10.1016/j.ecolind.2018.05.033
90. Wang L, Zheng S, Wang D, Wang L, Wang G. Thermomonas carbonis sp. nov., isolated from the soil of a coal mine. *Int J Syst Evol Micr.* (2014) 64:3631–5. doi: 10.1099/ijs.0.063800-0
91. Mergaert J, Cnockaert MC, Swings J. Thermomonas fusca sp. nov. and *Thermomonas brevis* sp. nov., two mesophilic species isolated from a denitrification reactor with poly(ε-caprolactone) plastic granules as fixed bed, and emended description of the genus Thermomonas. *Int J Syst Evol Micr.* (2003) 53:1961–6. doi: 10.1099/ijs.0.02684-0
92. Im WT, Liu QM, Lee KJ, Kim SY, Lee ST, Yi TH. Variovorax ginsengisoli sp. nov., a denitrifying bacterium isolated from soil of a ginseng field. *Int J Syst Evol Micro.* (2010) 60:1565–9. doi: 10.1099/ijs.0.014514-0

93. Fahrbach M, Kuever J, Remesch M, Huber BE, Kämpfer P, Dott W, et al. Steroidobacter denitrificans gen. nov., sp. nov., a steroidal hormone-degrading gammaproteobacterium. *Int J Syst Evol Micro.* (2008) 58:2215–23. doi: 10.1099/ijs.0.65342-0
94. Galazka A, Grzadziel J. Fungal genetics and functional diversity of microbial communities in the soil under long-term monoculture of maize using different cultivation techniques. *Front Microbiol.* (2018) 9:76. doi: 10.3389/fmicb.2018.00076
95. Wang QF, Ma MC, Jiang X, Guan DW, Wei D, Zhao BS, et al. Impact of 36 years of nitrogen fertilization on microbial community composition and soil carbon cycling-related enzyme activities in rhizospheres and bulk soils in northeast China. *Appl Soil Ecol.* (2019) 136:148–57. doi: 10.1016/j.apsoil.2018.12.019
96. Wang Q, Ma M, Jiang X, Zhou B, Guan D, Cao F, et al. Long-term N fertilization altered 13C-labeled fungal community composition but not diversity in wheat rhizosphere of Chinese black soil. *Soil Biol Biochem.* (2019) 135:117–26. doi: 10.1016/j.soilbio.2019.04.009
97. Rice AV, Currah RS. Two new species of pseudogymnoascus with geomyces anamorphs and their phylogenetic relationship with gymnostellatospora. *Mycologia.* (2006) 98:307–18. doi: 10.1080/15572536.2006.11832703
98. Marmeisse R, Girlanda M. 10 mycorrhizal fungi and the soil carbon and nutrient cycling. In: Druzhinina IS, Kubicek CP, editors. *Environmental and Microbial Relationships.* Cham: Springer International Publishing (2016). p. 189–203. doi: 10.1007/978-3-319-29532-9_10
99. Benjamin RK, Blackwell M, Chapela IH, Humber RA, Jones KG, Klepzig KD, et al. 18 - insect- and other arthropod-associated fungi. In: Mueller Gm, Bills GF, Foster MS, editors. *Biodiversity of Fungi.* Burlington: Academic Press (2004). p. 395–433. doi: 10.1016/B978-012509551-8/50021-0
100. Samerpitak K, Van Der Linde E, Choi HJ, Gerrits Van Den Ende AHG, Machouart M, Gueidan C, et al. Taxonomy of ochroconis, genus including opportunistic pathogens on humans and animals. *Fungal Divers.* (2014) 65:89–126. doi: 10.1007/s13225-013-0253-6
101. Huhndorf SM, Miller AN, Fernández FA. Molecular systematics of the sordariales: the order and the family lasiosphaeriaceae redefined. *Mycologia.* (2004) 96:368–87. doi: 10.2307/3762068
102. Teasdale SE, Beulke AK, Guy PL, Orlovich DA. Environmental barcoding of the ectomycorrhizal fungal genus cortinarius. *Fungal Divers.* (2013) 58:299–310. doi: 10.1007/s13225-012-0218-1
103. Li T, Liu MJ, Zhang XT, Zhang HB, Sha T, Zhao ZW. Improved tolerance of maize (Zea mays L.) to heavy metals by colonization of a dark septate endophyte (DSE) *Exophiala pisciphila*. *Sci Total Environ.* (2011) 409:1069–74. doi: 10.1016/j.scitotenv.2010.12.012
104. Carris LM, Glawe DA, Smyth CA, Edwards DI. Fungi associated with populations of heterodera glycines in two illinois soybean fields. *Mycologia.* (1989) 81:66–75. doi: 10.2307/3759452
105. Hu W, Strom N, Haarith D, Chen S, Bushley KE. Mycobiome of cysts of the soybean cyst nematode under long term crop rotation. *Front Microbiol.* (2018) 29:386. doi: 10.3389/fmicb.2018.00386
106. Kredics L, Hatvani L, Naeimi S, Körmöczi P, Manczinger L, Vágvölgyi C, et al. Chapter 1 - biodiversity of the genus hypocrea/trichoderma in different habitats. In: Gupta VK, Schmoll M, Herrera-Estrella A, Upadhyay RS, Druzhinina I, Tuohy MG, editors. *Biotechnology and Biology of Trichoderma.* Amsterdam: Elsevier (2014). p. 3–24. doi: 10.1016/B978-0-444-59576-8.00001-1
107. Chen H, Yu F, Shi W. Detection of N2O-producing fungi in environment using nitrite reductase gene (nirK)-targeting primers. *Fungal Biol.* (2016) 120:1479–92. doi: 10.1016/j.funbio.2016.07.012
108. Ibrahim SRM, Altyar AE, Mohamed SGA, Mohamed GA. Genus thielavia: phytochemicals, industrial importance and biological relevance. *Nat Product Res.* (2021) 1–16. doi: 10.1080/14786419.2021.1919105
109. Ibrahim SRM, Mohamed SGA, Altyar AE, Mohamed GA. Natural products of the fungal genus humicola: diversity, biological activity, industrial importance. *Curr Microbiol.* (2021) 78:2488–509. doi: 10.1007/s00284-021-02533-6
110. Letcher PM, Powell MJ, Chambers JG, Holznagel WE. Phylogenetic relationships among rhizophydium isolates from North America and Australia. *Mycologia.* (2004) 96:1339–51. doi: 10.2307/3762150
111. Farr DF, Rossman AY. *Fungal Databases, U.S. National Fungus Collections, ARS.* USDA (2021). Available online at: https://nt.ars-grin.gov/fungaldatabases/ (accessed July 23, 2021).
112. Bahram M, Hildebrand F, Forslund SK, Anderson JL, Soudzilovskaia NA, Bodegom PM, et al. Structure and function of the global topsoil microbiome. *Nature.* (2018) 560:233–7. doi: 10.1038/s41586-018-0386-6
113. Mothapo N, Chen H, Cubeta MA, Grossman JM, Fuller F, Shi W. Phylogenetic, taxonomic and functional diversity of fungal denitrifiers and associated N2O production efficacy. *Soil Biol Biochem.* (2015) 83:160–75. doi: 10.1016/j.soilbio.2015.02.001
114. Cabello P, Roldán MD, Moreno-Vivián C. Nitrate reduction and the nitrogen cycle in archaea. *Microbiology.* (2004) 150:3527–46. doi: 10.1099/mic.0.27303-0

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Soil Science

ORIGINAL RESEARCH
published: 10 February 2022
doi: 10.3389/fsoil.2022.824087

Post-termination Effects of Cover Crop Monocultures and Mixtures on Soil Inorganic Nitrogen and Microbial Communities on Two Organic Farms in Illinois

Eleanor E. Lucadamo[1], *Ashley A. Holmes*[2], *Sam E. Wortman*[2,3] *and Anthony C. Yannarell*[1]*

[1] *Department of Natural Resources and Environmental Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, United States,* [2] *Department of Crop Science, University of Illinois at Urbana-Champaign, Urbana, IL, United States,* [3] *Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, NE, United States*

Cover crops can continue to affect agricultural systems even after they have been terminated by influencing nitrogen dynamics and by altering soil microbial communities. These post-termination effects can influence soil fertility, weed pressure, and the dynamics of potential plant pathogens in the narrow window of time between cover crop termination and cash crop emergence. We evaluated the post-termination effects of 12 different spring-sown cover crop mixtures and monocultures on soil nitrogen and microbial communities on two different organic farms in Central Illinois (on Lawson silt loam soil) and Northern Illinois (on Virgil silt loam soil). In comparison to control plots with no cover crops, all cover crop treatments significantly reduced soil nitrate levels but increased the potentially mineralizable nitrogen pool following termination. Nitrate levels of cover crop plots approached those of controls after 2 and 4 weeks, respectively, but potentially mineralizable nitrogen levels in cover plots remained elevated for at least 4 weeks following termination. Monocultures of Brassica cover crops showed the greatest decrease in soil nitrate, while Brassicas and unplanted control plots containing high biomass of weeds showed the greatest increase in potentially mineralizable nitrogen in comparison to plant-free control plots. In contrast to their effect on soil nitrogen, cover crops had very limited impact on the composition of soil microbial communities. Overall microbial community composition varied across sites and years, and only soil fungi significantly responded to cover cropping treatments. Nevertheless, we found that some highly correlated groups of soil microbes showed significant responses to soil nitrate and to high plant biomass. Key members of these correlated groups included ammonia-oxidizing organisms and saprotrophic fungi. Our results suggest that cover crops may reduce the potential for springtime nitrogen leaching losses by retaining nitrogen in the soil organic pool, and they may also have impacts on the soil microbial community that are particularly relevant for nitrogen cycling and decomposition of plant residues.

Keywords: cover crops, nitrogen, microbial community, organic agriculture, early season pulse, nitrate, organic N, agricultural management

OPEN ACCESS

Edited by:
Nigel Victor Gale,
University of Toronto, Canada

Reviewed by:
Mahaveer P. Sharma,
ICAR Indian Institute of Soybean Research, India
Jun Shan,
Institute of Soil Science (CAS), China

***Correspondence:**
Anthony C. Yannarell
acyann@illinois.edu

Specialty section:
This article was submitted to Soil Biology, Ecosystems and Biodiversity, a section of the journal Frontiers in Soil Science

Received: *28 November 2021*
Accepted: *18 January 2022*
Published: *10 February 2022*

Citation:
Lucadamo EE, Holmes AA, Wortman SE and Yannarell AC (2022) Post-termination Effects of Cover Crop Monocultures and Mixtures on Soil Inorganic Nitrogen and Microbial Communities on Two Organic Farms in Illinois. Front. Soil Sci. 2:824087. doi: 10.3389/fsoil.2022.824087

INTRODUCTION

Cover crops are important tools employed in organic agriculture to improve soil quality and fertility (1–4). Living cover crops can prevent soil erosion during fallow periods and compete directly with weeds for sunlight and nutrients (5, 6). However, cover crops can continue to effect the soil system after they have been terminated, and these impacts can persist for hours, days, or weeks (7). For example, red clover cover crop residues suppressed weed seed germination for 30 days after termination through a combination of allelochemical release and stimulation of weed-suppressive microbial activity (8). These post-termination effects of cover crops can influence the soil microbial environment by altering nutrient pools, rates of residue decomposition, and relative abundance of plant mutualists and pathogens (9–18). Even if post-termination cover crop effects are short-lived, they can have important impacts on agriculture because they occur in a critical window of time when agroecosystems are subject to pressure from early season weeds and increased risk of soil nitrogen leaching losses (19, 20).

Living cover crops, their decaying "green manure" residues, and the soil microbial community can all influence the amount, timing, and the form of plant-available nitrogen (1, 21). These effects can vary widely across different combinations of plant and microbial species. Winter and spring cover crops can be used to take up excess or residual nitrates in the soil over the fallow season (22), reducing nitrate leaching and deprive early season weeds of nitrogen (5, 6). Grasses like oat (*Avena sativa*) and spring wheat (*Triticum aestivum*) are particularly good at this (2, 22–25). Legume species such as field pea (*Pisum sativum*) and fava bean (*Vicia faba*) can provide significant nitrogen contributions to agricultural systems via nitrogen fixation (26, 27). Plants of the family *Brassicaceae* can rapidly accumulate biomass to choke out weeds and sequester nitrogen (28), but they also produce allelopathic chemicals that can reduce plant growth and microbial activity (29, 30).

Upon termination, cover crop residues release nitrogen back to the soil, where the processes of decomposition, nitrogen mineralization, and nitrogen immobilization are governed by soil microorganisms interacting with plant tissues of varying qualities and composition. Soil microorganisms have a threshold carbon to nitrogen ratio (C:N) of 26:1. Plants with lower C:N ratios result in net nitrogen mineralization, while residues with higher C:N result in net nitrogen immobilization (31, 32). Legumes, which have low C:N ratios of 10–15 (33, 34), decompose rapidly as organic nitrogen is mineralized into plant-available forms (nitrate and ammonium) once microbial nitrogen demand is satisfied (35, 36). Grasses have high C:N ratios, ranging from 33 to 94 for oat and wheat, respectively (13, 31), and this can result in slower residue decomposition and net immobilization of nitrogen (37). The combination of high grass biomass and high C:N ratio results in less inorganic nitrogen made available for the crops that follow. Though there is variation among Brassicas, they vary between 10 and 31 C:N ratios of their plant tissues (38–40), so they are generally below the 26:1 microbial threshold. However, Brassica allelopathic secondary metabolites can suppress microbial decomposition and nitrogen mineralization (6, 21, 41, 42), which can result in slower overall conversion of organic nitrogen to plant-available forms (28).

In addition to their effect on soil nitrogen pools, cover crops can also affect soil-borne pathogen prevalence and promote plant-beneficial microbes. Cover cropping with canola (*Brassica napus* L.) was shown to reduce the incidence of disease caused by *Rhizoctonia solani* in potato (10) and apple (43). Many beneficial, pathogen-antagonistic, soil bacteria and fungi have also been identified to respond positively cover cropping (10, 15, 16). Wheat has also been found to enrich fungal diversity and reduce pathogen populations compared to oat (44). In general, more diverse microbial communities have been shown to experience a greater degree of resilience and are better equipped to suppress potentially pathogenic taxa (16, 45–47). However, fungi tend to cause more damage to agricultural crops than bacteria (46), so is not always the case that increased fungal diversity is a net benefit for crops. In order to fully understand how cover crops influence the soil in ways that can promote or hinder future crop growth, we need to identify specific microbial taxa that respond to cover cropping with different plant species.

Legumes, grasses, and simple mixtures of the two are most commonly used cover crops (5, 6, 48). Mustards and other plants from the *Brassicaceae* family can be used as short-season cover crops in the cooler climates of the upper Midwest (49), and they are sometimes included with other cover crop species as part of diverse mixtures (50). While there is considerable research showing that cover crops shape the soil microbial community (5, 6, 10, 15, 16), it is not well-understood if more diverse mixtures of grass, legume, and Brassica species yield increased benefits to soil quality and microbiology that may, in turn, improve subsequent crop growth. Planting diverse cover crop mixtures may allow us to take advantage of the myriad effects of different plant types on soil fertility and microbiology, and there is a growing popularity in the use of diverse cover crop mixtures, or "cocktails," in the organic farming community (3, 5, 24, 51–54). In this study, we aimed to investigate whether different multi-species cover crop mixtures had differential effects on soil nitrogen pools and soil microbial composition in the weeks following cover crop termination. By looking at six species of cover crops grown in monocultures and diverse, five-species mixtures, we sought to answer the following questions: (1) do the dynamics of soil nitrogen pools (nitrate, ammonium, and potentially mineralizable nitrogen) vary among different cover crop combinations; (2) how do soil microbial communities change as a result of cover cropping with different plant types (grasses, weeds, Brassicas, legumes, or mixtures); and (3) what are the most important drivers in determining the dynamics of microbial communities following cover crop termination?

MATERIALS AND METHODS

Field Study Design and Sample Collection

Our research took place as part of the same field experiment that has been previously described by Holmes and colleagues (55). Two organic vegetable farms participated in the experiment in 2015 and 2016: PrairiErth Farm in Atlanta, IL (40°13'N 89°13'W) and Kinnikinnick Farm in Caledonia, IL (42°27'N 88°52'W).

The soil type at PrairiErth farm was Lawson silt loam (fine-silty, mixed, superactive, mesic Aquic Cumulic Hapludoll), and the dominant soil type at Kinnikinnick farm was Virgil silt loam (fine-silty, mixed, superactive, mesic Udollic Endoaqualf). Cropping history at both sites was highly varied, including both vegetable and grain crops. Both farms were certified organic under the United States Department of Agriculture National Organic Program guidelines; Kinnikinnick Farm since 1994 and PrariErth Farm since 2004.

Spring-sown cover crops were planted in a randomized complete block design with four replicates of 12 treatments and two controls. Blocks were 4 m by 56 m in size, with each block accommodating fourteen 4 m by 4 m plots for the treatments (twelve cover crop plantings and two controls). Six cover crops were included in the study: two grasses (oat, *Avena sativa*, and spring wheat, *Triticum aestivum*), two legumes (field pea, *Pisum sativum*, and fava bean, *Vicia faba*) and two Brassicas (Yellow mustard, *Sinapis alba*, and purple top turnip, *Brassica campestris*). A "weedy" control treatment was included that received no cover crop seed but allowed volunteer weed growth, and the experiment also included a plant-free control maintained by hand-pulling. Cover crops were planted in monocultures and all possible five-species mixtures for a total of six monocultures and six mixture treatments with two controls. For subsequent analyses, the 14 cover crops will be referred to as "cover crop treatments." Cover crop diversity refers to whether the treatment was a mixture, monoculture, or control. Seeding application rates were as described by Holmes et al. (55). Cover crops were planted in early (PrariErth) or late (Kinnikinnick) April by hand-broadcasting, and seeds were lightly incorporated using gravel rakes and drag harrows. Cover crops grew for ∼2 months before termination by mowing and rotavation to a depth of 15 cm.

Aboveground cover crop biomass was measured from two randomly-tossed quadrats (45.7 cm by 61 cm) immediately before termination, as previously described (55). Weeds, which were treated as a single "species," were separated from cover crops and weighed separately. Dry weights were calculated for each cover crop species and used for subsequent analyses.

We sought to investigate the short-term impacts of cover crops in the period between termination and when typical cash crops would emerge. We collected soil samples from plots for three time points after cover crop termination: within 1 week (immediate effects), after 1 or 2 weeks (medium-term effects), and after 4 weeks (at typical crop emergence). Precise sampling dates varied for each site-year, depending on weather and soil conditions. In 2015, soils from each plot were collected at 3, 7, and 34 days post-termination at PrariErth and 6, 18, and 32 days post-termination at Kinnikinnick. In 2016, samples were collected 3, 17, and 33 days post-termination at PrairiErth and 5, 14, and 34 days post-termination at Kinnikinnick. From each plot, we collected 16 randomly-spaced soil cores down to depth of 10 cm, and we combined these cores to obtain a single composite sample for each plot. For microbial community composition (see below) a subsample of ∼20 g was collected immediately from each composite sample, and then frozen at −20°C and freeze-dried for DNA extraction. Approximately 50 g of the remaining soil was air-dried for subsequent nitrogen content analysis.

Soil Inorganic Nitrogen Analyses

Soil inorganic nitrogen content was assessed using standard methods for plant-available, exchangeable ammonium and nitrate through KCl-extraction (56) followed by colorimetric quantification of nitrate and ammonium. For each sample, two subsamples of 10 ± 0.05 g were weighed into 50 mL centrifuge tubes. One subsample was incubated anaerobically to quantify potentially mineralizable organic nitrogen (see below), while the other was processed immediately for inorganic nitrogen content.

For inorganic nitrogen extraction, 40 mL 1 M KCl was added and samples were shaken at approximately 240 rotations per minute at room temperature for 50 min. Nitrate and ammonium contents were quantified by colorimetric reactions. Nitrate analysis followed Doane and Horwath (57): a solution of sulfanilamide and N-(1-napththyl)-ethylenedaimine dihydrochloride in saturated vanadium (III) chloride was combined with each KCl extract and incubated in the dark for 4 h. Ammonium analysis followed Weatherburn (58): a solution of sodium salicylate, sodium citrate, sodium tartrate and sodium nitroprusside was combined with each KCl extract and with a 2% bleach:1.5 M sodium hydroxide solution and incubated for 50 min at room temperature. Absorbance values were measured at 540 nm for nitrate and 650 nm for ammonium to colorimetrically quantify nitrogen concentration using Epoch Biotek plate reader spectrophotometer and Gen5 software. Standard curves of known concentrations of KNO_3 and $(NH_4)_2SO_4$ were used to measure nitrate and ammonium concentrations, respectively. For all nitrogen assay results, concentrations were converted to mg/kg soil.

Potentially mineralizable nitrogen (PMN) was measured following protocols adapted from Drinkwater et al. (59) and Moebius-Clune et al. (60). The anaerobic incubation subsamples were combined with 10 mL ddH_2O and the headspace was cleared of O_2 with the addition of He gas to create a waterlogged, anaerobic environment in order to inhibit the oxidation of ammonium. These subsamples were incubate anaerobically at 37°C for 7 days in order to accumulate mineralized ammonium. Total PMN was determined by measuring the ammonium concentration following the protocol described above. PMN was calculated as the difference in ammonium concentration after and before the 7-day incubation.

DNA Extraction, Sequencing, and Analysis

Whole-community microbial DNA was extracted from freeze-dried soil samples using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH) following the manufacturer's protocol. Extracted DNA was purified at 65°C for 15 min with 1% cetyl-trimethylammonium bromide (CTAB) to remove humic acids. Samples were further extracted with 24:1 chloroform: alcohol to remove residual impurities. DNA was precipitated and washed three times with ethanol, then dried in a vacuum concentrator and dissolved in 1 x Tris-EDTA buffer. The purified DNA was adjusted to ∼20 ng/μL and stored at −80°C until further analysis.

To prepare samples for sequencing, 10 μL of each sample was added to a 96-well PCR plate and sequenced on a single flow cell using Illumina MiSeq V3 platform at W. M. Keck Center for Comparative and Functional Genomics at

the University of Illinois at Urbana-Champaign. For bacteria and archaea, the V4-V5 region of 16S rRNA was sequenced using primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACVSGGGTATCTAAT-3') (61). For fungi, the internal transcribed spacer (ITS) region between the 18S and large subunit rRNA genes was sequenced using primers ITS3-F (5'-GCATCGATGAAGAACGCAGC-3') and ITS4-R (5'-TCCTCCGCTTATTGATATGC-3') (62). Samples for 2015 and 2016 were sequenced separately and combined for downstream analyses. A total of 22,722,058 raw reads were obtained from samples in 2015 and 21,685,014 in 2016 from both bacterial and fungal sequences. Library size ranged from 3,979 to 112,830 sequences per sample for the fungal ITS region with a mean of 16,997 sequences per sample, and 3,551–102,839 sequences per sample from the bacterial V4 region with a mean of 12,280 sequences per sample.

Sequence files were obtained as fastq files. Paired-end 16S sequences were merged using Fast Length Adjustment of Short reads (FLASH) software (63). Quality filtering of fastq files was performed using the FASTX-Toolkit software; sequence reads with a quality score of <30 and with fewer than 90% of bases were removed (64). Sequences were binned into discrete operational taxonomic units (OTUs) based on 97% similarity using usearch (65). Quantitative Insight into Microbial Ecology (MacQIIME version 1.9.2) was used for aligning and assigning of sequences (66). Sequences were aligned using the basic local alignment search tool (BLAST), and taxonomy was assigned based on the Greengenes reference database for bacteria and archaea and the UNITE database for fungi (67–69). Sequences identified as plants, protists, chloroplasts, and mitochondria were removed. Read counts were rarefied to 5,100 for bacterial sequences and 2,900 for fungal sequences. After rarefying, there were 527 samples from which 16,069 unique bacterial and 112 unique archaeal OTUs and were detected from the 16S rRNA gene. For the fungal sequences, there were 560 samples from which 4,932 fungal OTUs were identified from the ITS region after rarifying. Sequences have been uploaded to GenBank, BioProject # PRJNA503856.

Data Analysis: Soil Nitrogen

Data sets and R code to perform the following analyses are available online at https://github.com/acyann/post-termination-cover-crops.

We examined whether soil nitrogen levels differed between mixtures, monocultures and controls. We combined cover crop treatments by functional group and/or mixture, and therefore these analyses used the following treatment groups: brassica monocultures (mustard and turnip), grass monocultures (wheat and oat), legume monocultures (pea and bean), mixtures, and controls (plant-free or weedy). We also examined whether these patterns changed over time after termination. For these analyses, we used three complete site-years and one partial site-year due to experimental problems at PrairiErth in 2016 (55). Each complete site-year included 56 plots (12 treatments x 4 blocks), sampled at three time points. For PrairiErth 2016, we only included soil data for brassica monocultures (mustard and turnip) and the two controls (4 plots x 4 blocks), sampled at three time points. All data were analyzed using R software version 4.1.1 (70). Linear mixed effects models were used to determine how cover crop type influenced measures of soil nitrogen and total soil phenolic content using the packaged *nlme* version 3.1-153 (71). We fit separate models for each of the three time points (1, 2, and 4 weeks following termination), and we evaluated each of the three nitrogen species (nitrate, ammonium, and PMN) separately. Cover crop type was treated as the fixed effect and year, site, and replicate as nested random effects. Models were fit using the maximum likelihood approach. To test for mean differences between treatment groups (i.e., cover crop type), Tukey's Honestly Significant Difference (HSD) *post-hoc* tests were run using the package *multcomp* version 1.4-18 (72). Results of the linear mixed models and Tukey HSD tests were considered significant at the level of alpha < 0.05.

Data Analysis: Microbiome

Data sets and R code to perform the following analyses are available online at https://github.com/acyann/post-termination-cover-crops.

To parallel the soil nitrogen analyses described above, we examined whether soil nitrogen levels differed between mixtures, monocultures and controls, and also over time following termination. We used the same samples as described above (three complete site-years, plus a limited set of samples from PrairiErth 2016), and we used the same treatment groups defined by plant functional group. We used permutational multivariate analysis of variance (PERMANOVA) with the function "adonis" from the R package *vegan* version 2.5-7 (73). All analyses used the Bray-Curtis distance matrix with 999 permuations to construct the null distribution. We first ran a PERMANOVA model including site, year, and their interaction in order to determine if these random effects influenced microbial communities. We then tested for the effect of time and cover crop treatment using a restricted permutation scheme by stratifying on the random effects site and year (for the test of time since termination) or site, year, and time (for the test of cover crop treatment). We visualized patterns in microbial community composition through two-dimensional non-metric multidimensional scaling of the Bray-Curtis matrix.

To provide a more in-depth analysis of microbial responses, we also examined correlations between microbial taxa and various environmental drivers in our data set, including soil nitrogen, site/year/time, and cover crop biomass. For this analysis, we conducted a weighted gene co-expression network analysis (WGCNA), which has previously been used for soil microbiome analysis in a variety of soil environments (74–77). We only used samples from the three complete site-years, because we did not have biomass data from PrairiErth in 2016 (55). We further restricted this analysis to the most abundant microbial taxa in our sample set. For both 16S and ITS, OTU tables were either filtered to exclude OTUs with a relative abundance of $<0.01\%$ or to include only the top 1000 OTUs, whichever method was more restrictive. The abundances of these top OTUs were normalized using the total sum scaling (TSS) method and then log2 transformed.

We then conducted a weighted gene co-expression network analysis (WGCNA) to determine patterns of co-occurrence among OTUS and between these OTU groups and our environmental data. WGCNA first uses network analysis to identify highly correlated "modules" of OTUs that respond in

concert, and then it seeks to identify environmental correlates for each of these modules. For this analysis, we used the package *WGCNA* version 1.70-3 (78, 79). The network was constructed based on the patterns of interactions across OTUs. A dendrogram was constructed, which creates a hierarchical topology for the network. From this point, a soft threshold was applied, which guides where the dendrogram is cut, and that cut separates the network into modules that display co-abundance. The location where the dendrogram is cut is determined by the topological features, expressed as the variable β, which is selected based on where graphs describing the scale independence and mean connectivity level off. This value sets the power for blockwise module construction (80). For the 16S network, $\beta = 5$ was used; for the ITS network, $\beta = 6$ was used. The minimum module size was set to 20 OTUs. This generated four modules for the 16S network and three modules for the ITS network. Correlation values between modules and environmental data (cover crop type, soil nitrogen, etc.) were generated on a heat map. Hub taxa were identified as the taxa in each module with a module correlation of $R > 0.70$ or $R < -0.70$. Taxonomy was visualized using *ggplot2* (81). Heatmaps were visualized using *WGCNA*.

RESULTS

Soil Nitrogen Dynamics

Across all site years, soil nitrate concentrations were greatest in the plant-free control plots within the 1st week after cover crop termination (**Figure 1A**). Soil nitrate in brassica plots was significantly lower than in the plant-free control plots during the 1st week, but there was no significant difference in soil nitrate across any of the cover crop monocultures or mixtures. Soil nitrate levels decreased over the 4-week post-termination period, particularly for the plant-free control plots, which were indistinguishable from cover crop plots by the 2nd week after termination (**Figure 1A**). By the 4th week, all soil nitrate levels were statistically indistinguishable across plots.

Soil ammonium levels were not statistically different across any cover crop treatment or controls at any time point after termination (**Figure 1B**). Soil ammonium levels tended to be highest during the 2nd week after termination, although the levels in plant-free controls were also high within the 1st week (**Figure 1B**).

Levels of potentially mineralizable organic nitrogen (PMN) were elevated in all cover crop plots relative plant-free controls, although this elevation was only statistically significant for cover crop mixtures (**Figure 1C**). All cover crop monocultures and mixtures had significantly higher PMN than plant-free controls by the 2nd week (**Figure 1C**), and PMN levels were highest overall at this time. By week four, only the brassica and weedy-control plots had significantly higher PMN than plant-free controls, with all other cover crop treatments having intermediate PMN values.

Overall Patterns in Soil Microbiome Composition

Site, year, and their interaction were significant predictors of soil bacterial and fungal community composition (**Table 1**), so we used stratification in subsequent models to test for effects of time and cover crop treatment. When stratified within site and year, time since termination was significant for both bacterial and fungal community composition (**Table 1**). When stratified within site, year, and time, cover crop type was a significant predictor of fungal community composition, but not of bacterial community composition (**Table 1**). Non-metric multidimensional scaling of microbial communities primarily reflected the overwhelming influence of site-year differences (**Supplementary Figures 1, 2**).

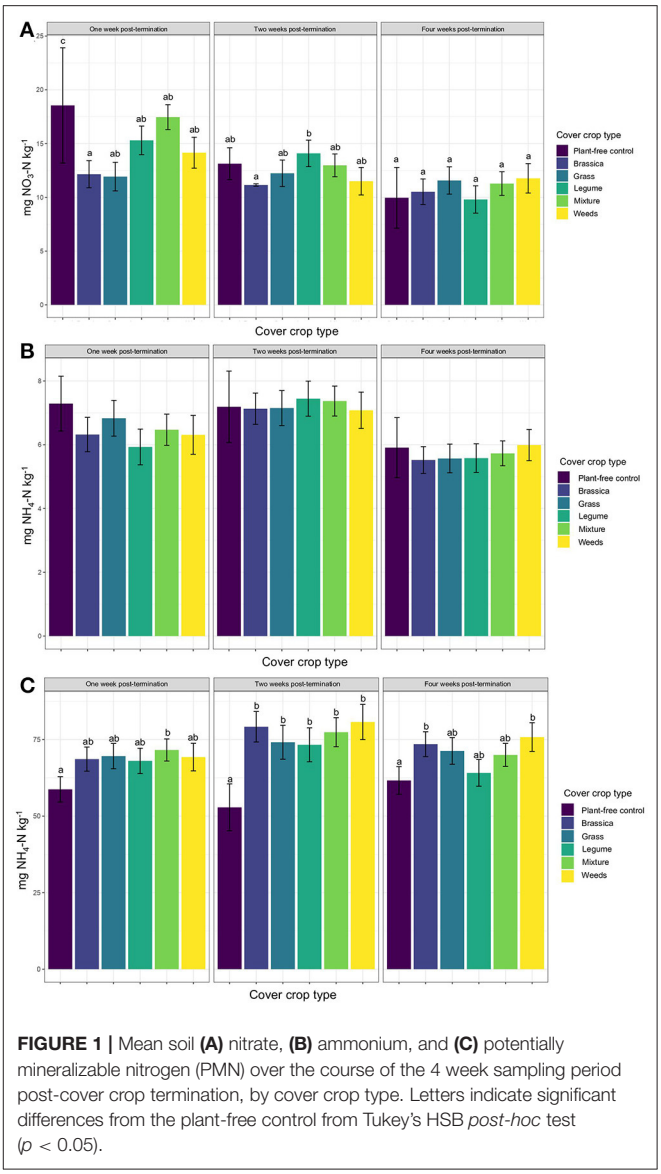


FIGURE 1 | Mean soil **(A)** nitrate, **(B)** ammonium, and **(C)** potentially mineralizable nitrogen (PMN) over the course of the 4 week sampling period post-cover crop termination, by cover crop type. Letters indicate significant differences from the plant-free control from Tukey's HSB *post-hoc* test ($p < 0.05$).

Coordinated Bacterial Responses: 16S WGCNA

A total of four modules were identified by the WGCNA analysis for 16S data (**Figure 2** and **Table 2**). The composition of the four modules was taxonomically distinct, and the relative proportions of phylum-level representation of the modules differed greatly

TABLE 1 | PERMANOVA tests were carried out on the entire dataset to evaluate the influences of site, year, cover crop type, cover crop diversity or sample date influenced bacterial and fungal community composition.

	Bacterial community				Fungal community			
	df	*F*	R^2	*p*	*df*	*F*	R^2	*p*
Site	1,526	31.67	0.051	0.001*	1,559	96.11	0.131	0.001*
Year	1,526	32.02	0.052	0.001*	1,559	50.50	0.069	0.001*
Site x year	1,526	29.03	0.047	0.001*	1,559	31.29	0.043	0.001*
Sample date *stratified by site and year	2,526	4.13	0.016	0.001*	2,559	13.37	0.046	0.001*
Cover crop type *stratified by site, year and sample date	5,526	1.46	0.014	0.087	5,559	2.11	0.019	0.001*

The Bray-Curtis distance method was applied to community data. df, degrees of freedom: numerator, total; F, F statistic; R^2, R^2-value; p, p-value. Results were considered significant at the $p < 0.05$ level and are indicated with an asterisk.

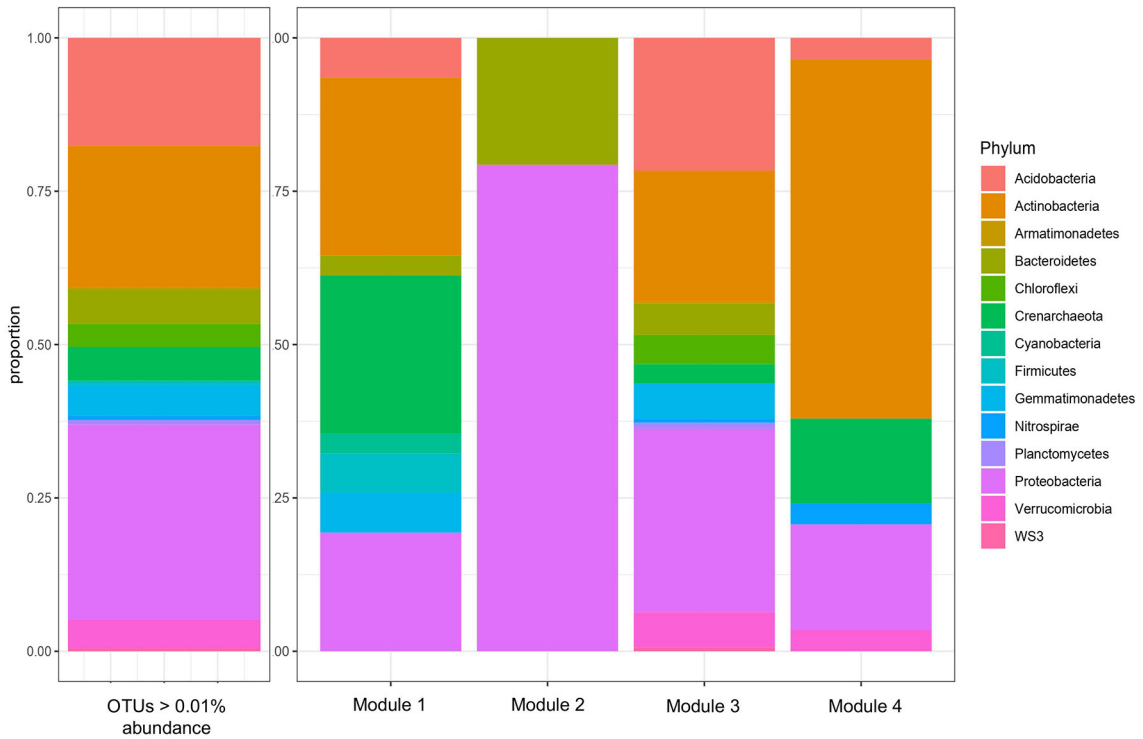


FIGURE 2 | Taxonomic composition, by phylum, of all 16S OTUs above 0.01% abundance, and of the four modules of 16S OTUs identified by WGCNA. The threshold of 0.01% abundance was used determine OTUs to include in the subsequent WGCNA.

with that of the overall soil microbiome (**Figure 2**). Module 1 was largely composed of Actinobacteria, Crenarcheaota, and Proteobacteria; Module 2 Proteobacteria and Bacteroidetes; Module 3 Acidobacteria, Actinobacteria, and Proteobacteria; and Module 4 Actinobacteria, Crenarchaeota, and Proteobacteria. Module 1 was most positively correlated with 2015 sampling year ($R = 0.19$, $p < 0.05$), Kinnikinnick farm ($R = 0.50$, $p < 0.05$), and weed biomass ($R = 0.31$, $p < 0.05$) (**Figure 3**). The hub taxa in Module 1 included mostly unclassified *Candidatus Nitrososphaera*, a type of ammonia-oxidizing Archaea (**Table 2**). Module 2 was most positively correlated with 2016 sampling year ($R = 0.64$, $p < 0.05$) and most negatively correlated with soil nitrate ($R = -0.36$, $p < 0.05$), soil ammonium ($R = -0.36$, $p < 0.05$), soil PMN ($R = -0.23$, $p < 0.05$), weed biomass ($R = -0.41$, $p < 0.05$), and total biomass ($R = -0.26$, $p < 0.05$). The hub taxa in Module 2 included both *Flavobacterium* spp. (Bacteroidetes) and Beta- and Gamma-proteobacteria. Module 3 was most positively correlated with PrariErth farm ($R = 0.86$, $p < 0.05$), 2015 sampling year ($R = 0.18$, $p < 0.05$), soil nitrate ($R = 0.38$, $p < 0.05$), and total cover crop biomass ($R = 0.36$, $p < 0.05$). This was the largest module and hub taxa were diverse: negative hub taxa included *Candidatus Nitrososphaera* spp. while positive hub taxa included mostly Proteobacteria, Actinobacteria, and Acidobacteria. Module 4 was most positively correlated with 2015 sampling year ($R = 0.29$, $p < 0.05$), PrariErth farm ($R = 0.58$, $p < 0.05$), soil nitrate ($R = 0.51$, $p < 0.05$), total cover crop

TABLE 2 | 16S hub taxa by module.

Module	16S OTU #	Taxonomy	Module correlation
Module 1	116	Archaea, Crenarchaeota, Thaumarchaeota, Nitrososphaerales, Nitrososphaeraceae, unclassified *Candidatus Nitrososphaera*	0.815
Module 1	1677	Archaea, Crenarchaeota, Thaumarchaeota, Nitrososphaerales, Nitrososphaeraceae, unclassified *Candidatus Nitrososphaera*	0.757
Module 1	407	Bacteria, Actinobacteria, Rubrobacteria, Rubrobacterales, Rubrobacteraceae, unclassified Rubrobacter	0.747
Module 1	134	Archaea, Crenarchaeota, Thaumarchaeota, Nitrososphaerales, Nitrososphaeraceae, unclassified *Candidatus Nitrososphaera*	0.743
Module 1	389	Bacteria, unclassified Gemmatimonadetes	0.717
Module 2	112	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, *Flavobacterium succinicans*	0.865
Module 2	2332	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, unclassified Xanthomonadaceae	0.854
Module 2	7145	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, unclassified *Flavobacterium*	0.786
Module 2	12739	Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, unclassified *Pseudomonas*	0.783
Module 2	451	Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, unclassified *Flavobacterium*	0.777
Module 2	91	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, unclassified Xanthomonadaceae	0.770
Module 2	20	Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, unclassified Oxalobacteraceae	0.753
Module 3	432	Bacteria, Chloroflexi, unclassified Gitt-GS-136	0.820
Module 3	49	Bacteria, Proteobacteria, Betaproteobacteria, unclassified MND1	0.819
Module 3	333	Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, unclassified Gaiellaceae	0.792
Module 3	14539	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, unclassified *Kaistobacter*	0.792
Module 3	78	Bacteria, Acidobacteria, [Chloracidobacteria], unclassified RB41	0.789
Module 3	67	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, unclassified Sinobacteraceae	0.785
Module 3	108	Bacteria, Acidobacteria, Acidobacteria-6, unclassified iii1-15	0.781
Module 3	6933	Bacteria, Actinobacteria, MB-A2-108, unclassified 0319-7L14	0.778
Module 3	110	Bacteria, Actinobacteria, Thermoleophilia, unclassified Solirubrobacterales	0.772
Module 3	151	Archaea, Crenarchaeota, Thaumarchaeota, Nitrososphaerales, Nitrososphaeraceae, unclassified *Candidatus Nitrososphaera*	0.759
Module 3	94	Bacteria, Acidobacteria, iii1-8, unclassified DS-18	0.747
Module 3	47	Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, unclassified Gaiellaceae	0.747
Module 3	120	Bacteria, Actinobacteria, MB-A2-108, unclassified 0319-7L14	0.741
Module 3	103	Bacteria, Acidobacteria, Acidobacteria-6, unclassified iii1-15	0.724
Module 3	90	Bacteria, Proteobacteria, Alphaproteobacteria, unclassified Rhizobiales	0.724
Module 3	27	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Bradyrhizobiaceae, unclassified *Balneimonas*	0.724
Module 3	14	Bacteria, Bacteroidetes, [Saprospirae], [Saprospirales], unclassified Chitinophagaceae	0.721
Module 3	2600	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Bradyrhizobiaceae, unclassified *Balneimonas*	0.717
Module 3	207	Bacteria, Gemmatimonadetes, unclassified Gemm-1	0.711
Module 3	8571	Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, unclassified Gaiellaceae	0.703
Module 3	155	Bacteria, Actinobacteria, Actinobacteria, Actinomycetales, Microbacteriaceae, unclassified *Agromyces*	0.702
Module 3	822	Bacteria, Verrucomicrobia, [Spartobacteria], [Chthoniobacterales], [Chthoniobacteraceae], unclassified *DA101*	−0.701
Module 3	1882	Bacteria, Verrucomicrobia, [Spartobacteria], [Chthoniobacterales], [Chthoniobacteraceae], unclassified *DA101*	−0.703
Module 3	2583	Bacteria, Acidobacteria, [Chloracidobacteria], unclassified RB41	−0.705
Module 3	683	Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, unclassified Gaiellaceae	−0.711
Module 3	1255	Bacteria, Actinobacteria, Thermoleophilia, unclassified Gaiellales	−0.713
Module 3	148	Bacteria, Acidobacteria, Solibacteres, unclassified Solibacterales	−0.713
Module 3	17862	Archaea, Crenarchaeota, Thaumarchaeota, Nitrososphaerales, Nitrososphaeraceae, *Candidatus Nitrososphaera SCA1170*	−0.716
Module 3	458	Bacteria, Acidobacteria, Acidobacteriia, Acidobacteriales, Koribacteraceae, unclassified *Candidatus Koribacter*	−0.731

(Continued)

TABLE 2 | Continued

Module	16S OTU #	Taxonomy	Module correlation
Module 3	3221	Bacteria, Gemmatimonadetes, Gemmatimonadetes, unclassified Ellin5290	−0.733
Module 3	243	Bacteria, Gemmatimonadetes, Gemmatimonadetes, unclassified Ellin5290	−0.734
Module 3	1145	Bacteria, Proteobacteria, Alphaproteobacteria, unclassified Ellin329	−0.734
Module 3	122	Bacteria, Bacteroidetes, [Saprospirae], [Saprospirales], unclassified Chitinophagaceae	−0.734
Module 3	255	Bacteria, Acidobacteria, Solibacteres, Solibacterales, Solibacteraceae, unclassified *Candidatus Solibacter*	−0.735
Module 3	880	Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, unclassified Gaiellaceae	−0.750
Module 3	5425	Bacteria, Gemmatimonadetes, Gemmatimonadetes, unclassified N1423WL	−0.760
Module 3	3517	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, unclassified *Kaistobacter*	−0.762
Module 3	10	Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, unclassified *Kaistobacter*	−0.779
Module 3	1805	Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, unclassified Gaiellaceae	−0.784
Module 3	118	Bacteria, Acidobacteria, Acidobacteriia, Acidobacteriales, unclassified Koribacteraceae	−0.787
Module 3	17124	Bacteria, Verrucomicrobia, [Spartobacteria], [Chthoniobacterales], [Chthoniobacteraceae], unclassified *DA101*	−0.789
Module 3	1302	Bacteria, Acidobacteria, Acidobacteriia, Acidobacteriales, unclassified Koribacteraceae	−0.792
Module 3	552	Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, unclassified Gaiellaceae	−0.795
Module 3	384	Bacteria, Proteobacteria, Alphaproteobacteria, unclassified Ellin329	−0.796
Module 3	3	Bacteria, Verrucomicrobia, [Spartobacteria], [Chthoniobacterales], [Chthoniobacteraceae], unclassified *DA101*	−0.812
Module 3	11121	Bacteria, Verrucomicrobia, [Spartobacteria], [Chthoniobacterales], [Chthoniobacteraceae], unclassified *DA101*	−0.814
Module 3	421	Bacteria, Acidobacteria, Acidobacteriia, Acidobacteriales, unclassified Koribacteraceae	−0.814
Module 3	96	Bacteria, Proteobacteria, Betaproteobacteria, A21b, unclassified EB1003	−0.822
Module 3	289	Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, unclassified Gaiellaceae	−0.826
Module 3	66	Archaea, Crenarchaeota, Thaumarchaeota, Nitrososphaerales, Nitrososphaeraceae, *Candidatus Nitrososphaera*	−0.836
Module 3	77	Archaea, Crenarchaeota, Thaumarchaeota, Nitrososphaerales, Nitrososphaeraceae, *Candidatus Nitrososphaera*	−0.859
Module 3	168	Bacteria, Acidobacteria, Acidobacteriia, Acidobacteriales, unclassified Koribacteraceae	−0.869
Module 4	374	Bacteria, Actinobacteria, Actinobacteria, unclassified Actinomycetales	0.842
Module 4	1704	Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, unclassified Gaiellaceae	0.829
Module 4	738	Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, unclassified Gaiellaceae	0.782
Module 4	128	Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, unclassified Gaiellaceae	0.777
Module 4	338	Bacteria, Actinobacteria, MB-A2-108, unclassified 0319-7L14	0.776
Module 4	8090	Archaea, Crenarchaeota, Thaumarchaeota, Nitrososphaerales, Nitrososphaeraceae, unclassified *Candidatus Nitrososphaera*	0.755
Module 4	366	Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, unclassified Gaiellaceae	0.748
Module 4	199	Bacteria, Actinobacteria, Thermoleophilia, unclassified Solirubrobacterales	0.747
Module 4	13312	Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, unclassified *Rhodoplanes*	0.715
Module 4	46	Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, unclassified Gaiellaceae	0.711
Module 4	102	Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, unclassified Gaiellaceae	0.706

Correlation > 0.70 or < -0.70.

biomass ($R = 0.32$, $p < 0.05$), and most negatively correlated with sampling days post-cover crop termination ($R = -0.27$, $p < 0.05$). Hub taxa for Module 4 were comprised mostly of unclassified Gaiellaceae.

Coordinated Fungal Responses: ITS WGCNA

Three modules were identified among ITS OTUs in the WGCNA analysis (**Figure 4**). At the class level, the three modules were broadly similar in their relative taxonomic composition. They were also compositionally similar to the overall fungal community, although Sordariomycetes and Dothidiomycetes tended to be over-represented in modules relative to the overall community, and Agaricomycetes tended to be under-represented

in modules. Module 1 was most positively correlated with 2015 ($R = 0.42$, $p < 0.05$), PrariErth farm ($R = 0.97$, $p < 0.05$), soil nitrate ($R = 0.52$, $p < 0.05$), and total cover crop biomass ($R = 0.43$, $p < 0.05$) (**Figure 5**). Hub taxa in this module included Sordariomyctes and Leotiomycetes (**Table 3**). Module 2 was most positively correlated with 2016 ($R = 0.96$, $p < 0.05$) and Kinnikinnick farm ($R = 0.46$, $p < 0.05$) and most negatively correlated with soil nitrate ($R = -0.61$, $p < 0.05$), soil ammonium ($R = -0.33$, $p < 0.05$), weed biomass ($R = -0.46$, $p < 0.05$), and total cover crop biomass ($R = -0.48$, $p < 0.05$). The top hub taxa in Module 2 included *Cystolepiota adulterine* and Dothidiomycetes. Module 3 generally had much weaker correlations to environmental variables, and it was positively correlated with 2016 ($R = 0.36$, $p < 0.05$) and Kinnikinnick farm ($R = 0.21$, $p < 0.05$) and negative correlated with soil nitrate ($R = -0.20$, $p < 0.05$) and total cover crop biomass ($R = -0.20$, $p < 0.05$). The hub taxa in Module 3 included *Mortierella capitate*, *Trichocladium asperum*, and other Sordariomyctes.

DISCUSSION

Compared to plant-free controls, cover cropping had a significant impact on soil nitrogen levels in these short-term, springtime trials, but we found very few overall differences between plots that used different functional groups of cover crops (brassicas vs. grasses vs. legumes). By far, the most important differences appeared to be between plots that contained some kind of plant cover (including weeds) and the plant-free control plots. Plots with plant cover had lower soil nitrate and higher potentially mineralizable nitrogen than plant-free controls in the first few weeks following termination, although these differences largely disappeared by the 4th week. Microbial community composition in our study was largely driven by site and year, suggesting that large scale spatial and temporal effects are the primary determinants of soil microbial species pools. Nevertheless, our network analysis revealed coordinated responses in highly-correlated modules of soil bacteria and fungi in cover cropped systems, and we discuss these in more detail below.

Soil Nitrogen by Cover Crop Type

Soil nitrate concentrations were initially greater under the plant-free control plots than any plots with cover crops. The inclusion of cover crops in this system should therefore minimize risk of nitrate leaching in the weeks following termination, as has been previously observed (20, 38, 40). Significant mineral nitrogen uptake by brassicas and weeds during the growing season may have further supported greater PMN concentrations during the fallow period prior to subsequent crop establishment (20). From an ecological and environmental sustainability perspective, lower concentrations of nitrate in the spring, during times of heavy rainfall and increased risk for leaching, may be advantageous. The five-species mixtures all contained at least one brassica, one legume, and one grass species, in addition to volunteer weed growth. It was, therefore, not surprising that the post-termination effects of mixtures on soil mineral nitrogen were consistently moderate. Holmes et al. (55) found that mixtures were consistently productive throughout the study, generating neither the most nor least biomass. Like the other more productive cover crops (brassicas, weeds, and grasses), nitrate losses were low under mixtures following termination. Similar to grasses, ammonium concentrations following mixtures declined steadily, though less dramatically.

Under mixtures, PMN concentrations were also moderate, and the relative dominance of brassicas in some of the mixtures (55) could have influenced the post-termination effects of those mixtures. PMN concentrations have been reported to decrease in mixtures with increasing proportions of grasses like rye or rye grass (82), and the mixtures in this study were heavily influenced by high biomass producers such as brassicas and weeds instead of grasses (55). This difference in PMN may also be reflected by the lower C:N ratios of legumes than grasses, and contribute to higher PMN due to more easily mineralizable content form tissues (20). Since mixtures contained tissues with variable C:N ratios, decomposition was occurring at different rates during the four-week sampling period. Organic nitrogen mineralization from legumes was likely more rapid due to low C:N ratios (83), lowering the overall PMN content when averaged across the 4-week sampling period. The quick release of nitrogen from legumes likely contributed to increased soil nitrate and slightly decreased soil PMN under mixtures as compared to monocultures like brassicas.

Organic farmers must prioritize their goals for planting spring-sewn cover crops. If the objectives are to reduce potential nitrate leaching and increase the potential for nitrogen mineralization throughout the upcoming growing season, then our study shows that a brassica monoculture such as Idagold mustard would accomplish this goal. However, while low soil nitrate concentrations post-cover crop incorporation may be beneficial for suppressing weed establishment, low mineral nitrogen could potentially hinder future crop growth if nitrogen demands are not met. If the goal of cover cropping is to increase nitrogen fixation, and subsequently inorganic nitrogen supply for crops, then a legume monoculture or mixture would allow for increased inorganic nitrogen available to subsequent crops while reducing the growth and establishment of weeds in legume monocultures. Determining the correct legume to use and ensuring that it will establish effectively when planted in a mixture will also be necessary to ensure successful establishment of all species.

Soil Microbial Community Response

Year and site were the strongest drivers of microbial community composition, which was illustrated in the PERMANOVA analysis. Therefore, it wasn't surprising to find that year and farm were often the strongest drivers in the WGCNA analysis. For the bacterial and archaeal OTUs, module 2 was strongly associated with the year 2016, and subsequently Kinnikinnick since biomass data that year was only available from that farm. Likewise, module 3 was strongly influenced by PrariErth farm. Castle et al. (84) also found that site-specific controls were most influential on short-term responses of soil bacterial communities under different cover crop treatments. The other correlations (nitrate, PMN, cover crop biomass) were considerably weaker, and thus challenging to disentangle the overarching strong effects

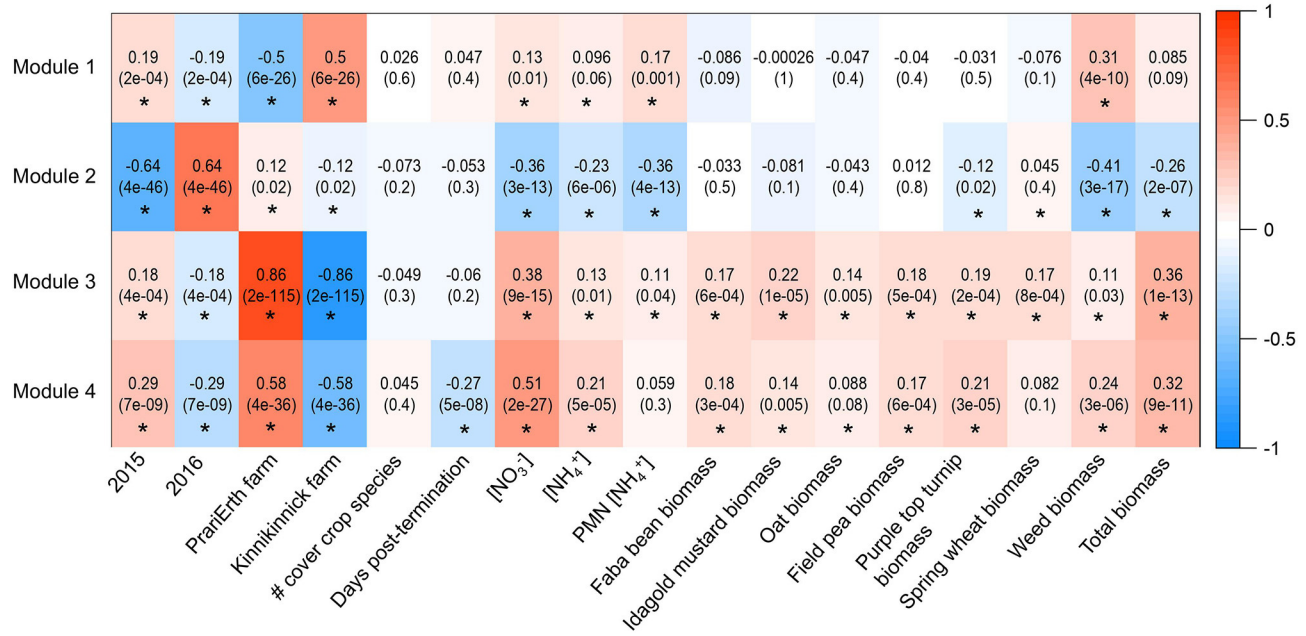


FIGURE 3 | Heatmap generated for 16S WGCNA analysis. Values are correlations and *p*-values are in parentheses. Asterisks indicate significant correlations at the level of $p < 0.05$.

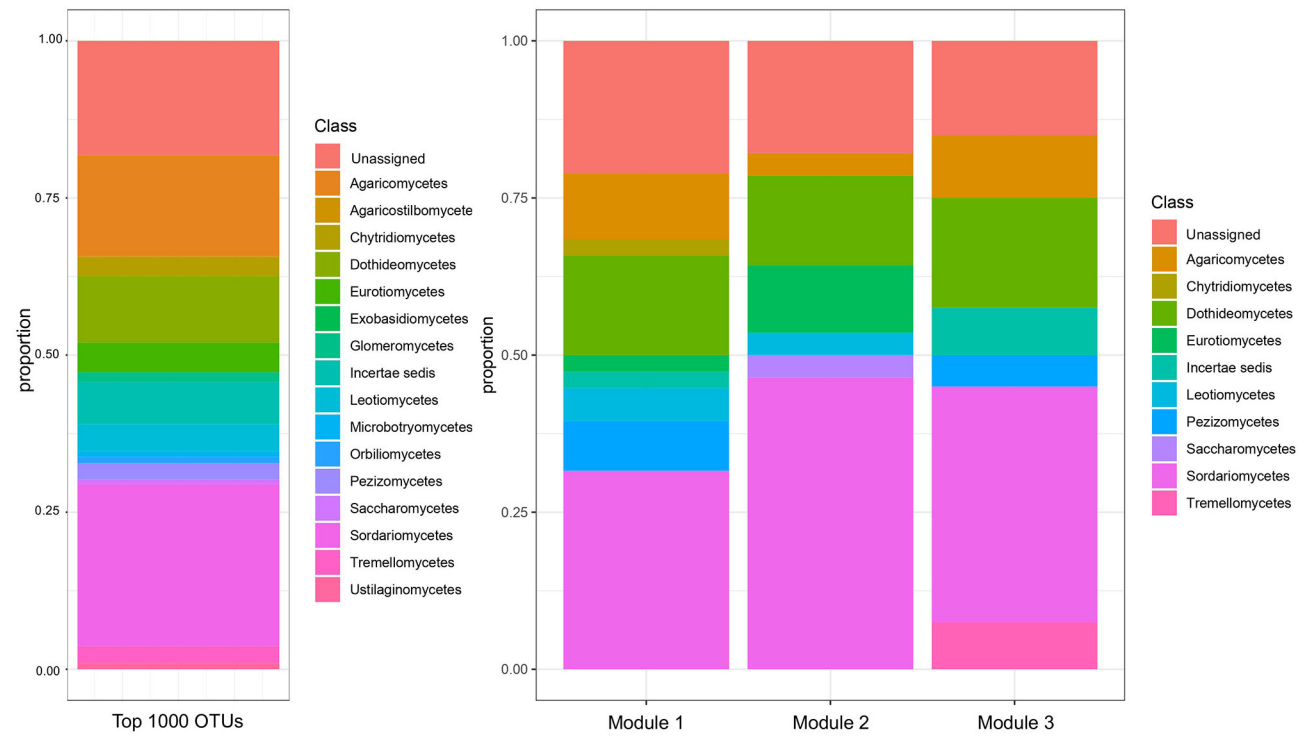


FIGURE 4 | Taxonomic composition, by class, of the top 1000 ITS OTUs and of the three modules of ITS OTUs identified by WGCNA. The threshold of top 1000 OTUs was applied for the subsequent WGCNA analysis.

of site and year. For the fungal OTUs, module 1 was very strongly influenced by PrariErth farm and module two by the year 2016. It is important to recognize these strong site and year effects, which reflect large scale temporal and spatial drivers of soil microbial communities. However, because these effects are idiosyncratic to our study, they hinder our ability to speculate about how cover

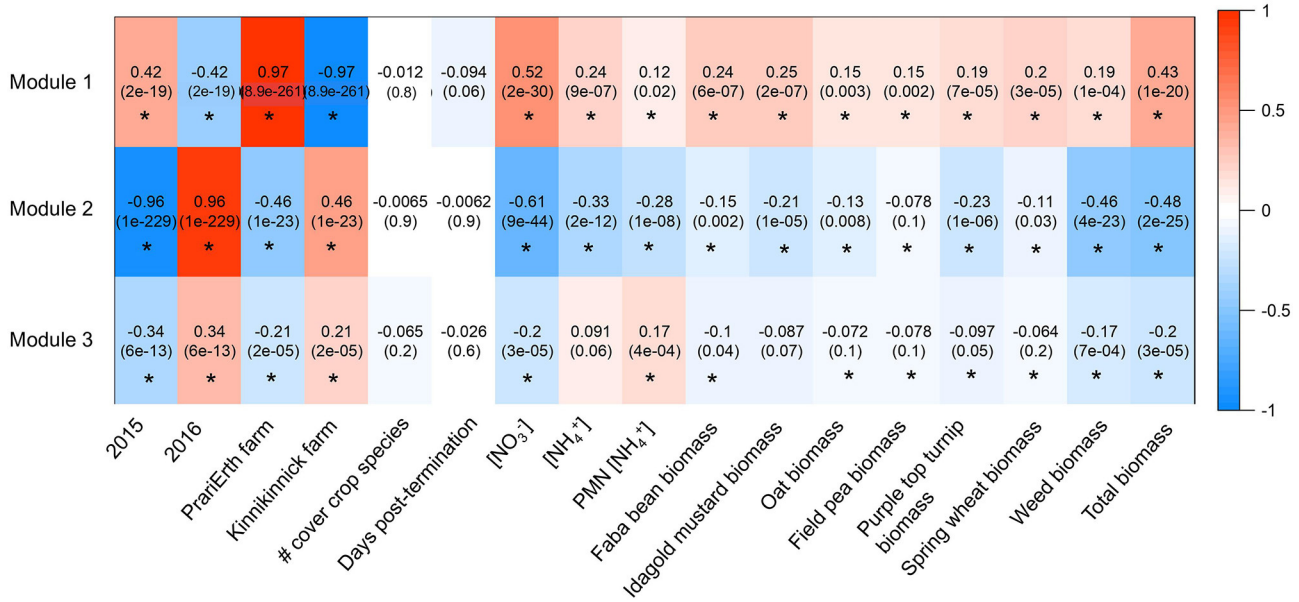


FIGURE 5 | Heatmap generated for ITS WGCNA analysis. Values are correlations and *p*-values are in parentheses. Asterisks indicate significant correlations at the level of $p < 0.05$.

cropping may affect microbial communities more generally. Therefore, we focus the remainder of our discussion on the remaining modules that showed much weaker correlations with site and year, and may therefore better reflect more general features of microbial response to cover cropping.

In the analysis of bacterial and archaeal OTUs, module 1 had a relatively strong positive correlation with weed biomass. There is a prevalence of ammonia-oxidizing organisms within the hub taxa from this module. The most abundant taxa from 16S module 1 were ammonia-oxidizing archaea of the genus *Candidus Nitrosphaera*. Individual ammonia-oxidizing bacterial and archaeal OTUs displayed individualistic responses to cover crop biomass, for example they were found in other modules of the analysis (module 3, module 4). In a concurrent study at this site, similar concentrations of soil ammonium across all cover crop types were reported, so detection of ammonia-oxidizing microorganisms across various cover crop biomasses was not entirely surprising. Though archaea made up <1% of the total "bacterial" 16S sequences that were analyzed in this study, they are ubiquitous in soils and are generally resistant to changing environmental conditions (85–87).

Ammonia-oxidizing bacteria and archaea are responsible for the first step of nitrification, conversion of ammonium to nitrite. This pathway is particularly important in agricultural systems, where nitrogen loss via nitrification decreases the pool of available inorganic nitrogen for subsequent crop uptake (12). The positive correlation between these ammonia-oxidizing OTUs and weed biomass may suggest that weeds, when at high biomass, can further support nitrification in soils. This may be an important discovery in the effort to reduce inorganic nitrogen losses in agriculture, which is a major concern in the Midwest.

Other hub taxa of note in 16S module included a single Rubrobacter, which are widely distributed in soils, such as grasslands, prairies, and pastures (88). Hub taxa for this module also included a Gammatimonadetes OTU, a taxon that has been found may be adapted to low soil moisture (89).

16S module 4 had negative correlation with sampling date (positive week 1), positive correlation with soil nitrate (nitrate levels also higher in week 1, across all cover crop types) and positive correlation with total cover crop biomass. The hub taxa in this module were overwhelmingly unclassified OTUs of the family Gaiellaceae (phylum: Actinobacteria). OTUs of the order Gaiellales have been shown to predominate in extreme environment, including saline-alkaline soils (90), wastewater treatments plants (91), and marine ecosystems (92). There was a single Actinomycetales (phylum: Actinobateria). Members of this order are often found in soil habitats and can support plant growth via biological nitrogen fixation (93, 94). There was one Rhizobiales (phylum: Proteobacteria), which also includes nitrogen-fixing associative taxa (95–98). There was also an ammonia-oxiziding archaea in this module, a potential producer of increased soil nitrate levels, which module 4 was also positively with. Rhodoplanes was also identified as a hub taxa in this module, and taxa of this family are photosynthetic with denitrification properties (99).

Fungal module 3 was negatively correlated with soil nitrate and total cover crop biomass, which likely links it to the plant-free control plots or the cover crop treatments with lower successful establishment. One of the hub taxa was *Mortierella capitate* (phylum: Zygomycota), which has been found to promote crop growth (100). There were two taxa identified as *Trichocladium asperum*, a polyphyletic genus of the family Chaetomiaceae

TABLE 3 | ITS hub taxa by module.

Module	ITS OTU #	Classification	Module correlation
Module 1	98	Fungi, Ascomycota, Leotiomycetes, unclassified Helotiales	0.852
Module 1	72	Fungi, Ascomycota, Sordariomycetes, Sordariales, unclassified Lasiosphaeriaceae	0.805
Module 1	257	Fungi, Basidiomycota, Agaricomycetes, Sebacinales, Sebacinales Group B, unclassified *Serendipita*	0.800
Module 1	2669	Fungi, Ascomycota, Sordariomycetes, Hypocreales, unclassified Nectriaceae	0.761
Module 1	65	Fungi, unclassified Ascomycota	0.760
Module 1	181	unclassified Fungi	0.752
Module 1	153	Fungi, Ascomycota, Leotiomycetes, Helotiales, Incertae sedis, *Pyrenopeziza revincta*	0.719
Module 1	46	Fungi, Ascomycota, Eurotiomycetes, Eurotiales, Trichocomaceae, *Aspergillus fischeri*	0.711
Module 1	2872	Fungi, Ascomycota, Sordariomycetes, Hypocreales, unclassified Nectriaceae	0.702
Module 2	526	Fungi, Basidiomycota, Agaricomycetes, Agaricales, Agaricaceae, *Cystolepiota adulterina*	0.829
Module 2	316	Fungi, Ascomycota, Dothideomycetes, unclassified Pleosporales	0.798
Module 2	398	Fungi, unclassified Rozellomycota	0.741
Module 2	1216	Fungi, Ascomycota, Eurotiomycetes, Onygenales, Incertae sedis, unclassified *Myceliophthora*	0.727
Module 2	444	Fungi, Ascomycota, unclassified Leotiomycetes	0.721
Module 2	542	Fungi, Ascomycota, Sordariomycetes, Xylariales, Xylariaceae, unclassified *Xylaria*	0.721
Module 2	634	Fungi, Ascomycota, Dothideomycetes, unclassified Pleosporales	0.721
Module 2	853	Fungi, unclassified Ascomycota	0.721
Module 2	1017	Fungi, Ascomycota, Saccharomycetes, Saccharomycetales, Trichomonascaceae, unclassified *Blastobotrys*	0.721
Module 2	1616	Fungi, Ascomycota, Dothideomycetes, Tubeufiales, Tubeufiaceae, unclassified *Helicoma*	0.721
Module 3	100	Fungi, Zygomycota, Incertae sedis, Mortierellales, Mortierellaceae, *Mortierella capitata*	0.927
Module 3	5870	Fungi, Ascomycota, Sordariomycetes, Sordariales, Chaetomiaceae, *Trichocladium asperum*	0.828
Module 3	3	Fungi, Ascomycota, Sordariomycetes, Sordariales, Chaetomiaceae, *Trichocladium asperum*	0.808
Module 3	86	Fungi, unclassified Ascomycota	0.795
Module 3	11	Fungi, Ascomycota, Sordariomycetes, Hypocreales, Nectriaceae, unclassified *Fusarium*	0.782
Module 3	5999	Fungi, Ascomycota, Sordariomycetes, Hypocreales, Clavicipitaceae, *Metarhizium marquandii*	0.740
Module 3	4674	Fungi, unclassified Ascomycota	0.732
Module 3	131	Fungi, Basidiomycota, Tremellomycetes, Cystofilobasidiales, Cystofilobasidiaceae, *Mrakia frigida*	0.721
Module 3	53	Fungi, Ascomycota, Sordariomycetes, unclassified Sordariales	−0.711
Module 3	39	Fungi, Ascomycota, Sordariomycetes, Hypocreales, Nectriaceae, unclassified *Fusarium*	−0.750

Correlation > 0.70 or < -0.70.

that has been found a number of habitats, including soils and decomposing plant material (101). Other members of the family are commonly found in decomposing plant material and play a role in plant degradation (102). Some other species in the family have causes neurological disease in humans (103). Another hub taxa belongs to the family Nectriaceae, which also includes important human and plant pathogens (104). Hub taxa *Metarhizium marquandii* includes plant-growth promoting fungi (105).

Taken together, changes in these 16S and ITS modules suggest that the more general coordinated responses of soil microbial communities in our study were primarily associated with changes in soil nitrate concentrations and overall plant biomass. Given that plant cover was also a key driver of soil nitrate in our study, we conclude that a major impact of springtime cover cropping is to drive changes in soil nitrate levels, and that soil nitrate, in turn, is a key driver of microbial community composition, particularly for bacteria. Cover cropping also promoted an increase in potentially mineralizable nitrogen pools in soils, and the presence of saprotrophic fungi in the hub taxa of module 3 suggests that the decomposition of cover crop residues may drive subsequent soil microbial changes over time.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, BioProject # PRJNA503856.

AUTHOR CONTRIBUTIONS

AH, SW, and AY designed the research. AH maintained the experimental research plots. AH and AY collected the samples. EL produced the data. EL and AY conducted the data analysis and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by AFRI Foundational and Applied Science Program grant no. 2015-67013-22948 and by the Hatch Act of 1887 project accession nos. 1004684 and 1021769 from the USDA National Institute of Food and Agriculture. This material is based upon work supported by the National Science Foundation under BII Grant No. 2022049. This work was also supported by the Ceres Trust Organic Research Initiative through Grant no. Ceres 2015-01667.

ACKNOWLEDGMENTS

We gratefully acknowledge Dave Bishop of PrairiErth Farm and David Cleverdon of Kinnikinnick Farm for their invaluable support of this research, which was conducted on their land. We also acknowledge A. W. Kent, S. Kuwayama, M. O'Loughlin, T. Ying, A. Gatdula, and S. Guillmot for research support and A. S. Davis and C. Pittelkow for advice in preparation of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsoil.2022.824087/full#supplementary-material

Supplementary Figure 1 | NMDS plot of bacterial communities representing all cover crop treatments. Each point represents a single sample and the bacterial community from that sample. Due to the significant effects of site and year, points are labeled by their site-year interactions. The Bray-Curtis distance method was used to perform the NMDS, with a stress level of 0.164. Ellipses represent the 95% confidence interval around the centroid for the given site-year.

Supplementary Figure 2 | NMDS plot of fungal communities representing all cover crop treatments. Each point represents a single sample and the fungal community from that sample. Due to the significant effects of site and year, points are labeled by their site-year interactions. Bray-Curtis distances were used to perform the NMDS, with a stress level of 0.214. Ellipses represent the 95% confidence interval around the centroid for the given site-year.

REFERENCES

1. Doran JW, Smith MS. Role of cover crops in ntirogen cycling. In: Hargrove WL, editor. *Cover Crops for Clean Water*. Ankeny, IA: SWCS (1991).
2. Tonitto C, David MB, Drinkwater LE. Replacing bare fallows with cover crops in fertilizer-intensive cropping systems: A meta-analysis of crop yields and N dynamics. *Agric Ecosyst Environ.* (2006) 112:58–72. doi: 10.1016/j.agee.2005.07.003
3. Wortman SE, Francis CA, Lindquist JL. Cover crop mixtures for the western corn belt: Opportunities for increased productivity and stability. *Agron J.* (2012) 104:699–705. doi: 10.2134/agronj2011.0422
4. van der Putten WH, Bardgett RD, Bever JD, Bezemer TM, Casper BB, Fukami T, et al. Plant-soil feedbacks: The past, the present and future challenges. *J Ecol.* (2013) 101:265–76. doi: 10.1111/1365-2745.12054
5. Akemo MC, Regnier EE, Bennett MA. Weed suppression in spring-sown rye (Secale cereale)–pea (Pisum sativum) cover crop mixes. *Weed Technol.* (2000) 14:545–9. doi: 10.1614/0890-037X(2000)014[0545:WSISSR]2.0.CO;2
6. Kumar V, Brainard DC, Bellinder RR. Effects of spring-sown cover crops on establishment and growth of hairy galinsoga (Galinsoga ciliata) and four vegetable crops. *HortScience.* (2009) 44:730–6. doi: 10.21273/HORTSCI.44.3.730
7. Wurst S, Ohgushi T, Allen E. Do plant- and soil-mediated legacy effects impact future biotic interactions? *Funct Ecol.* (2015) 29:1373–82. doi: 10.1111/1365-2435.12456
8. Lou Y, Davis AS, Yannarell AC. Interactions between allelochemicals and the microbial community affect weed suppression following cover crop residue incorporation into soil. *Plant Soil.* (2016) 399:357–71. doi: 10.1007/s11104-015-2698-8
9. Madden NM, Mitchell JP, Lanini WT, Cahn MD, Herrero EV, Park S, et al. Evaluation of conservation tillage and cover crop systems for organic processing tomato production. *Horttechnology.* (2004) 14:243–50. doi: 10.21273/HORTTECH.14.2.0243
10. Larkin RP, Honeycutt W. Effects of different 3-year cropping systems on soil microbial communities and rhizoctonia diseases of potato. *Phytopathology.* (2006) 96:68–79. doi: 10.1094/PHYTO-96-0068
11. Carrera LM, Buyer JS, Vinyard B, Abdul-Baki AA, Sikora LJ, Teasdale JR. Effects of cover crops, compost, and manure amendments on soil microbial community structure in tomato production systems. *Appl Soil Ecol.* (2007) 37:247–55. doi: 10.1016/j.apsoil.2007.08.003
12. van der Heijden MG, Bardgett RD, van Straalen MN. The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecol Lett.* (2008) 11:296–310. doi: 10.1111/j.1461-0248.2007.01139.x
13. Pascault N, Cecillon L, Mathieu O, Henault C, Sarr A, Leveque J, et al. In situ dynamics of microbial communities during decomposition of wheat, rape, alfalfa residues. *Microb Ecol.* (2010) 60:816–28. doi: 10.1007/s00248-010-9705-7
14. Fernandez AL, Sheaffer CC, Wyse DL, Staley C, Gould TJ, Sadowsky MJ. Structure of bacterial communities in soil following cover crop and organic fertilizer incorporation. *Appl Microbiol Biotechnol.* (2016) 100:9331–41. doi: 10.1007/s00253-016-7736-9
15. Vukicevich E, Lowery T, Bowen P, Úrbez-Torres JR, Hart M. Cover crops to increase soil microbial diversity and mitigate decline in perennial agriculture. A review. *Agronomy Sustain Dev.* (2016) 36:385. doi: 10.1007/s13593-016-0385-7
16. Brennan EB, Acosta-Martinez V. Cover cropping frequency is the main driver of soil microbial changes during six years of organic vegetable production. *Soil Biol Biochem.* (2017) 109:188–204. doi: 10.1016/j.soilbio.2017.01.014
17. Finney DM, Buyer JS, Kaye JP. Living cover crops have immediate impacts on soil microbial community structure and function. *J Soil Water Conserv.* (2017) 72:361–73. doi: 10.2489/jswc.72.4.361
18. Liu J, Yu Z, Yao Q, Hu X, Zhang W, Mi G, et al. Distinct soil bacterial communities in response to the cropping system in a Mollisol of northeast China. *Appl Soil Ecol.* (2017) 119:407–16. doi: 10.1016/j.apsoil.2017.07.013
19. Kruidhof HM, Bastiaans L, Kropff MJ. Cover crop residue management for optimizing weed control. *Plant Soil.* (2009) 318:169–84. doi: 10.1007/s11104-008-9827-6
20. Tribouillois H, Cohan JP, Justes E. Cover crop mixtures including legume produce ecosystem services of nitrate capture and green manuring: Assessment combining experimentation and modelling. *Plant Soil.* (2015) 401:347–64. doi: 10.1007/s11104-015-2734-8
21. Bending GD, Turner MK, Burns IG. Fate of nitrogen from crop residues as affected by biochemical quality and the microbial biomass. *Soil Biol Biochem.* (1998) 30:2055–65. doi: 10.1016/S0038-0717(98)00081-9
22. Brandi-Dohrn FM, Hess M, Selker JS, Dick RP, Kauffman SM, Hemphill DDJ. Nitrate leaching under a cereal rye cover crop. *J Environ Qual.* (1997) 26:181–6. doi: 10.2134/jeq1997.00472425002600010026x
23. Kaspar TC, Singer JW. The use of cover crops to manage soil. In: Hattfield JL, Sauer TJ, editors. *Soil Management: Building a Stable Base for Agriculture*. Madison, WI: American Society of Agronomy and Soil Science Society of America (2011).
24. Wortman SE, Francis CA, Bernards ML, Drijber RA, Lindquist JL. Optimizing cover crop benefits with diverse mixtures and an alternative termination method. *Agron J.* (2012) 104:1425. doi: 10.2134/agronj2012.0185

25. O'Connell S, Shi W, Grossman JM, Hoyt GD, Fager KL, Creamer NG. Short-term nitrogen mineralization from warm-season cover crops in organic farming systems. *Plant Soil.* (2015) 396:353–67. doi: 10.1007/s11104-015-2594-2
26. Wagger MG, Cabrera ML, Ranells NN. Nitrogen and carbon cycling in relation to cover crop residue quality. *J Soil Water Conserv.* (1998) 53:214–8.
27. Luscher A, Mueller-Harvey I, Soussana JF, Rees RM, Peyraud JL. Potential of legume-based grassland-livestock systems in Europe: A review. *Grass Forage Sci.* (2014) 69:206–28. doi: 10.1111/gfs.12124
28. Jackson L, Wyland L, Stivers L. Winter cover crops to minimize nitrate losses in intensive lettuce production. *J Agric Sci.* (1993) 121:55–62. doi: 10.1017/S0021859600076796
29. Brown PD, Morra MJ. Control of soil-borne plant pests using glucosinolate-containing plants. *Adv Agronomy.* (1997) 61:167–231. doi: 10.1016/S0065-2113(08)60664-1
30. Haramoto ER, Gallandt ER. Brassica cover cropping for weed management: A review. *Renewable Agricul Food Syst.* (2004) 19:187–98. doi: 10.1079/RAFS200490
31. Hu S, Grunwald NJ, van Bruggen AHC, Gamble GR, Drinkwater LE, Shennan C, et al. Short-term effects of cover crop incorporation on soil carbon pools and nitrogen availability. *Soil Sci Soc Am J.* (1997) 61:901–11. doi: 10.2136/sssaj1997.03615995006100030027x
32. USDA. *Carbon to Nitrogen Ratios in Cropping Systems*. Greensboro, NC (2011).
33. Yousef AN, Sprent JI. Effects of NaCl on growth, nitrogen incorporation and chemical composition of incoculated and NH4NO3 fertilized *Vicia faba* (L.) plants. *J Exp Bot.* (1983) 34:941–50. doi: 10.1093/jxb/34.8.941
34. Jensen ES. Nitrogen immobilization and mineralization during initial decomposition of 15N-labelled pea and barley residues. *Biol Fertil Soils.* (1997) 24:39–44. doi: 10.1007/BF01420218
35. Stevenson FJ, Cole MA. *The Internal Cycle of Nitrogen in Soil. Cycles of Soil: Carbon, Nitrogen, Phosphorus, Sulfur, and Micronutrients*. New York, NY: Wiley (1985).
36. Wagger MG. Time of desiccation effects on plant composition and subsequent nitrogen release from several winter annual cover crops. *Agron J.* (1989) 81:236–41. doi: 10.2134/agronj1989.00021962008100020020x
37. Teasdale JR, Abdul-Baki AA. Comparison of mixtures vs. monocultures of cover crops for fresh-market tomato production with and without herbicide. *HortScience.* (1998) 33:1163–6. doi: 10.21273/HORTSCI.33.7.1163
38. Chaves B, DeNeve S, Hofman GP, Boeckx, Van Cleemput. O. Nitrogen mineralization of the vegetable root residues and green manures as related to their (bio)chemical composition. *Eur J Agronomy.* (2004) 21:161–70. doi: 10.1016/j.eja.2003.07.001
39. USDA. *Manhattan Plant Material Center Cover Crop Study*. U.S. Department of Agriculture, Natural Resources Conservation Service Kansas (2013).
40. Brennan EB, Smith RF. Mustard cover crop growth and weed suppression in organic, strawberry furrows in California. *HortScience.* (2018) 53:432–40. doi: 10.21273/HORTSCI12576-17
41. Ohno T, First PR. Assessment of the Folin and Ciocalteu's method for determining soil phenolic carbon. *J Environ Q.* (1998) 27:776. doi: 10.2134/jeq1998.00472425002700040008x
42. Gao J, Xie Y, Jin H, Liu Y, Bai X, Ma D, et al. Nitrous oxide emission and denitrifier abundance in two agricultural soils amended with crop residues and urea in the North China plain. *PLoS ONE.* (2016) 11:e0154773. doi: 10.1371/journal.pone.0154773
43. Mazzola M, Mullinix K. Comparative field efficacy of management strategies containing Brassica napus seed meal or green manure for the control of apple replant disease. *Plant Dis.* (2005) 89:1207–13. doi: 10.1094/PD-89-1207
44. Benitez MS, Taheri WI, Lehman RM. Selection of fungi by candidate cover crops. *Appl Soil Ecol.* (2016) 103:72–82. doi: 10.1016/j.apsoil.2016.03.016
45. Reynolds HL, Packer A, Bever JD, Clay K. Grassroots ecology: Plant-microbe-soil interactions as drivers of plant community structure and dynamics. *Ecology.* (2003) 84:2281–91. doi: 10.1890/02-0298
46. Brussaard L, de Ruiter PC, Brown GG. Soil biodiversity for agricultural sustainability. *Agric Ecosyst Environ.* (2007) 121:233–44. doi: 10.1016/j.agee.2006.12.013
47. Lehman RM, Acosta-Martinez V, Buyer JS, Cambardella CA, Collins HP, Ducey TF, et al. Soil biology for resilient, healthy soil. *J. Soil Water Conserv.* (2015) 70:12A—8. doi: 10.2489/jswc.70.1.12A
48. Akemo MC, Bennett MA, Regnier EE. Tomato growth in spring-sown cover crops. *HortScience.* (2000) 35:843–8. doi: 10.21273/HORTSCI.35.5.843
49. Björkman T, Lowry C, Shail JW, Brainard DC, Anderson DS, Masiunas JB. Mustard cover crops for biomass production and weed suppression in the Great Lakes region. *Agron J.* (2015) 107:1235–49. doi: 10.2134/agronj14.0461
50. Chu M, Jagadamma S, Walker FR, Eash NS, Buschermohle MJ, Duncan LA. Effects of multispecies cover crop mixture on soil properties and crop yield. *Agricul Environ Lett.* (2017) 2:170030. doi: 10.2134/ael2017.09.0030
51. Creamer NG, Baldwin KR. An evaluation of summer cover crops for use in vegetable production systems in North Carolina. *HortScience.* (2000) 35:600–3. doi: 10.21273/HORTSCI.35.4.600
52. Cardinale BJ, Wright JP, Cadotte MW, Carroll IT, Hector A, Srivastava DS, et al. Impacts of plant diversity on biomass production increase through time because of species complementarity. *PNAS.* (2007) 104:18123–8. doi: 10.1073/pnas.0709069104
53. Wortman SE, Francis CA, Bernards MA, Blankenship EE, Lindquist JL. Mechanical termination of diverse cover crop mixtures for improved weed suppression in organic cropping systems. *Weed Sci.* (2013) 61:162–70. doi: 10.1614/WS-D-12-00066.1
54. Smith RG, Atwood LW, Warren ND. Increased productivity of a cover crop mixture is not associated with enhanced agroecosystem services. *PLoS ONE.* (2014) 9:e97351. doi: 10.1371/journal.pone.0097351
55. Holmes AA, Thompson AA, Wortman SE. Species-specific contributions to productivity and weed suppression in cover crop mixtures. *Agron J.* (2017) 109:2808. doi: 10.2134/agronj2017.06.0309
56. Mulvaney RL. Chapter 38: Nitrogen — inorganic forms. In: Sparks DL, Page AL, Helmke PA, Loeppert RH, Soltanpour PN, Tabatabai MA, editors. *Methods of Soil Analysis. Part 3. Chemical Methods*. Madison, WI: Soil Science Society of America, Inc. and American Society of Agronomy, Inc. (1996).
57. Doane TA, Horwáth WR. Spectrophotometric determination of nitrate with a single reagent. *Anal Lett.* (2003) 36:2713–22. doi: 10.1081/AL-120024647
58. Weatherburn WM. Phenol-hypochlorite reaction for determination of ammonia. *Anal Chem.* (1967) 39:971–4. doi: 10.1021/ac60252a045
59. Drinkwater LE, Cambardella CA, Reeder JD, Rice CW. Potentially mineralizable nitrogen as an indicator of biologically active soil nitrogen. *Soil Sci Soc Am J.* (1996) 49:217–29. doi: 10.2136/sssaspecpub49.c13
60. Moebius-Clune BN, Moebius-Clune BK, Gugino BK, Idowu OJ, Schindelbeck RR, Ristow AJ, et al. *Potentially Minerablizable Nitrogen*. Geneva, NY: Cornell University (2016).
61. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Nat Acad Sci USA.* (2011) 108:4516–22. doi: 10.1073/pnas.1000080107
62. White TJ, Bruns T, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. *PCR Protocols: A Guide to Methods and Applications*. New York, NY: Academic Press (1990). doi: 10.1016/B978-0-12-372180-8.50042-1
63. Magoč T, Salzberg SL. FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics.* (2011) 21:2957–63. doi: 10.1093/bioinformatics/btr507
64. Gordon A, Hannan GJ. *FastX Tool Kit*. (2010). Available online at: http://hannonlab.cshl.edu/fastx_toolkit
65. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics.* (2010) 26:2460–1. doi: 10.1093/bioinformatics/btq461
66. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods.* (2010) 7:335–6. doi: 10.1038/nmeth.f.303
67. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol.* (1990) 3:403–10. doi: 10.1016/S0022-2836(05)80360-2
68. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol.* (2006) 72:6069–5072. doi: 10.1128/AEM.03006-05
69. Urmas K, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AF, Bahram M, et al. Toward a unified paradigm for sequence-based identification of fungi. *Mol Ecol.* (2013) 22:5271–7. doi: 10.1111/mec.12481
70. Team RC. *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing (2017).

71. Pinheiro J, BatesD, DebRoy S, Sarkar D, Team RC. *Nlme: Linear and Nonlinear Mixed Effect Models*. (2017). Available online at: https://CRAN.R-project.org/package=nlme
72. Hothorn T, Bretz F, Westfall P. Simultaneous inference in general parametric models. *Biom J*. (2008) 50:346–63. doi: 10.1002/bimj.200810425
73. Oksanen JF, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. *Vegan: Community Ecology Package*. (2017). Available online at: https://CRAN.R-project.org/package=vegan
74. Castillo JD, Vivanco JM, Manter DK. Bacterial microbiome and nematode occurrence in different potato agricultural soils. *Soil Microbiol.* (2017) 74:888–900. doi: 10.1007/s00248-017-0990-2
75. Hewavitharana SS, Klarer E, Reed AJ, Leisso R, Poirier B, Honaas L, et al. Temporal Dynamics of the Soil Metabolome and Microbiome During Simulated Anaerobic Soil Disinfestation. *Front Microbiol.* (2019) 10:2365. doi: 10.3389/fmicb.2019.02365
76. Yue Y, Shao T, Long X, He T, Gao T, Zhou Z, et al. Microbiome structure and function in rhizosphere Jerusalem artichoke grown in saline land. *Sci Total Environ.* (2020) 724:138259. doi: 10.1016/j.scitotenv.2020.138259
77. Favela A, Bohn MO, Kent AD. Maize germplasm chronosequence shows crop breeding history impacts recruitment of rhizospher microbiome. *ISME J.* (2021) 15:2454–64. doi: 10.1038/s41396-021-00923-z
78. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinform.* (2008) 9:559. doi: 10.1186/1471-2105-9-559
79. Langfelder P, Horvath S. Fast R functions for robust correlations and hierarchical clustering. *J Statistical Sofware.* (2012) 46:1–17. doi: 10.18637/jss.v046.i11
80. Ghazalpour A, Doss S, Zhang B, Wang S, Plaisier C, Castellanos R, et al. Integrating genetic and network analysis to characterize genes related to mouse weight. *PLoS Genet.* (2006) 2:e130. doi: 10.1371/journal.pgen.0020130
81. Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Spring-Verlag (2009). doi: 10.1007/978-0-387-98141-3
82. Kuo S, Sainju UM. Nitrogen mineralization and availability of mixed leguminous and non-leguminous cover crop residues in soil. *Biol Fertil Soils.* (1998) 26:346–53. doi: 10.1007/s003740050387
83. Brennan EB, Boyd NS, Smith RF. Winter cover crop seeding rate and variety affects during eight years of organic vegetables: III cover crop residue quality and nitrogen mineralization. *Agron J.* (2013) 105:171–82. Available online at: https://www.ars.usda.gov/ARSUserFiles/21904/BrennanPeerRevPdfs/Agron%20J%20105p171.pdf
84. Castle SC, Samac DA, Gutknecht JL, Sadowsky MJ, Rosen CJ, Schlatter D, Kinkel LL. Impacts of cover crops and nitrogen fertilization on agricultural soil fungal and bacterial communities. *Plant Soil*. (2021) 466:139–50. doi: 10.1007/s11104-021-04976-z
85. Simon HM, Dodsworth JA, Goodman RM. Crenarchaeota colonize terrestrial plant roots. *Environ Microbiol.* (2000) 2:495–505. doi: 10.1046/j.1462-2920.2000.00131.x
86. Bates ST, Berg-Lyons D, Caporaso JG, Walters WA, Knight R, Fierer N. Examining the global distribution of dominant archaeal populations in soil. *ISME J.* (2011) 5:908–17. doi: 10.1038/ismej.2010.171
87. Maul JE, Buyer JS, Lehman RM, Culman S, Blackwood CB, Roberts DP, et al. Microbial community structure and abundance in the rhizosphere and bulk soil of a tomato cropping system that includes cover crops. *Appl Soil Ecol.* (2014) 77:42–50. doi: 10.1016/j.apsoil.2014.01.002
88. DeBruyn JM, Nixon LT, Fawaz MN, Johnson AM, Radosevich M. Global biogeography and quantitative seasonal dynamics of Gemmatimonadetes in soil. *Appl Environ Microbiol.* (2011) 77:6295–300. doi: 10.1128/AEM.05005-11
89. Fawaz MN. *Revealing the Ecological Role of Gemmatimonadetes Through Cultivation and Molecular Analysis of Agricultural Soils*. Master's Thesis. University of Tennessee (2013)
90. Peng M, Jia H, Wang Q. The effect of land use on bacterial communities in saline alkali soil. *Curr Microbiol*. (2017) 74:325–33. doi: 10.1007/s00284-017-1195-0
91. Shu D, He Y, Yue H, Wang Q. Microbial structures and community functions of anaerobic sludge in six full-scale wastewater treatment plants as revealed by 454 high throughput pyrosequencing. *Bioresour Technol*. (2015). 186:163–72. doi: 10.1016/j.biortech.2015.03.072
92. Chen R, He Y, Cui L, Li C, Shi S, Long L, Tian X. Diversity and distribution of uncultured and cultured gaiellales and rubrobacterales south china sea sediments. *Front Microbiol*. (2021) 12:657072. doi: 10.3389/fmicb.2021.6570752
93. Buckley DH, Huangyutitham V, Hsu SF, Nelson TA. Stable isotope probing with $^{15}N_2$ reveals novel noncultivated diazotrophs in soil. *Appl Environ Microbiol.* (2007) 73:3196–204. doi: 10.1128/AEM.02610-06
94. Yadav AN, Verma P, Kumar S, Kumar V, Kumar M, Sugitha TCK, et al. Chapter 2: Actinobacteria from rhizosphere: molecular diversity, distributions, and potential biotechnological applications. In: Singh BP, Gupta VK, Passari K, editors. *New and Future Developments in Microbial Biotechnology and Bioengineering*. Boston, MA: Elsevier (2018). doi: 10.1016/B978-0-444-63994-3.00002-3
95. Ivanova EG, Doronina NV, Shepelyakovskaya, AO, Laman AG, Brovko FA, Trotsenko YA. Facultative and obligate aerobic methylobacteria synthesize cytokinins. *Microbiol*. (2000). 69:646–51. doi: 10.1023/A:1026693805653
96. Delmotte N, Knief C, Chaffron S, Innerebner G, Roschitzki B, Schlapbach R, von Mering C, Vorholt JA. Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proc Natl Acad Sci USA*. (2009) 106:16428–33. doi: 10.1073/pnas.0905240106
97. Verginer M, Siegmund B, Cardinale M, Müller H, Choi Y, Míguez CB, Leitner E, Berg G. Monitoring the plant epiphyte Methylobacterium extorquens DSM 21961 by real–time PCR and its influence on the strawberry flavor. *FEMS Microbiol Ecol*. (2010). 74:136–45. doi: 10.1111/j.1574-6941.2010.00942.x
98. Erlacher A, Cernava T, Cardinale M, Soh J, Sensen CW, Grube M, et al. Rhizobiales as funtional and endosymbiontic members of the lichen symbiosis of Lobaria pulmonaria L. *Front Microb*. (2015) 6:53. doi: 10.3389/fmicb.2015.00053
99. Hiraishi A, Imhoff JF. Rhodoplanes. In: BSM Trust, editor. *Bergey's Manual of Systematics of Archaea and Bacteria*. Hoboken, NJ: John Wiley & Sons, Inc. (2021). doi: 10.1002/9781118960608.gbm00826.pub2
100. Li F, Zhang S, Wang Y, Li Y, Li P, Chen L, et al. Rare fungus, Mortierella capitata, promotes crop growth by stimulating primary metabolisms related genes and reshaing rhizosphere bacterial community. *Soil Biol Biochem*. (2020) 151:108017. doi: 10.1016/j.soilbio.2020.108017
101. Hernández-Restrepo M, Gené J, Casañeda-Ruiz RF, Mena-Portales J, Crous PW, Guarro J. Phylogeny of saprobic microfungi from Southern Europe. *Stud Mycol.* (2017) 86:53–97. doi: 10.1016/j.simyco.2017.05.002
102. Guppy KH, Thomas C, Thomas K. Cerebral fungal infections in the immunocompromised host: a literature review and a new pathogen—Chaetomium atrobrunneum: Case report. *Neurosurgery*. (1998) 43:1463–69. doi: 10.1097/00006123-199812000-00122
103. Abbott SP, Sigler L, McAleer R. Fatal cerebral mycoses caused by the ascomycete Chaetomium strumarium. *J Clin Microbiol*. (1995) 33:2692–8.
104. Lombard L, van der Merwe NA, Groenewald JZ, Crous PW. Generic concepts in netriaceae. *Stud Mycol*. (2015) 80:189–245. doi: 10.1016/j.simyco.2014.12.002
105. Baron NC, de Souza Pollo A, Rigobelo EC. Purpureocillium lilacinum and metarhizium marquandii as plant growth-promoting fungi. *Peer J*. (2020) 8:e9005. doi: 10.7717/peerj.9005

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Soil Science

ORIGINAL RESEARCH
published: 11 March 2022
doi: 10.3389/fsoil.2022.835849

Agriculture by Irrigation Modifies Microbial Communities and Soil Functions Associated With Enhancing C Uptake of a Steppe Semi-Arid Soil in Northern Patagonia

Juan P. Frene[1], Valeria Faggioli[2†], Julieta Covelli[1†], Dalila Reyna[1†], Luciano A. Gabbarini[1†], Patricio Sobrero[1], Alejandro Ferrari[1], Magalí Gutierrez[3] and Luis G. Wall[1]*

[1] Laboratory of Soil Biochemistry and Microbiology, Center for Soil Biochemistry and Microbiology, National University of Quilmes, Buenos Aires, Argentina, [2] Instituto Nacional de Tecnología Agropecuaria (INTA) Marcos Juárez Agricultural Experiment Station, Córdoba, Argentina, [3] Gerente Técnico de Desarrollo (GTD) Proyecto Chacra Valle Irrigado Norte Patagónico (VINPA), Asociación Argentina de Productores en Siembra Directa, Santa Fe, Argentina

OPEN ACCESS

Edited by:
Patricia Dorr De Quadros,
University of Waterloo, Canada

Reviewed by:
Sebastian Loeppmann,
Christian-Albrechts-Universität zu Kiel, Germany
Malak M. Tfaily,
University of Arizona, United States

***Correspondence:**
Luis G. Wall
wall.luisgabriel@gmail.com

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Soil Biology, Ecosystems and Biodiversity, a section of the journal Frontiers in Soil Science

Received: 15 December 2021
Accepted: 07 February 2022
Published: 11 March 2022

Citation:
Frene JP, Faggioli V, Covelli J, Reyna D, Gabbarini LA, Sobrero P, Ferrari A, Gutierrez M and Wall LG (2022) Agriculture by Irrigation Modifies Microbial Communities and Soil Functions Associated With Enhancing C Uptake of a Steppe Semi-Arid Soil in Northern Patagonia. Front. Soil Sci. 2:835849. doi: 10.3389/fsoil.2022.835849

The transformation of the semiarid steppe soil after 5 years of intensive irrigated agriculture in Northern Patagonia was analyzed in an on-farm study. The private grower venture used conservative practices, including no-till to maintain soil structure, high crop rotation and cover crops. To characterize steppe soil changes by irrigated agriculture, we analyzed the enzymatic activities involved in the biogeochemical cycles (carbon, nitrogen, phosphorus and sulfur), the whole soil fatty acids profile, the state of soil aggregation, and the bacterial and fungal microbiota through DNA sequencing methods. After 5 years of management, irrigated agriculture soil increased organic matter (25–33%), enzymatic activities -Cellobiose-hydrolase (60–250%), Phosphatase (35–60%), Xylanase (101–185%), Aryl-sulphatase (32–100%), Chitinase (85%), β-Glucosidase (61–128%), Leucine-aminopeptidase (138%)—depending on soil series, and macro-aggregate formation at the expense of the abundance of micro-aggregates in the first 0–5 cm of soil. Whole soil fatty acids profiles changed, enhancing mono-unsaturated, branched, cyclic and methylated fatty acids. Microbial communities showed significant differences between irrigated agriculture sites and pristine valleys. The richness-based alpha-diversity established increased bacterial communities but decreased fungal communities in cultivated soil. Indicators selected using the LEfSe method revealed the bacterial taxa *Acidothermus, Conexibacter* and *Thermoleophilum,* associated with semiarid steppe soil while *Asticcacaulis, Aquicella* and *Acromobacter* with irrigated agriculture. Ascomycota Phylum changed its community composition, being both taxa *Aspergillus* and *Alternaria* reduced while *Stagonospora* and *Metarhizium* were enhanced in irrigated agriculture. Taxa belonging to *Acidobacteria, Chloroflexi*, and *Betaproteobacteria*, that were enriched in irrigated agriculture soils, were associated with higher capture of C but smaller values of aggregation, while taxa abundant on steppe soils belonging to *Actinobacteria, Alphaproteobacteria,* and *Firmicutes* were positively associated with soil aggregation but negatively with C uptake.

Keywords: soil enzymes, soil lipids, soils use change, soil microbiota, soil aggregates, irrigated agriculture

INTRODUCTION

Transforming non-productive areas of semiarid soil into productive agriculture fields by irrigation is an option that depends on its business balance and the challenge of improving food production without affecting soil biodiversity.

Developing a better understanding of soil biodiversity and its modification by agriculture is crucial for the rational exploitation of beneficial microbial communities to improve crop performance under water shortage conditions, which is anticipated to become even less consistent as global climate changes (1). Soil biodiversity is linked to soil organic matter (SOM), which composition, and origin are a matter of debate and ongoing studies (2, 3). It is accepted that both microbial necromass (4) and plant rhizo-deposition (5) are critical factors in SOM build-up and C sequestration. Both microbial necromass and microbial-derived compounds explain soil mineral-associated (MAOM) and particulate (POM) organic matter aggregation as a result of microbial activity (5). Soil organic matter (SOM) changes and losses have commonly been associated with soil cultivation, especially conventional agriculture practices (6). A substantial fraction of SOM is composed by extracellular polymeric substances (EPS), including polysaccharides, proteins, lipids and nucleic acids, resulting from substrate transformation by soil microbes (4, 7). EPS is claimed to be critical for initial soil particles aggregation (7) that finally determined soil structure as the result of the soil trophic interactions (8). Both bacteria and fungi showed a relevant function on soil macro-aggregate (2000-250 μm) formation, where Proteobacteria, Cyanobacteria, and Glomeromycota are key phylum for this activity (9). Fungal traits have been associated with this soil aggregation activity (10). Soil biodiversity is also associated with the maintenance of soil multifunctionality, which can be summarized by multiple ecosystem functions and services (11, 12), highlighting the importance of microbial biodiversity associated with C uptake and sequestration, both vital processes for the soil (13). Finally, within bacteria diversity, it remains the question of any bacteria, or particular taxa are most relevant for the soil ecological services (14).

In Argentina, a group of farmers succeeded to implement irrigated agriculture in the semiarid steppe in the northern part of Patagonia. They obtained promising results in terms of goods productivity in a relatively short period (7 years). We run an on-farm study analyzing the soil of the cultivated fields of this private initiative. This paper aimed to investigate the response of soil microbial communities and associated ecosystems functions to irrigated agriculture. For this, we evaluated three agricultural sites under drip irrigation for 7 years in the north Patagonia dryland region. Soil enzyme activities covering C, N, P, and S nutrient cycles and whole soil fatty acids (WSFA) were used to partially characterize soil biochemistry. Illumina high-throughput sequencing of 16S rRNA and ITS1 genes were used to estimate microbial community structures, and soil aggregation stability was used to estimate soil physical structure and biological integrated activity, since aggregation is considered the result of soil trophic interactions (7, 9). We hypothesize that (1) irrigated agriculture impacts the diversity and composition of soil bacterial and fungal communities; (2) the effects of irrigated agriculture on bacterial and fungal communities influence soil functions, changing soil enzymes profile; and (3) changes in microbial communities would modified extracellular polymeric substances production and soil trophic interactions affecting soil aggregation. The result of this study could improve our understanding of the effect of irrigated agriculture on soil microbes and ecosystems functions.

MATERIALS AND METHODS

Site Description and Sampling

The on-farm study was conducted in three agricultural sites located north of the Argentinean Patagonian region, with similar histories of irrigated agriculture. The first two sites, called "Rio Negro" (RN) and "La Victoria" (LV), were located side by side, belonging to two different soil series within a circle of irrigation at the agricultural farm "Kaitaco" (39°53'3"S; 64°53'25"O) (**Supplementary Figure 1**). The third site, called "Chocorí" (CH), was located on the other margin of the Rio Negro river (39°59'08"S; 64°55'07"O), and both farms were located near to the city of General Conesa (Rio Negro province, Argentina) (**Supplementary Figure 1**). Both farms, "Kaitaco" and "Chocorí" belong to the project: "North Patagonian Irrigated Valley Farm" (VINPA) of the Argentine Association of No-Till Farmers (AAPRESID). The soils of the study area range from loamy sandy to loamy; therefore, the permeability of most soils is adequate for irrigation. The annual precipitation is about 260 mm, and the annual mean temperature is 15.6°C. The irrigation from sprinklers simulated growers' water application in the same area. The soil type, description, history of use and crop rotations can be found in **Supplementary Table 1**. The steppe soil reference plots were taken near the agricultural plots, 50–100 m outside the irrigation effect zone (**Supplementary Figure 1**). The plots were characterized as a Patagonian semi-arid steppe region, which is typically species-rich in forbs, grasses and lignified shrubs, with one grass (*Festuca pallescens*) and one shrub (*Mulinum spinosum*) contributing a large part of the total plant cover, with some species as *Prosopis strombullifera* characteristic for semi-arid and salted soils (15).

The soil samples were collected from two depths (0–5 and 0–20 cm) in August 2018. Ten plots were sampled in each site, five irrigated and five non-irrigated. Each plot has a 10 m^2 area separated by 40 m buffer zones (**Supplementary Figure 1**). Twenty subsamples were randomly taken by soil sample auger in each plot, composed as a single sample and immediately sealed in plastic bags, placed in coolers, and transported to the laboratory. According to biological analysis, the soil samples were sieved (2 mm) and stored at 4°C for enzyme analyses, and at −20°C for WSFA and DNA analysis, respectively. The 0–20 cm samples were sent to the laboratory for chemical and physical analysis.

Chemical and Agronomic Parameters

The chemical analysis measured in the soil samples were soil organic matter (SOM), electrical conductivity (EC), sodium adsorption relationship (SAR), and pH. Briefly, SOM was determined by the potassium dichromate method based on

Walkley and Black method (16). For EC: a filtrate was extracted from a 1:5 mixture of soil:water to measure the electrical conductivity (DSM^{-1}). Soil pH was measured at a soil-water ratio of 1:2.5, using a compound electrode (Accumet, Westford, MA, USA). To measure SAR, we calculated the sodium (Na) concentration by flame spectrometry and the concentration of magnesium (Mg) and calcium (Ca) by colorimetry. SAR was calculated using the following equation:

$$SAR = \frac{Na^{+}}{\{[Ca^{2+}] + [Mg^{2+}]/2\}1/2}$$

Particle-Size Fractionation and Mean Weight Diameter

Soil was particle-size fractionated into five particle size classes (2000-63, 250-63, 63-20, 20-2, 2-0.1 μm) following Neumann et al. (17). Briefly, a total of 10 g dry weight was suspended in distilled water (soil/water ratio 1:5 w/v) and ultrasonicated with an energy input of 30 J mL^{-1}. The ultrasonication was performed using an Ultrasonic Cleaner ultrasonicator (Testlab S.R.L., Argentina). The sand fraction (2000-250 and 250-63 μm) was separated from the other fractions by wet-sieving. The flow-through consisting of particles <63 μm was aliquoted to four glass tubes and centrifuged at 50 g for 15 min at 4°C. To separate clay size (<2 μm) from silt size (63-2 μm) particles, the supernatant containing the clay particles was decanted and collected in a 50 ml tube. The remaining pellets in the centrifugation tubes were resuspended in distilled water and centrifuged again. The centrifugation and suspension steps were repeated seven times with decreasing centrifugation times, 15, 13, 12, and 11 min, each twice, respectively. The last resuspended pellet was wet-sieved (by 20 μm), separating the coarse silt (63-20 μm) from the fine silt fractions (2–20 μm) in the flow-through. To enhance the flocculation of clay particles in the < 2 μm supernatant, $MgCl_2$ (final concentration 3.3 mM) was added to the 3-L beaker and kept at 4°C overnight. After decantation, the sedimented clay particles were further concentrated by centrifugation for 10 min at 2,400 g. All fractions were dried at 40°C and weighted.

The index of aggregate stability, mean weight diameter (MWD), is based on a weighted average of the five aggregate size classes, which was calculated using the following equation (18):

$$MWD = \sum_{i=1}^{5} Pi^{*}Si$$

Where Si is the average diameter (μm) for particles in their fraction and Pi is the weight percentage of the fraction in the bulk soil.

Soil Enzymes Activities

Enzymes activities were measured according to Marx et al. (19) method, based on the use of fluorogenic MUB-substrates and microplates (20). The bulk soil was analyzed for β-cellobiohydrolase (CEL), N-acetyl-β-glucosaminidase (NAG), β-glucosidase (BGLU), α-glucosidase (AGLU), phosphomonoesterase (PME), xylanase (XYL), leucine aminopeptidase (LAP), and arylsulfatase (SUL) using 4-MUB-β-d-cellobioside, 4- MUB -N-acetyl-β-glucosaminide, 4-MUB-β-d-glucoside, 4-MUB-α-d-glucoside, 4-MUB-phosphate, 4-MUB-β-1,4-xylosidase, l-Leucine-7-amino-4-methyl coumarin, and 4-MUB-sulfatase as substrates, respectively.

Briefly, 0.1 g of soil material was mixed with 10 ml of sterile 0.1 M MES buffer (2-(N-morpholino) ethanesulfonic acid) adjusted to pH 6.1 in a 20 ml tube with five steel balls homogenizing the soil material with a shaker for 5 min at 200 rpm. Soil slurry was immediately dispensed into 96-well microplates (Thermo Scientific Nunc) with buffer, sample, reference, and substrate following a strict order and position on the plate (ISO-TS-22939, 2010). The final substrate concentration in each well was 200 μM, that was a saturating concentration for all the enzymes (this was tested in preliminary assays in our lab). All chemicals supply by Sigma-Aldrich. Fluorescence intensity was read with an excitation of 355 nm and an emission of 460 nm on a POLARstar Omega automatic microplate fluorimeter (BMGLabtech, Ortenberg, Germany) for 20 cycles of 60 s at 30 °C. Enzyme activities were calculated based on three technical replicates by each soil sample and expressed as nmol h^{-1} g^{-1} (21).

Soil Whole Fatty Acids Lipidic Profile and Estimation of Microbial Structure Biomass

Whole soil fatty acids analysis (WSFA) was run according to Ferrari et al. (22). In brief, 1 g of freeze-dried and liquid N_2 milled soil sample was saponified with a NaOH-methanol mixture and methylated with HCl-methanol. After extraction with hexane/methyl tert-butyl ether (MTBE) and amendment with 33.75 μg of nanodecanoic (19:0) methyl ester as internal standard, the extract was washed with NaOH, evaporated under N_2 stream, resuspended in 100 μl of hexane and injected into an Agilent 6850 gas chromatography. The oven temperature was increased from 170 to 260°C with a five °C/min ramp, followed by another ramp (40°C/min) until a final temperature of 310°C. Hydrogen and nitrogen were used as carrier and make-up gases, respectively. A phenyl-siloxane (2.5%) column was used (25 m long, 200 μm ID, 0.33 μm film) with a flame ionization detector, fed by a hydrogen-air mixture. Fatty acids were analyzed through the MIDI microbial identification protocol (Sherlock® Microbial Identification System, version 6.2 and the RTSBA6 aerobe library). Nomenclature of fatty acids and grouping by chemical functions were done according to Ferrari et al. (22). The concentration of each fatty acid identified by the MIDI software was assessed concerning the 19:0 standard and expressed as nmol g^{-1}. The following fatty acids were used as biological marker to estimate bacteria and fungi biomass, knowing the practical limitations of this estimation, according to Ferrari et al. (22, 23): Bacteria (15:0 iso, 15:0 anteiso, 17:0iso, 17:0 anteiso, 17:0 10-Methyl); Fungi (16:1 w5c, 18:1w9c, 18:3 w6c).

DNA Extraction and Sequencing

According to the manufacturer's instructions, soil microbial DNA was extracted and purified from soil subsamples (0.5 g) using the FastDNA TM SPIN Kit for Soil (MP

Biomedicals, Solon, OH, USA). Quantification and quality of the extracted DNA were specified using the NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific, USA) and electrophoresis gel, respectively.

Soil DNA was submitted to NOVOGEN SA for amplicon sequencing. A fragment of approximately 250 bp spanning the V3-V4 region of 16S rRNA was amplified by PCR using primers 341F-806R. Sequencing of fungi was conducted using the ITS1-5F region of the ITS rRNA gene with primers ITS5-1737F and ITS2-2043R. Libraries were sequenced using an Illumina MiSeq system, generating 250 bp paired-end amplicon reads. The amplicon data were multiplexed using dual barcode combinations for each sample. Amplicons were mixed at roughly equivalent ratios based on electrophoretic band intensity and purified using Agencourt Ampure XP magnetic bead purification kit (Beckman Coulter, CA, USA).

Bioinformatics Analysis

The 16S raw reads were processed with the mothur v.1.35.0 software (24). Reads were trimmed with the following criteria: minimum length: 425 PB; minimum quality score: 25; degree of mismatching allowed: 1 mismatch to the primer and no mismatch to the barcode; homopolymers no longer than 10. Reads with ambiguous bases and singletons were removed. Chimera was checked with Uchime implemented in mothur (25) and removed from the dataset. 16S rDNA sequences were aligned and classified against SILVA bacterial SSU reference database v119. Denoised sequences were clustered into operational taxonomic units (OTUs) employing the average neighbor-clustering algorithm implemented in mothur at 97% sequence identity. The richness and diversity indexes were normalized, considering the number of sequences obtained from the smallest sample. Fungal reads were truncated to 200 bp and trimmed following the criteria described for bacterial reads using Usearch v.11 (26). Remaining sequences were collapsed into unique sequence types on a per-sample basis while preserving read counts and excluding singletons. These sequences served as the input for OTU clustering at a 97% similarity, while simultaneously removing putatively chimeras using Usearch v.11. Representative sequences of the OTUs were subjected to a similarity search against the UNITE database (27). Raw reads were deposited in the NCBI Short-Read Archive under accession number for bacteria SUB10673264 and fungi SUB10791814, BioProject PRJNA784308: Norpatagonia Agriculture project, Locus Tag Prefixes: LQ767 (SAMN23482046).

Statistical Analysis

To evaluate soil enzymatic activities and soil lipids (WSFA) analyses, permutational multivariate analysis of variance (PERMANOVA) was employed to quantitatively evaluate the effects of Site on Treatment and for calculated relative abundance data using "Adonis" function in the vegan package based on 999 permutations and method Bray-Curtis (28). Statistical analyses were performed by using R. Two-way analysis of variance (ANOVA) with Fisher's Least Significant Difference (LDS) pairwise comparisons at $p < 0.05$ were used to assess differences between soil uses and sites. Two multivariate methods of ordination were used to analyze enzymatic profiles and WSFA profile; LDA (Linear discriminant analysis) and PCoA (Principal component analysis) were used to assess which parameters contribute mostly to the separation of treatments. Two different data set of fatty acids were used: one built with those fatty acids present in at least 20% of all samples, in order to eliminate very rare fatty acids from the data set; the second data set comprised only those fatty acids that did not show interaction between use and site effects (**Supplementary Table 2**).

To evaluate the DNA data, statistical analyses were performed by using R. Two-way analysis of variance (ANOVA) with Fisher's Least Significant Difference (LDS) pairwise comparisons at $p < 0.05$ were used to assess differences between soil use and sites. Interactions between the main effects were evaluated using different packages (vegan, agricolae, gplots, ggplots2, RColorBrewer, edger, phyloseq). PERMANOVA analysis was based on Bray-Curtis dissimilarity using 10.000 permutations calculated from OTUs abundances. Alpha-diversity indexes (Observed richness and Shannon) were calculated per sample based on 100 times randomly subsampled read count data. The non-metric multidimensional scaling (NMDS) was also performed to visualize the most relevant patterns in microbial communities. Additionally, we tested for differential OTU abundance between agriculture and steppe communities (same thresholder OTU tables) of both kingdoms using likelihood ratio tests (LRT) with the R package edgeR (29). To graphically display the abundance distribution of the 50 most abundant genera across the different treatments, a heatmap based on centered and scaled log10-transformed relative abundances from taxon was drawn. LEfSe (Linear discriminant analysis Effect Size) analysis was conducted using the online analyzer at http://huttenhower.sph.harvard.edu/galaxy (30), to find the representative biomarker (specific abundance taxa) of different group treatment. The factorial Kruskal-Wallis test among different treatments was conducted with an α value of 0.05. The threshold on the logarithmic LDA (Linear discriminant analysis) score for discriminative features was 3.0 for bacterial and fungal communities. The strategy of "all-against-all" was used for multi-class analysis. Mantel test and Spearman correlation were used to correlating the bacterial/archaeal and fungal communities with chemical, physical, and biochemical parameters.

RESULTS

Chemical and Physical Analysis

The physicochemical properties of the soil samples are shown in **Table 1**. In general, sites were significantly different according to soil properties ($p < 0.001$). The irrigated agriculture modified physicochemical soil properties compared to the steppe. SOM was increased in CH (25%) and LV (33%), but not statistically significant ($p > 0.05$). The soil pH significant decreased in CH ($p = 0.0106$) while the other sites showed a non-significant decrease ($p > 0.05$). The EC showed three times significant increase in CH ($p < 0.001$), while the other sites showed a non-significant decrease ($p > 0.05$). SAR showed a decrease in three sites (18% in CH, 17% in LV and 30% in RN) but was not statically significant in any site (**Table 1**).

The soil aggregation showed significant changes between the semiarid steppe and agricultural one. The MWD showed a significant increase in the three irrigated agriculture sites ($p < 0.001$) (**Table 2**). This parameter was reflected in the significant increase of the macroaggregate fraction (2000-250 μm), which also significantly increased 36%, 168%, and 188% in CH, LV, and RN, respectively ($P < 0.001$). The aggregate fractions 63-20, 20-2, and 2-0.1 μm presented a significant decrease in cultivated soils in CH and LV (**Table 2**).

Soil Enzyme Activities

Soil enzymatic profiles were different between agricultural and steppe soil, according to a PCA multivariate analysis (**Figure 1** and **Supplementary Table 2**), being enzymes activities higher in agricultural soil than in the semiarid steppe. The increments of activities varied with sites. Average increments were observed as: SUL (32-100%); NAG (85%); XYL (101-185%); CEL (60-250%); BGLU; (61-128%); PME (35-60%) and LAP (138%) (**Supplementary Table 2**). Besides soil management, enzymatic profiles are also grouped according to soils series or sites under study (**Figure 1**). Despite similar agricultural management on two different soil series, LV and RN being part of the same agriculture irrigation circle (**Supplementary Figure 1**), the most relevant enzymatic activities characterizing each soil series differed. NAG and SUL activities were associated with RN soil. In contrast, all the other activities were associated with LV (**Figure 1**). The site CH, geographically apart from the other two and with different agricultural strategies as an alfalfa irrigated pasture instead of crop rotation, generally showed lower enzymatic activity (**Figure 1** and **Supplementary Table 2**).

Soil Lipidic Structure Measured by WSFA

The irrigated agriculture significantly increased the abundance of total WSFA, which summarize the total microbial biomass in the soil, total bacteria, and total fungi by 206.42, 145.28, and 135.50%, respectively ($p < 0.01$) (**Figure 2**). In contrast, the fungal-bacterial ratio decreased from steppe to irrigated agriculture, but

TABLE 1 | Soil physic and chemical properties from steppe and agricultural irrigated soils.

Site	Treatment	SOM	pH	CE	SAR
CH	Steppe	1.06a	7.28a	0.22b	8.18a
	Agriculture	1.32a	6.92b	0.76a	6.78a
LV	Steppe	1.55a	8.17a	0.586a	3.14a
	Agriculture	2.070a	7.68a	0.46a	2.75a
RN	Steppe	2.37a	7.34a	0.52a	1.50a
	Agriculture	2.32a	7.24a	0.34a	1.05a
ANOVA	Treatment	n.s.	*	n.s.	n.s.
	Site	***	***	n.s.	***
	Treatment x site	n.s.	n.s.	***	n.s.

*SOM, soil organic matter; CE, cation exchange; SAR, sodium absorption ratio. Data were tested for significance by one- and two-way ANOVA followed by Fisher's LSD test. Different letters denote significant differences between irrigation agriculture and steppe ($p < 0.05$) for each site. *$p < 0.05$; ***$p < 0.001$; n.s., non-significant.*

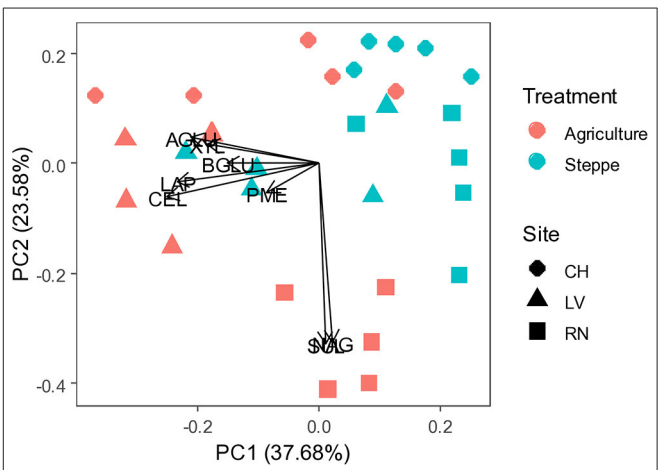


FIGURE 1 | Principal component analysis (PCA) of soil enzymatic pattern response to irrigated agriculture and steppe soils. CEL, β-cellobiohydrolase; NAG, N-acetyl-β-glucosaminidase; BGLU, β-glucosidase; AGLU, α-glucosidase; PME, phosphomonoesterase; XYL, xylanase; LAP, leucine aminopeptidase; SUL, arylsulfatase.

TABLE 2 | Soil aggregate fractions determined by wet sieving.

Site	Treatment	>250 μm	250-60 μm	60-20 μm	20-2 μm	<2 μm	MWD
CH	Steppe	34.42b	39.71b	17.60a	6.62a	1.62a	343.08b
	Agriculture	47.71a	43.20a	5.37b	2.64b	1.06b	459.85a
LV	Steppe	12.72b	32.44a	19.04a	31.0a	4.70a	136.57b
	Agriculture	34.16a	31.41a	8.26b	23.83b	2.30b	305.75a
RN	Steppe	7.28b	50.46a	18.56a	13.93a	2.02a	116.17b
	Agriculture	20.80a	44.31a	12.82a	14.24a	2.52a	227.55a
ANOVA	Treatment	***	n.s.	***	**	**	***
	Site	***	***	*	***	***	***
	Treatment x site	*	n.s.	n.s.	*	***	*

*Data were tested for significance by one- and two-way ANOVA followed by Fisher's LSD test. Different letters denote significant differences between irrigation agriculture and steppe ($p < 0.05$) for each site. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s., non-significant.*

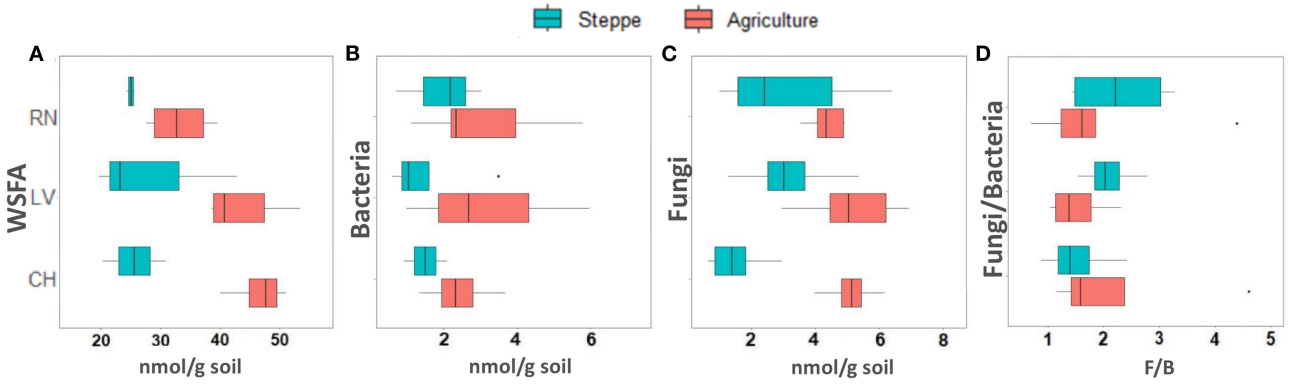


FIGURE 2 | Boxplots showing **(A)** total WSFA, **(B)** total bacteria, **(C)** total fungi, and **(D)** fungi-bacteria ratio estimated from WSFA.

the decrease was non-significant. Most relevant is the change in the whole soil lipidic composition. Out of a hundred detected fatty acids, the list was reduced to 49, deleting the rarest ones (**Supplementary Table 3**). A PERMANOVA analysis of this list showed significant effect by treatment (*P* 0.001) and by the site (*P* 0.002) and showed significant site-treatment interaction (**Table 3A**). An LDA analysis showed clear discrimination among soil use and management and sites (**Figure 3A**). The fatty acid with more significant loading on LDA showed a different pattern between soil use. MUFAs (14:1ω5c, 16:1ω7c alcohol, 17:1ω5c, 17:1ANTEISOω9c, 17:1ANTEISO A, 17:1ω7c, 18:1ω7c, 18:1ω9c characterized cultivated soil, while linear fatty acids (14:0, 16:0, 17:0), PUFA 20:4ω6,9,12,15c -usually considered marker for mesofauna- and 16:1ω5c -marker for arbuscular mycorrhiza- characterized steppe native soil. Analyzing the site-treatment interaction for each fatty acid in the WSFA profile, it was possible to reduce the WSFA list to a group of 31 fatty acids that did not show a significant site-treatment interaction (**Supplementary Table 3**). The PERMANOVA run on this new data set showed significant treatment effect (P 0.001) and site effect (P 0.003) without site/treatment interaction (**Table 3B**). LDA or PCA multivariate analyses showed clear separation of samples between irrigated agriculture and steppe soils (**Figure 3B**). This separation, suggesting a different lipidic structure between soils, was sustained by PCA when the fatty acids were grouped by their chemical function [see M&M and (22)]. The PC1 and PC2 of this new analysis explained 36.5 and 16.5% of the total variation, respectively. The samples were separated by treatment according to axis PC1, while the sites were mainly separated according to PC2 (**Figure 3B**).

TABLE 3 | PERMANOVA analysis on **(A)**, trimmed list of detected WSFA eliminating the rare ones -present in less than 20% of the analyzed samples- (see M&M) ($n = 49$); and on **(B)**, trimmed list of detected WSFA that did not show site-treatment interaction.

	Df	Sums of Sqs	Mean Sqs	F. Model	R^2	P(>F)
(A)						
Site	2	0.800	0.400	4.613	0.1650	0.002
Treatment	1	1.288	1.288	14.846	0.2655	0.001
Site: treatment	2	0.680	0.340	3.918	0.1401	0.001
Residuals	24	2.082	0.087	#N/A	0.4292	#N/A
Total	29	4.852	#N/A	#N/A	1	#N/A
(B)						
Site	2	0.664	0.332	3.184	0.154	0.003
Treatment	1	0.821	0.821	7.880	0.191	0.001
Site: treatment	2	0.318	0.159	1.526	0.074	0.113
Residuals	24	2.502	0.104	#N/A	0.581	#N/A
Total	29	4.305	#N/A	#N/A	1.000	#N/A

Soil Microbial Structure Measured by Soil DNA Sequencing

Bacterial and Fungal Community Diversity

Bacterial/archaeal diversity showed significant differences between irrigated agriculture and steppe treatments (**Figures 4A,B**). The agricultural treatment increased the diversity in both indices, richness, measured by Observed index ($p = 0.016$) and Shannon ($p = 0.001$) indexes. The Shannon index showed significant differences for the site ($p < 0.001$) and the interaction among both factors ($p = 0.03$), too. Only RN presented significant differences between treatments when the Shannon index was analyzed in each site ($p < 0.001$). For the fungal community, the diversity showed significant differences between treatments ($p = 0.0152$) and interactions among treatment and site ($p = 0.0125$) for richness, with a tendency to reduce diversity by irrigated agriculture. Only CH showed significantly higher diversity in the steppe ($p < 0.001$) (**Figure 4C**). In contrast, the Shannon index did not show significant differences between treatments or sites ($p > 0.05$) (**Figure 4D**).

We visualized and quantified the differences between microbial communities (β-diversity) using unconstrained non-metric multidimensional scaling (NMDS) and permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis dissimilarities. The PERMANOVA showed similar differences between treatments ($p < 0.001$) and sites ($p < 0.001$). The PERMANOVA results showed that sites explained 23.2% for

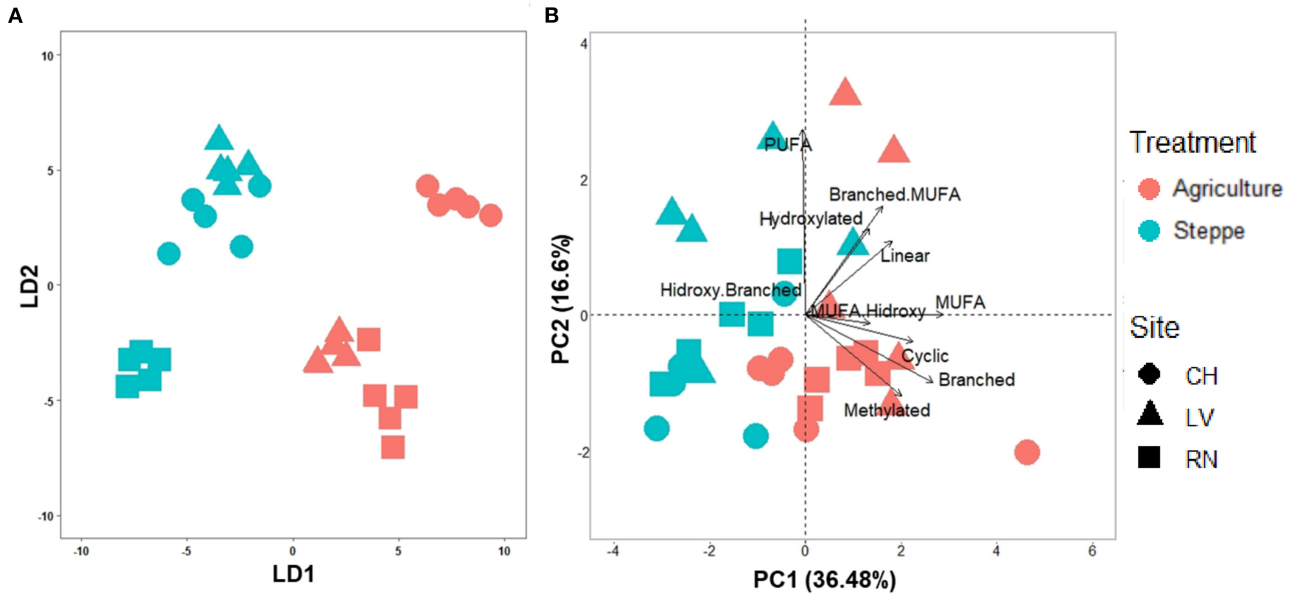


FIGURE 3 | (A) Lineal discriminant analysis (LDA) plots for the soil samples considering the trimmed fatty acids profile (49 fatty acids), and **(B)** Principal component analysis (PCA) plot of soil samples based on fatty acids grouped by their chemical functions for irrigated agriculture and steppe soils.

bacteria and 10.9% for fungi's variation while the treatment explained 35.6 and 28.4% of the variation between samples for bacteria and fungi, respectively (**Supplementary Table 4**). The NMDS analysis showed that the communities were spatially structured along with the soil treatment, followed by the effects of sites (**Figures 5A,B**). Similarly, the Edger analysis showed that irrigated agriculture and steppe presented different microbial habitats with specific sets of microbes (**Figures 5C,D**). Irrigated agriculture and steppe soils shared 90.74 and 52.84% of the OTUs for bacteria/archaea and fungi, respectively. The steppe presented more unique fungi sequences than agriculture, while the opposite trend appeared for bacteria/archaea (**Supplementary Figures 2A,B**).

Bacterial and Fungal Community Structure

The analysis of the taxonomic composition on bacterial phylum level revealed that *Proteobacteria* (34.67%), *Actinobacteria* (32.02%), *Bacteroidetes* (9.33%), *Acidobacteria* (9.13%), *Gemmatimonadetes* (4.25%), and *Firmicutes* (4.20%) were dominating in all soil samples. These taxa accounted for >93% of the bacterial sequence in all treatments (**Supplementary Figure 3A**). *Proteobacteria*, *Bacteroidetes*, *TM7*, and *Nitrospira* showed a significantly higher abundance in agricultural treatment ($p < 0.05$), while *Actinobacteria*, *Gemmatimonadetes*, *Verrucomicrobia*, *Planctomycetes*, and *Armatimonadetes* were significantly higher in the steppe ($p < 0.05$). *Acidobacteria* only showed a significant difference in CH, being higher in agriculture. Finally, *Firmicutes* did not present any significant differences. All the bacterial phylum presented significant differences between sites ($P < 0.05$), except for *Verrucomicrobia*, *Planctomycetes*, and *Armatimonadetes*.

The fungal community showed that the most abundant phyla were *Ascomycota* (53.48%), *Basidiomycota* (21.17%), *fungi unclassified* (21.07%), and *Mortierellomycota* (2.41%). These taxa accounted for >96% of the fungal sequences in all treatments. Considering the average of the relative phyla abundances, *Mortierellomycota* and *Aphelidiomycota* quantities significantly increased (226 and 100%, respectively), while the *Basidiomycota* and *Chytridiomycota* decreased (−55 and −60%, respectively), in irrigated agricultural soils compared to steppe values (**Supplementary Figure 3B**). Fungal phyla did not present statistical differences in abundance between sites, except for the phylum *Kickxellomycota* ($p = 0.02517$).

For bacterial/archaeal and fungal analysis, the analysis based on genus cluster heatmap (**Figure 6**) showed a similar pattern as NMDS (**Figures 5A,B**), where the samples clustered according to treatment and then by the site. The genus analysis showed significant differences between soil treatments for *Actinobacteria*, *Rubrobacter* and *Rhizobiales unclassified*, and *Acidobacteria* (*Solibacterales*, *GP3*, *GP4*, *Gp6*, *Gp7*, and *Gp10*) (**Figure 6A**). Most of the detected genera were unclassified. Those enhanced in steppe were related to functional traits that support desiccation and can sporulate; meanwhile, those enhanced in irrigated agriculture belong to Proteobacteria genera known to be rhizospheric or plant-associated bacteria, probably an interaction enhanced in the irrigated agricultural plots. Some fungal genera identified with steppe treatment were *Fusariella*, *Exophiala*, *Gelasinospora*, *Rhizophlyctis*, *Lectera*, *Blumeria*, *Cyphellophora*, *Chaetonium*, *Penicillium*, while agriculture treatment enhanced *Byssochlamys*, *Aspergillus*, *Alternaria*, *Trichoderma*, *Nosetophorma*, *Ascobolum*, and *Coriochaeta* (**Figure 6B**).

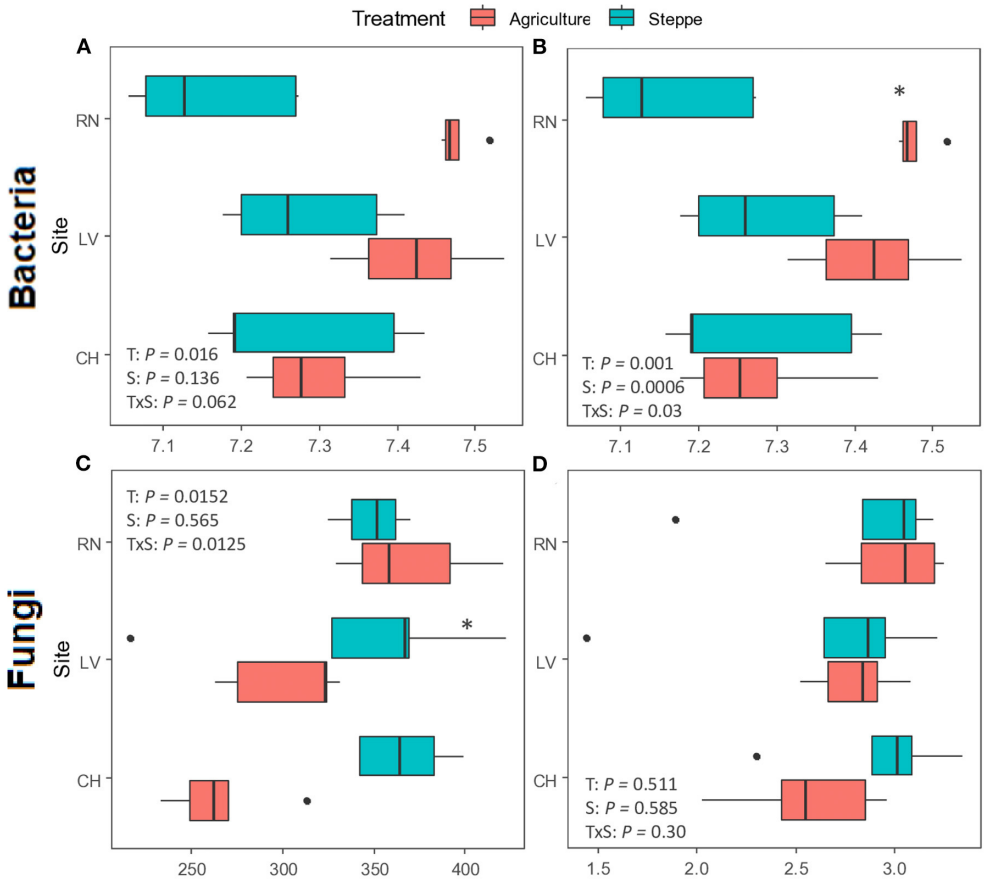


FIGURE 4 | Alpha diversity measures of the bacteria **(A,B)** and fungi **(C,D)** communities in irrigated agriculture and steppe. Richness (observed OTUs) and diversity (Shannon-Wiener) indices were evaluated in both treatments ($n = 5$). Boxes represent 25–75% of the data, solid lines the median, dots in the box mean, the tips represent the minimum and maximum values excluding the outliers (1.5 times lesser or greater than the lower or upper quantiles) represented by dots outside of the boxes. Data were tested for significance by two-way ANOVA followed by Fisher's LSD test. *Denote significant differences between irrigation agriculture and steppe ($p < 0.05$).

Bacterial and Fungal Community Indicators

After LefSe analysis, a total of 4 and 8 bacterial taxa with LDA scores of >3 were selected as biomarkers of steppe and agriculture treatments, respectively (**Figure 7A**). The bacterial taxa associated with steppe were *Acidothermus*, *Conexibacter*, *Thermoleophilum* (all *Actinobacteria*), *Terrimonas* (*Bacteroidetes*), and *Chelatococcus* (*Alpha-proteobacteria*). In contrast, agriculture biomarkers were associated with *Ammoniphilus* (*Firmicutes*), *Sphingomonas* and *Asticcacaulis* (both *Alpha-proteobacteria*), *Xanthomonas* and *Aquicella* (both *Gamma-proteobacteria*), and *Achromobacter* (*Beta-proteobacteria*) (**Figure 7A**). Regarding the 28 fungal biomarkers detected, the predominant fungal phylum was *Ascomycota*, with 25/28 representatives. The steppe soil was associated with 15 fungal biomarkers like *Darksidea*, *Alternaria*, *Lophiostoma*, *Lapidomyces*, *Neostagonospora*, *Bipolaris*, *Neodidymelliopsis* (*Dothideomycetes*, *Ascomycota*), *Byssochlamys*, *Aspergillus*, *Penicillium* (*Eurotiomycetes*, *Ascomycota*), *Xylaria*, *Coniochaeta*, *Apiosordaria* (*Sordariomycetes*, *Ascomycota*), *Clarireedia* (*Leotiomycetes*, *Ascomycota*), and *Cortinarius* (*Agaromycetes*, *Basidiomycota*). For agricultural irrigated soil, the biomarkers identified (13) were *Macroconia*, *Alfaria*, *Plectosphaerella*, *Fusarium*, *Lectera*, *Fusariella* (*Sordariomycetes*, *Ascomycota*), *Cladosporium*, *Neosetophoma*, *AureobasidIum* (*Dothideomycetes*, *Ascomycota*), *Cyphellophora* (*Eurotiomycetes*, *Ascomycota*), *Entoloma* (*Basidiomycota*), and *Rhizophlyctis* (*Chytridiomycota*) (**Figure 7B**).

Correlation Analysis

Mantel test showed that soil variables significantly influenced the soil microbial community structures. MWD ($F = 0.294$, $p = 0.001$), SOM ($F = 0.199$, $p = 0.004$), and SAR ($F = 0.197$, $p = 0.009$) jointly shaped the bacterial community structure, while MWD ($F = 0.192$, $p = 0.003$) has significantly affected the fungal community structure (**Table 4**).

The SOM significantly positively correlated with *Proteobacteria*, *Gemmatimonadetes*, *Nitrospira*, *OP1*, and *OD1* while negatively correlated with *Chloroflexi* (**Figure 8A**). The SAR significantly correlated with *Proteobacteria*, *Acidobacteria*, *Gemmatimonadetes*, *Nitrospira*, *OP1*, and *BCR1* while negatively

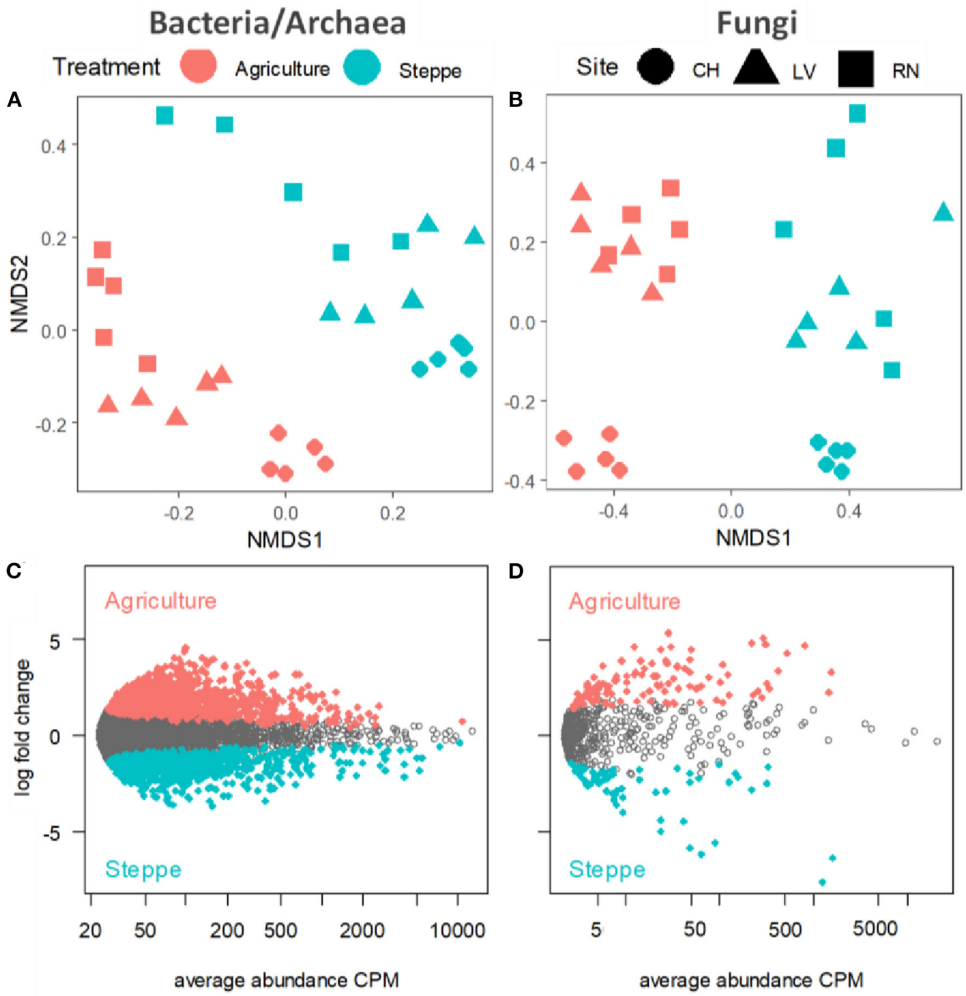


FIGURE 5 | Non-metric multi-dimensional scaling (NMDS) plots of community composition in irrigated agriculture and steppe plots in each site for **(A)** bacteria/archaea and **(B)** fungi. Plots display the abundance patterns of **(C)** bacteria and **(D)** fungi in irrigated agriculture and steppe soil. X-axis reports average OTU abundance (as counts per million, CPM), and Y-axis log2-fold change. Agriculture and steppe-specific OTUs were colored in redish and greenish, respectively, and non-differentially abundant OTUs are in gray (likelihood ratio test, $p < 0.05$, FDR corrected).

correlated with *Chloroflexi* (**Figure 8A**). MWD showed a positive correlation with *Actinobacteria*, *Chloroflexi*, *WS3*, and *TM7*. MWD negatively impacted on *Acidobacteria*, *Chlorobi*, *Deinococcus-Thermus*, *Planctomycetes*, and *Spirochaetes*. Some Bacteria phyla correlated positively with different enzymes ($p < 0.05$): *Proteobacteria* with LAP and NAG, *Actinobacteria* with BGLU; *Acidobacteria* with NA; *Verrucomicrobia* with BGLU; *Firmicutes* with XYL and CEL; *OP11* with LAP, NAG and SUL; *TM7* with CEL, SUL and PME; and *WS3* with BGLU and SUL. Other phyla show negative correlations with enzymes ($p < 0.05$): *Acidobacteria* with XYL and BGLU, *Deinococcus-Thermus* with BGLU and SUL, *Chloroflexi* with LAP and NAG, *Chlorobi* with SUL, *Chlamydiae* with CEL; and *Spirochaetes* with BGLU and PME (**Figure 8A**). Within the fungal phylum, SOM positively correlated with *Mortierollomycota*, *Chytridiomycota*, and *Kickxellomycota* (**Figure 8B**). *Mortierollomycota* and *Kickxellomycota* negatively correlated with SAR. MWD positively correlated with *Rozellomycota* and negatively correlated with *Basidiomycota*. Different phyla positively correlated with different soil enzyme activities: *Ascomycota* with CEL; Chytridiomycota with LAP, Mortierollomycota with LAP, NAG and CEL; *Rozellomycota* with XYL, BGLU and SUL; and *Zoopagomycota* with BGLU. *Basidiomycota* was the only fungal phylum that negatively correlated with CEL and BGLU (**Figure 8B**).

Finally, we plot the bacteria/archaeal (**Figure 8C**) and fungal (**Figure 8D**) genus, which significantly correlated with SOM and MWD. The phylum and classes *Beta-Proteobacteria* (3), *Chloroflexi* (2), *Acidobacteria* (2), and *Actinobacteria* (1), which were more abundant in agriculture, presented a positive correlation with SOM and negatively correlated with MWD. While *Actinobacteria* (9), *Alpha-proteobacteria* (6), *Firmicutes* (4), and *Bacteroidetes* (1) negatively correlated with SOM and positively with MWD (**Figure 8C**). At the fungal level,

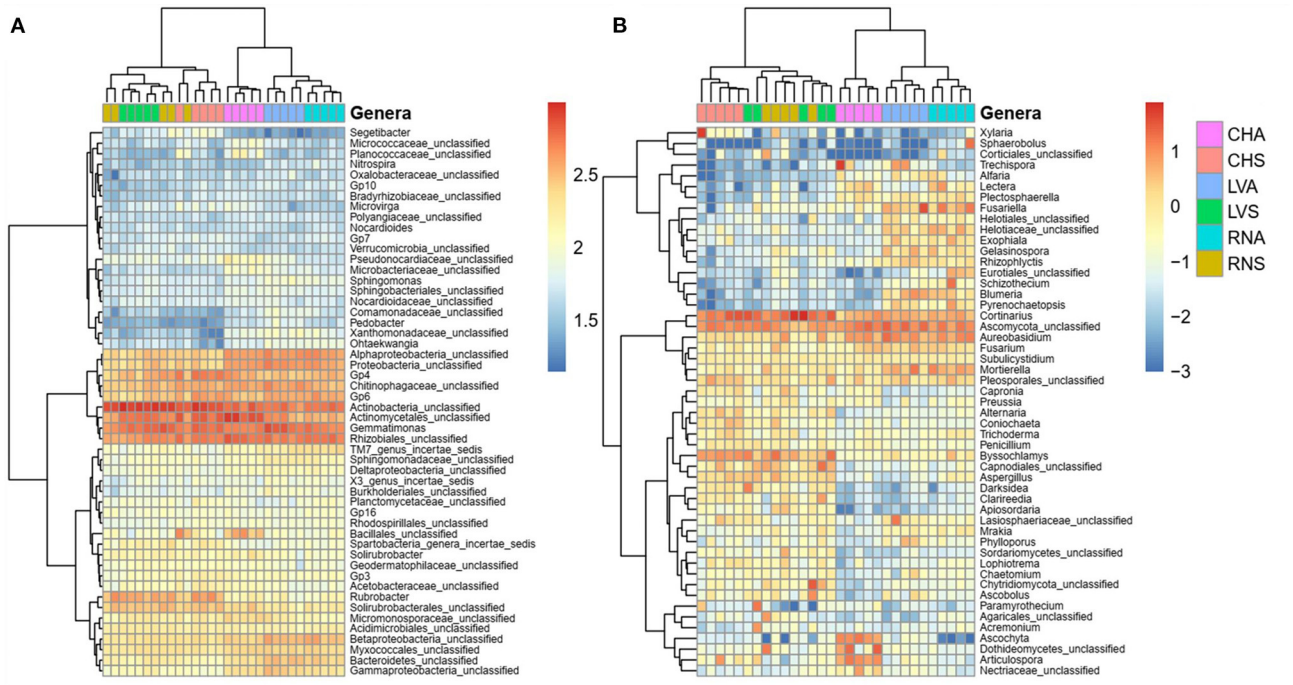


FIGURE 6 | Heatmap showing the abundances of the 50 most abundant **(A)** bacteria/archaea and **(B)** fungi genera in irrigated agriculture and steppe soils under. Data were centered and scaled to the mean of each taxon's log-transformed relative abundance. Top dendrogram cluster samples according to the community composition. Side dendrogram cluster taxa according to their relative abundance distribution across samples.

Ascomycota, *Basidiomycota*, and *Rozellomycota* genus correlated positively and negatively with SOM and MWD (**Figure 8D**). Additionally, one *Chytridiomycota* genus positively correlated with SOM and negatively with MWD.

DISCUSSION

This study examined the soil biochemistry and biological diversity of bacterial/archaeal and fungi after converting semiarid steppe in the north of Patagonia into cropland by no-till irrigated agricultural systems. A general soil microbial structure estimated from WSFA showed a shift toward bacterial dominance in irrigated agriculture soils. According to irrigated agriculture, the modification observed in the microbial community structure strongly shifted enzymatic profiles. An apparent increase of biological activities was observed for hydrolases as SUL, XYL, CEL, BGLU, PME, most probably related to water availability and crop rhizodeposition.

Monounsaturated, branched cyclic and methylated fatty acids were enhanced by irrigated agriculture in SOM. This modification of fatty acids profile seems to indicate soil health improvement (Wall et al., unpublished). Additionally, the changes observed in the WSFA profile also suggested changes in soil organic matter processed by the soil microbiota, as suggested by Liang et al. (3, 4). The enhancement of soil aggregation found at the macroaggregate fraction (2000-250 mm) could also be related to the change in the WSFA profile since WSFA would be part of soil EPS involved in soil aggregation (7).

The introduction mentioned that biodiversity is crucial in sustainable agroecosystems to maintain soil ecosystems services (12). The microbiome structure obtained from soil DNA sequencing confirmed that semiarid steppe and irrigated agriculture soil significantly differed on bacterial and fungal community abundance, showing two different niches probably due to new physical and chemical conditions. Irrigated agriculture had a different impact according to the microbial kingdom at alpha-diversity indexes (richness and Shannon), increasing bacterial diversity while the fungal diversity tent not to change or decrease (31). Irrigated agriculture significantly altered soil-borne fungi resources and environment, affecting diversity patterns (32–34). Indeed, decrement in fungal diversity due to fertilization has been broadly reported (32, 35), and such response is associated with the alteration of soil nutrient and carbon conditions (32, 36–38). Our study is consistent with other studies that showed a change in bacterial diversity associated with the amount of soil water (39).

Proteobacteria and *Bacteroidetes* are two significant phyla that positively respond to cover vegetation (40–42), while *Mortierellomycota* can be saprophytic on the residual limbs of plants and animals and decompose their remains (43). *Proteobacteria* and *Bacteroidetes* from bacteria and *Mortierellomycota* from fungi significantly increased in irrigated agricultural plots. Furthermore, *Proteobacteria* showed a significant correlation with SOM in LV and RN sites. On the opposite, *Actinobacteria*, *Gemmatimonadetes*, *Verrucomicrobia*, *Planctomycetes*, and *Armatimonadetes* for bacteria and *Basidiomycota* for fungi were higher in the semiarid

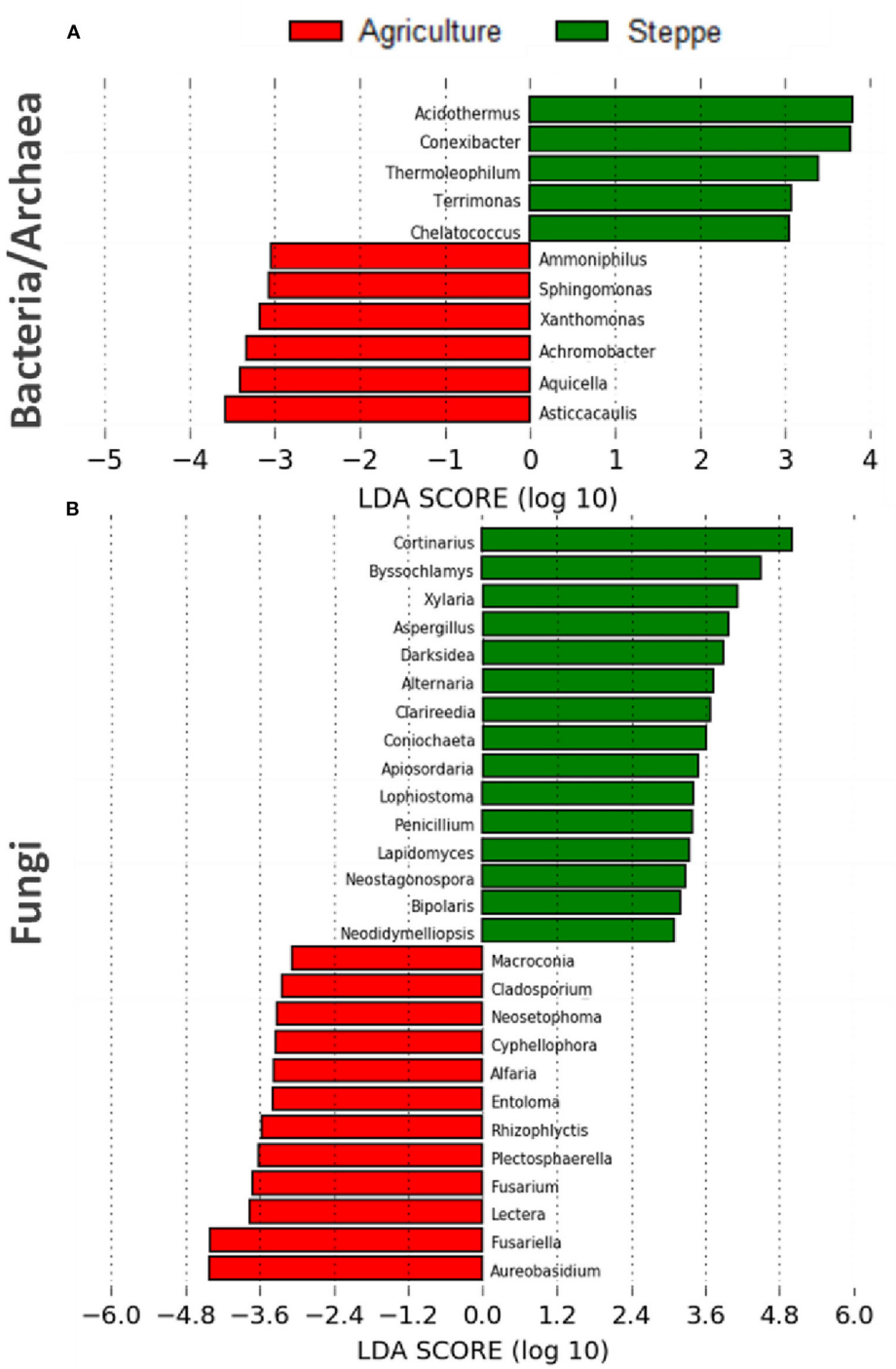


FIGURE 7 | Linear discriminant analysis effect size (LEfSe) analysis showing **(A)** bacterial/archaea and **(B)** fungal microbiota changes between the irrigated agriculture and steppe ($n = 15$).

steppe and decreased in irrigated agriculture. These phyla have been recognized as oligotrophy with a slow growth rate (23, 44) and had been displaced by copiotroph bacteria like *Proteobacteria* and *Bacteroidetes* (45) in the cultivated plots.

Agricultural practices can substantially impact symbiotic species more than saprotrophic fungi (46). According to a study conducted by Chen et al. (47) along a chronosequence after converting a desert area to irrigated agriculture, the shift in the fungal community composition can be extended for decades. While species belonging to *Ascomycota* are mainly ubiquitous, *Basidiomycota* and *Zygomycota* are less abundant in cropping ecosystems (36, 47, 48). The LefSe analyses in the

fungal community showed that *Cortinarius (Basidiomycota)*, the largest and most species-rich genus of ectomycorrhizal fungi in Patagonia (49), appeared as an indicator of the semiarid steppe soil but not of the agricultural sites. Before agriculture adoption, the pristine steppe was characterized by lignified shrubs, putative hosts for *Cortinarius* ectomycorrhizae. Therefore, we infer that the reduction of *Basidiomycota* abundances in irrigated agricultural sites can be related to substituting of autochthonous vegetation, crucial hosts for symbiotic *Basidiomycota* species.

The bacterial biomarkers identified by LEfSe in agricultural soils belong mainly to *Proteobacteria*, which are associated with the soil degradation and decomposition of plant residues (50). These results agree with Bastida et al. (39) and Frenk et al. (51). The bacterial biomarkers associated with the steppe, such as *Terrimonas*, *Acidothermus*, and *Conexibacter*, have been associated with well-developed (52) and enriched soil (50), probably related in our case to the development and evolution of these alluvial soils along the Valley of Río Negro. *Actinobacteria* played a vital role in the desert (12) related to this semiarid steppe environment. These results were confirmed by the heatmaps analysis, showing the most remarkable differences at *Actinobacteria* phylum. On the other side, LefSe analyses in the fungal community showed *Cortinarius* as an indicator of the semiarid steppe soil, which is considered the largest and most species-rich genus of the ectomycorrhizal fungi in Patagonia (49). The steppe was characterized by lignified shrubs, putative hosts for those ectomycorrhizae. Likewise, *Apiosordaria*, *Lophiostoma* and *Penicillium* have been identified as putative

TABLE 4 | Mantel correlation between bacteria/archaea and fungi with soil physicochemical properties.

Soil properties	Bacteria		Fungi	
	Mantel r	***p*-value**	**Mantel r**	***p*-value**
MO	0.1996	0.009	−0.047	0.668
SAR	0.1979	0.004	−0.016	0.546
MWD	0.2941	0.001	0.1923	0.003
pH	0.0093	0.392	0.0778	0.173
CE	−0.006	0.448	0.1083	0.11

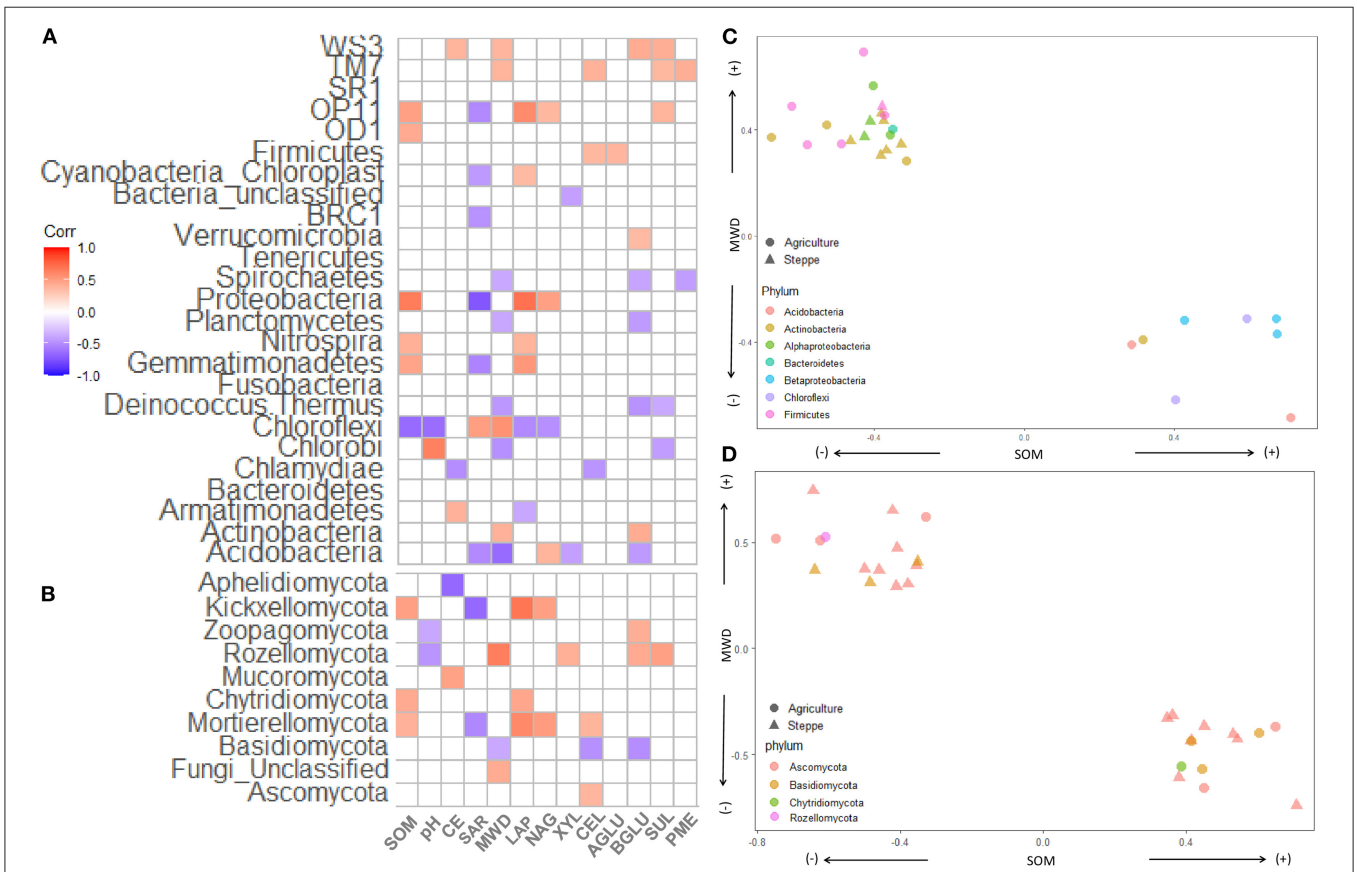


FIGURE 8 | Correlation heatmap of **(A)** bacterial/archaea and **(B)** fungal and soil properties. The shade of color indicates the correlation between relative abundance of each microbiota and soil physical and chemical parameters. **(C,D)** Showing spearman correlation between bacteria/archaea or fungi, respectively, with MWD in the y axis and SOM in the x-axis. SOM, soil organic matter; CE, cation exchange; SAR, sodium absorption ratio; CEL, β-cellobiohydrolase; NAG, N-acetyl-β-glucosaminidase; BGLU, β-glucosidase; AGLU, α-glucosidase; PME, phosphomonoesterase; XYL, XYLanase; LAP, leucine aminopeptidase; SUL, arylsulfatase.

drought tolerance endophytes (53–55) were also indicators of the steppe soil. On the other hand, the differential abundance of fungi in the irrigated agriculture situation was represented by taxa belonging to generalist saprotrophic genera such as *Fusarium*, commonly found in croplands (48, 56). The improvement in soil water conditions under irrigated fields was also indicated by the significant increase of *Rhizophlyctis* abundance, which augmented sporulation in aqueous soil extracts (57).

Bacteria and Fungi have been described as the critical factor in soil aggregation, particularly at the macroaggregate (2000-250 mm) level (9). Soil aggregation is an important property involved in C sequestration and stabilization (2), where macro-aggregates enhanced these processes, either protecting C stock from degradation (58) or creating the niche for C transformation and final uptake (3). Soil aggregation, measured by Mean Weight Diameter (MWD), was more important than other properties in the shaping of bacterial and fungal communities. Regarding bacteria, *Chloroflexi*, *Actinobacteria*, *WS3* and *TM7* taxa were positively associated with soil aggregation, in agreement with a previous works that showed a significant role of *Actinobacteria* and *Proteobacteria* in soil aggregate formation (9). Edaphic properties are among the main drivers of fungal diversity and community composition (59). For example, moderately acidic soils have been identified as more favorable for saprotrofic (59) and pathogenic fungi (60). Fungal hyphae adhere to and physically entangle soil particles and stabilize micro-and macro-aggregates by releasing extracellular compounds (61). *Basidiomycota* displayed an insufficient capacity to form aggregates compared to other main phyla due to their low, dense mycelia (10). Hence, this trait can be associated with the negative correlation between MWD and *Basidiomycota* observed in this study.

In our study, we found negative correlations between soil pH and *Rozellomycota* and *Zoopagomycota* abundances. Considering that both phyla are mainly obligate mycoparasites and predators of nematode, amebae and protists (62), they might have responded indirectly to habitat preferences of host organisms. Probably, the shift within the whole fungal community driven by specific mycoparasitism by *Rozellomycota* may have reduced the secretion of fungal compounds that detrimentally affect soil aggregation (10, 63). However, in the absence of direct experimental evidence, we cannot explain the positive associations between SOM and the saprotrophic decomposers phyla *Kickxellomycota*, *Chitridiomycota*, and *Morteriellomycota*. Despite our observations being in line with previous studies in agricultural soils (64, 65), the causal mechanism behind the correlations remains uncertain, and further evidence is necessary to disentangle the role of minor phyla in soil C cycling, EPS synthesis and aggregate formation.

CONCLUSIONS

Agricultural systems adopting no-tillage, cover crops, and crop rotations, and in this particular case, sustained by irrigation, improved ecosystem biodiversity and soil organic matter content. Consequently, enhanced natural soil fertility and the biodiversity of microbial communities. Our detection of particular taxa related to those soil improvements suggests that similar analyses worldwide would help finding new soil health indicators based on biological data obtained from the study of the soil microbiome in agricultural systems. Understanding the microbial and biochemical soil changes associated with agriculture managements would help find keystone microbial markers and soil biological indicators that would allow farmers to make decisions on the sustainability of the productive processes while keeping soil healthy.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, SAMN23482046.

AUTHOR CONTRIBUTIONS

JF: bacteria/archaea community analysis and correlations between soil variables—manuscript writing. VF: fungi community analyses—contribution to manuscript writing. JC and DR: soil lipids determination and data analysis. LG: soil sampling, soil enzymes activities determination and data analysis, and manuscript revision. MG: field assay supervision, chemical analyses, and guide for soil sampling. LW: general idea and study design, laboratory analyses and data supervision, discussion of results, and Manuscript writing and edition. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants PUNQ EXPTE 1306/19 and project PICT 2803/17 of the Argentinean National Agency for Scientific and Technological Promotion (ANPCyT). LW and LG are members of the Argentinean National Council for Scientific and Technical Research (CONICET).

ACKNOWLEDGMENTS

The authors are especially grateful to Chacra VINPA-AAPRESID in Argentina, that manage the agricultural production by irrigation and allowed this on farm study, contributing with the chemical soil data. Farms Kaitaco, La Julia and Chocorí also contributed with this work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsoil.2022.835849/full#supplementary-material

REFERENCES

1. de Vries FT, Liiri ME, Bjørnlund L, Bowker MA, Christensen S, Setälä HM, et al. Land use alters the resistance and resilience of soil food webs to drought. *Nat Clim Chang.* (2012) 2:276–80. doi: 10.1038/nclimate1368
2. Cotrufo MF, Soong JL, Horton AJ, Campbell EE, Haddix ML, Wall DH, et al. Formation of soil organic matter via biochemical and physical pathways of litter mass loss. *Nat Geosci.* (2015) 8:776–9. doi: 10.1038/ngeo2520
3. Liang C, Kästner M, Joergensen RG. Microbial necromass on the rise: the growing focus on its role in soil organic matter development. *Soil Biol Biochem.* (2020) 2020:108000. doi: 10.1016/j.soilbio.2020.108000
4. Liang BC, VandenBygaart AJ, MacDonald JD, Cerkowniak D, McConkey BG, Desjardins RL, et al. Revisiting no-till's impact on soil organic carbon storage in Canada. *Soil Tillage Res.* (2020) 198:104529. doi: 10.1016/j.still.2019.104529
5. Villarino SH, Pinto P, Jackson RB, Piñeiro G. Plant rhizodeposition: A key factor for soil organic matter formation in stable fractions. *Sci Adv.* (2021) 7:eabd3176. doi: 10.1126/sciadv.abd3176
6. Bot A, Benitez J. *The Importance of Soil Organic Matter. Key to Drought-Resistant Soil and Sustained Food Production*. Rome: FAO Soils Bulletin (2005).
7. Costa OYA, Raaijmakers JM, Kuramae EE. Microbial extracellular polymeric substances: ecological function and impact on soil aggregation. *Front Microbiol.* (2018) 9:1636. doi: 10.3389/fmicb.2018.01636
8. Erktan A, Or D, Scheu S. The physical structure of soil: Determinant and consequence of trophic interactions. *Soil Biol Biochem.* (2020) 2020:107876. doi: 10.1016/j.soilbio.2020.107876
9. Lehmann A, Zheng W, Rillig MC. Soil biota contributions to soil aggregation. *Nat Ecol Evol.* (2017) 1:1828–35. doi: 10.1038/s41559-017-0344-y
10. Lehmann A, Zheng W, Ryo M, Soutschek K, Roy J, Rongstock R, et al. Fungal traits important for soil aggregation. *Front Microbiol.* (2020) 10:2904. doi: 10.3389/fmicb.2019.02904
11. Hector A, Bagchi R. Biodiversity and ecosystem multifunctionality. *Nature.* (2007) 448:188–90. doi: 10.1038/nature05947
12. Wagg C, Dudenhöffer J, Widmer F, van der Heijden MGA. Linking diversity, synchrony and stability in soil microbial communities. *Func Ecol.* (2019) 32:1280–92. doi: 10.1111/1365-2435.13056
13. Domeignoz-Horta LA, Pold G, Liu XJA, Frey SD, Melillo JM, et al. Microbial diversity drives carbon use efficiency in a model soil. *Nat Comm.* (2020) 11:1–10. doi: 10.1038/s41467-020-17502-z
14. Hartman K, van der Heijden MGA, Wittwer RA, et al. Cropping practices manipulate abundance patterns of root and soil microbiome members paving the way to smart farming. *Microbiome.* (2018) 6:14. doi: 10.1186/s40168-017-0389-9
15. Aguiar MR, Paruelo JM, Sala OE, Lauenroth WK. Ecosystem responses to changes in plant functional type composition: An example from the Patagonian steppe. *J. Veget. Sci.* (1996) 7:381–90. doi: 10.2307/3236281
16. Jackson ML. *Análisis Químico de Suelos, 1st ed.*; Omega SA: Barcelona, España (1976).
17. Neumann D, Heuer A, Hemkemeyer M, Martens R, Tebbe CC. Response of microbial communities to long-term fertilization depends on their microhabitat. *FEMS ME.* (2013) 86:71–84. doi: 10.1111/1574-6941.12092
18. van Bavel CHM. Mean Weight-Diameter of soil aggregates as a statistical index of aggregation. *Soil Sci Soc Am J.* (1950) 14:20–3. doi: 10.2136/sssaj1950.03615995001400C0005x
19. Marx MC, Wood M, Jarvis SC. A microplate fluorometric assay for the study of enzyme diversity in soils. *Soil Biol Biochem.* (2001) 33:1633–40. doi: 10.1016/S0038-0717(01)00079-7
20. Truong C, Gabbarini LA, Corrales A, Mujic AB, Escobar JM, Moretto A, et al. Ectomycorrhizal fungi and soil enzymes exhibit contrasting patterns along elevation gradients in southern Patagonia. *New Phytol.* (2019) 222:1936–50. doi: 10.1111/nph.15714
21. DeForest JL. The influence of time, storage temperature, and substrate age on potential soil enzyme activity in acidic forest soils using MUB-linked substrates and L-DOPA. *Soil Biol Biochem.* (2009) 41:1180–6. doi: 10.1016/j.soilbio.2009.02.029
22. Ferrari AE, Ravnskov S, Wall LG. Crop rotation in no-till soils modifies the soil fatty acids signature. *Soil Use Manag.* (2018) 34:427–36. doi: 10.1111/sum.12440
23. Fierer N, Bradford MA, Jackson RB. Toward an ecological classification of soil bacteria. *Ecology.* (2007) 88:1354–64. doi: 10.1890/05-1839
24. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol.* (2009) 75:7537–741. doi: 10.1128/AEM.01541-09
25. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics.* (2011) 27:2194–200. doi: 10.1093/bioinformatics/btr381
26. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics.* (2010) 26:2460–1. doi: 10.1093/bioinformatics/btq461
27. Nilsson RH, Larsson, K.-H., Taylor AFS, Bengtsson-Palme J, Jeppesen TS, et al. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Res.* (2018) 47:D259–64. doi: 10.1093/nar/gky1022
28. Oksanen J, Guillaume Blanchet F, Friendly M, Kindt R, Legendre P, McGlinn D, et al. *Vegan: Community Ecology Package*. R package Version 2.4-3 (2017). Available online at: https://CRAN.R-project.org/package=vegan
29. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* (2010) 26:139–40. doi: 10.1093/bioinformatics/btp616
30. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. *Genome Biol.* (2011) 12:R60. doi: 10.1186/gb-2011-12-6-r60
31. Chen Y, Xu Z, Feng K, Yang G, Fu W, Chen B. Nitrogen and water addition regulate soil fungal diversity and co-occurrence networks. *J Soil Sediments.* (2020) 20:3192–203. doi: 10.1007/s11368-020-02629-9
32. Sun R, Dsouza M, Gilbert JA. Fungal community composition in soils subjected to long-term chemical fertilization is most influenced by the type of organic matter. *Environ Microbiol.* (2016) 18:5137–50. doi: 10.1111/1462-2920.13512
33. García-Delgado C, Barba-Vicente V, Marín-Benito JM, Igual J, Sánchez-Martín MJ, Rodríguez-Cruz SM. Influence of different agricultural management practices on soil microbial community over dissipation time of two herbicides. *Sci Total Environ.* (2019) 646:1478–88. doi: 10.1016/j.scitotenv.2018.07.395
34. Vukicevich E, Lowery T, Bennett JA, Hart M. Influence of groundcover vegetation, soil physicochemical properties, and irrigation practices on soil fungi in semi-arid vineyards. *Front Ecol Evol.* (2019) 7:1–10. doi: 10.3389/fevo.2019.00118
35. Nie S, Lei X, Zhao L. Fungal communities and functions response to long-term fertilization in paddy soils. *Appl Soil Ecol.* (2018) 130:251–8. doi: 10.1016/j.apsoil.2018.06.008
36. Yang X, Ma L, Ji L. Long-term nitrogen fertilization indirectly affects soil fungi community structure by changing soil and pruned litter in a subtropical tea (*Camellia sinensis* L.) plantation in China. *Plant Soil.* (2019) 444:409–26. doi: 10.1007/s11104-019-04291-8
37. Zhu L, Wang X, Chen F, Li C, Wu L. Effects of the successive planting of Eucalyptus urophylla on soil bacterial and fungal community structure, diversity, microbial biomass, enzyme activity. *Degrad Dev.* (2019) 30:636–46. doi: 10.1002/ldr.3249
38. Ning Q, Chen L, Zhang C. Saprotrophic fungal communities in arable soils are strongly associated with soil fertility and stoichiometry. *Appl Soil Ecol.* (2021) 159:103843. doi: 10.1016/j.apsoil.2020.103843
39. Bastida F, Torres IF, Romero-Trigueros C, Baldrian P, Větrovský T, et al. Combined effects of reduced irrigation and water quality on the soil microbial community of a citrus orchard under semi-arid conditions. *Soil Biol Biochem.* (2017) 104:226–37. doi: 10.1016/j.soilbio.2016.10.024
40. Mavrodi DV, Mavrodi OV, Elbourne LDH, Tetu S, Bonsall RF, Parejko J, et al. Long-term irrigation affects the dynamics and activity of the wheat rhizosphere microbiome. *Front Plant Sci.* (2018) 9:345. doi: 10.3389/fpls.2018.00345
41. Gao Y, Xu X, Ding J, Bao F, De Costa YG, Zhuang W, et al. The responses to long-term water addition of soil bacterial, archaeal,

and fungal communities in a desert ecosystem. *Microorganisms.* (2021) 9:981. doi: 10.3390/microorganisms9050981
42. Zhang H, Wu C, Wang F, Wang H, Chen G. Wheat yellow mosaic enhances bacterial deterministic processes in a plant-soil system. *Sci. Total Environ.* (2021) 2021:151430. doi: 10.1016/j.scitotenv.2021.151430
43. Zhang L, Xie Z, Zhao R, Zhang Y. Plant, microbial community and soil property responses to an experimental precipitation gradient in a desert grassland. *Appl Soil Ecol.* (2018) 127:87–95. doi: 10.1016/j.apsoil.2018.02.005
44. Dang Q, Tan W, Zhao X, Li D, Li Y, Yang T, et al. Linking the response of soil microbial community structure in soils to long-term wastewater irrigation and soil depth. *Sci Total Environ.* (2019) 688:26–36. doi: 10.1016/j.scitotenv.2019.06.138
45. Hartmann M, Brunner I, Hagedorn F, Bardgett RD, Stierli B, Herzog C, et al. A decade of irrigation transforms the soil microbiome of a semi-arid pine forest. *Mol Ecol.* (2017) 26:1190–206. doi: 10.1111/mec.13995
46. Liao L, Wang X, Wang J, Liu G, Zhang C. Nitrogen fertilization increases fungal diversity and abundance of saprotrophs while reducing nitrogen fixation potential in a semiarid grassland. *Plant Soil.* (2021) 465:515–32. doi: 10.1007/s11104-021-05012-w
47. Chen J, Wu Q, Li S, Ge J, Liang C, Qin H, et al. Diversity and function of soil bacterial communities in response to long-term intensive management in a subtropical bamboo forest. *Geoderma.* (2019) 354:113894. doi: 10.1016/j.geoderma.2019.113894
48. Wang JT, Shen JP, Zhang LM. Generalist taxa shape fungal community structure in cropping ecosystems. *Front Microbiol.* (2021) 12:e678290. doi: 10.3389/fmicb.2021.678290
49. Truong C, Mujic AB, Healy R. How to know the fungi: combining field inventories and DNA-barcoding to document fungal diversity. *New Phytol.* (2017) 214:913–9. doi: 10.1111/nph.14509
50. Fu H, Li H, Yin P, Mei H, Li J, Zhou P. Integrated application of rapeseed cake and green manure enhances soil nutrients and microbial communities in tea garden soil. *Sustainability.* (2021) 13:2967. doi: 10.3390/su13052967
51. Frenk S, Hadar Y, Minz D. Resilience of soil bacterial community to irrigation with water of different qualities under Mediterranean climate. *Environ Microbiol.* (2014) 16:559–69 doi: 10.1111/1462-2920.12183
52. Sánchez-Marañón M, Miralles I, Aguirre-Garrido JF, Anguita-Maeso M, Millán V, Ortega R, et al. Changes in the soil bacterial community along a pedogenic gradient. *Sci Rep*. (2017) 7:14593. doi: 10.1038/s41598-017-15133-x
53. Murphy BR, Martin Nieto L, Doohan FM, Hodkinson TR. Fungal endophytes enhance agronomically important traits in severely drought-stressed barley. *J Agron Crop Sci.* (2015) 201:419–27. doi: 10.1111/jac.12139
54. Sadeghi F, Samsampour D, Askari Seyahooei M, Bagheri A, Soltani J. Fungal endophytes alleviate drought-induced oxidative stress in mandarin (*Citrus reticulata* L.): Toward regulating the ascorbate–glutathione cycle. *Sci Hortic.* (2020) 261:108991. doi: 10.1016/j.scienta.2019.108991
55. Soares Fortes NG, dos Santos MAL, Vitoria NS. Apiosordaria nigeriensis (Ascomycota): a new record for the Americas. *Rodriguesia.* (2020) 71:1092. doi: 10.1590/2175-7860202071092
56. Panelli S, Capelli E, Comandatore F, Landinez-Torres A, Granata MU, Tosi S, et al. A metagenomic-based, cross-seasonal picture of fungal consortia associated with Italian soils subjected to different agricultural managements. *Fungal Ecol.* (2017) 30:1–9. doi: 10.1016/j.funeco.2017.07.005
57. Willoughby LG. The activity of Rhizophlyctis rosea in soil: Some deductions from laboratory observations. *Mycologist.* (2001) 15:113–7. doi: 10.1016/S0269-915X(01)80032-X
58. Six J, Bossuyt H, Degryze S, Denef K. A history of research on the link between (micro) aggregates, soil biota, and soil organic matter dynamics. *Soil Till Res.* (2004) 79:7–31. doi: 10.1016/j.still.2004.03.008
59. Tedersoo L, Bahram M, Põlme S, Kõljalg U, Yorou NS, Wijesundera R, et al. Global diversity and geography of soil fungi. *Science.* (2014) 346:e1256688. doi: 10.1126/science.1256688
60. Lekberg Y, Arnillas CA, Borer ET, Bullington LS, Fierer N, Kennedy PG, et al. Nitrogen and phosphorus fertilization consistently favor pathogenic over mutualistic fungi in grassland soils. *Nat Comm.* (2021) 12:1–8. doi: 10.1038/s41467-021-23605-y
61. Lehmann A, Rillig MC. Understanding mechanisms of soil biota involvement in soil aggregation: A way forward with saprobic fungi? *Soil Biol Biochem.* (2015) 88:298–302. doi: 10.1016/j.soilbio.2015.06.006
62. Tedersoo L, Sánchez-Ramírez S, Kõljalg U, Bahram M, Döring M, Schigel D, et al. High-level classification of the Fungi and a tool for evolutionary ecological analyses. *Fungal Divers.* (2018) 90:135–59. doi: 10.1007/s13225-018-0401-0
63. Walker JK, Cohen H, Higgins LM, Kennedy PG. Testing the link between community structure and function for ectomycorrhizal fungi involved in a global tripartite symbiosis. *New Phytol.* (2014) 202:287–96. doi: 10.1111/nph.12638
64. Jiao S, Lu Y. Abundant fungi adapt to broader environmental gradients than rare fungi in agricultural fields. *Glob Chang Biol.* (2020) 26:4506–20. doi: 10.1111/gcb.15130
65. Orrù L, Canfora L, Trinchera A, Migliore M, Pennelli B, Marcucci A, et al. How tillage and crop rotation change the distribution pattern of fungi. *Front Microbiol.* (2021) 12:1469. doi: 10.3389/fmicb.2021.634325

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Soil Science

ORIGINAL RESEARCH
published: 14 March 2022
doi: 10.3389/fsoil.2022.838497

Long Term Influence of Fertility and Rotation on Soil Nitrification Potential and Nitrifier Communities

*Sierra S. Raglin, Chinmay Soman†, Yanjun Ma† and Angela D. Kent**

Microbial Ecology Laboratory, Department of Natural Resources and Environmental Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, United States

OPEN ACCESS

Edited by:
Patricia Dorr De Quadros, University of Waterloo, Canada

Reviewed by:
Bharati Kollah, Indian Institute of Soil Science (ICAR), India
Yang Ouyang, University of Oklahoma, United States
Shigeto Otsuka, The University of Tokyo, Japan

****Correspondence:***
Angela D. Kent
akent@illinois.edu

†Present addresses:
Chinmay Soman, EarthSense, Inc., Urbana, IL, United States
Yanjun Ma, DeWitt, LLP, Madison, WI, United States

Specialty section:
This article was submitted to Soil Biology, Ecosystems and Biodiversity, a section of the journal Frontiers in Soil Science

Received: *17 December 2021*
Accepted: *07 February 2022*
Published: *14 March 2022*

Citation:
Raglin SS, Soman C, Ma Y and Kent AD (2022) Long Term Influence of Fertility and Rotation on Soil Nitrification Potential and Nitrifier Communities. Front. Soil Sci. 2:838497. doi: 10.3389/fsoil.2022.838497

The agricultural imprints on soil microbial processes manifest at various timescales, leaving many temporal patterns to present slowly. Unfortunately, the lack of long-term continuous agricultural field sites in North America has left gaps in our understanding of agricultural management on biogeochemical processes and their controlling microbiota. Nitrification, ammonium oxidation by bacteria and archaea, is a critical control point in terrestrial nitrogen fluxes by oxidizing cationic ammonium to anionic nitrate, promoting nitrate leaching. Moreover, nitrous oxide is produced during nitrification, contributing to massive nitrous oxide emissions from fertilized agroecosystems. Nitrification is sensitive to many macro and micro-ecological filters, as nitrifiers are obligate aerobes and are sensitive to numerous non-growth substrates and metal ions. This study sought to understand the long-term implications of various rotation and fertilizer regimes on nitrification potential and nitrifying bacterial communities in the Morrow Plots (Urbana, IL). The Morrow Plots was established in 1876 and are the longest continuous field experiments in North America, making it the only site in America capable of assessing the impact of over 140 years of agricultural management on nitrification. The Morrow Plots contrasts fertilizer (manure, inorganic, unfertilized) and rotation (continuous corn, corn-soy, corn-oat alfalfa), allowing us to explore how conventional vs. regenerative agriculture practices impact nitrifier communities. The results of this study suggest that fertilizer and rotation interact to promote distinct bacterial nitrifier communities. Nitrification potential is highest in manure corn-oat-alfalfa plots, suggesting ammonium availability is not solely responsible for active nitrifier communities. Various soil chemical variables, like CEC, Mg, and Ca, significantly influenced nitrifier community beta-diversity, using 16S rRNA amplicon sequencing, suggesting long-term accumulation of specific cations diverge microbial community assembly. While this study only uses nitrification potential enzyme activity instead of isotope analyses, it sheds light on the importance of various physiochemical drivers on nitrification potential and communities. The results support the need for a more precise exploration of the mechanisms controlling field-scale nitrification rates over large temporal scales. Put together, this study supports the importance of long-term field sites for understanding agricultural manipulations of microbial biogeochemical cycling and sheds light on the micronutrients influencing nitrifier communities and potential activity.

Keywords: crop rotation, microbial community composition, nitrification, fertilizer treatment, ammonium oxidizing bacteria (AOB), ammonium oxidizing archaea (AOA), nitrite-oxidizing bacteria (NOB)

INTRODUCTION

Soil nitrification, the aerobic oxidation of NH_4^+ to NO_2^- and NO_3^-, is a critical control point in terrestrial nitrogen (N) cycling by modulating N-loss capacity from fertilized soils. Agronomic management practices may directly impact nitrifiers, through manipulation of nitrifier growth, or indirectly through the alteration in their soil physiochemical habitat. However, soil matrix integrity shifts temporally as the impacts of management on soil structure manifest slowly over decades (1). The lack of long-term agricultural experiments within North America which contrast regenerative (organic fertilizers and tri-rotational regimes) and intensive (synthetic fertilizers and monocultures) practices, limits the ability to investigate long-term management-driven shifts in nitrifying communities and activities. The Morrow Plots long-term agricultural experiment, established in 1867 at the University of Illinois Urbana-Champaign, contrasts conventional with regenerative practices presenting the opportunity to study the relationship between soil nitrifying potential, agronomic management strategies, and soil physiochemical factors. Long-term factorial experiments can be used to evaluate the impacts of fertilizer and rotational management strategies on nitrogen biogeochemistry in order to understand if nitrogen losses in industrial systems can be mitigated with regenerative practices. Understanding anthropogenic disruption in nitrification will assist in the optimization of agronomic management strategies, ensuring the sustainability and protection of soil resources.

Nitrifying microorganisms consists of ammonium oxidizing bacteria (AOB) and archaea (AOA), nitrite-oxidizing bacteria (NOB), and comammox bacteria (2). Nitrification involves the stepwise oxidation of ammonium to nitrite by AOB and AOA, with subsequent oxidation of NO_2^- to NO_3^- by NOB. The primary step is catalyzed by ammonium monooxygenase (AMO), a Cu-containing membrane-bound monooxygenase (3), and is coupled to the reduction of oxygen to water, producing hydroxylamine. Hydroxylamine is further oxidized by hydroxylamine oxidoreductase (HAO), producing NO_2^-. NOB catalyze the second step of nitrification, the oxidation of NO_2^- to NO_3^- *via* nitrite oxidoreductase (NXR). In addition to variations in the enzymatic and coordination chemistry of nitrification redox enzymes, nitrifier genera utilize several different carbon fixation strategies. Nitrifiers use the Calvin-Benson cycle (AOB: *Nitrosomonas;* NOB: *Nitrospirae*), 3-hydroxypropionate−4-hydroxybutyrate cycle (AOA: aerobic *Crenarcheota*), reductive tricarboxylic acid pathway (NOB: *Nitrospirae*), and the dicarboxylate 4-hydroxybutyrate pathway (AOA: anaerobic *Crenarcheota*) (4–6). The variation in environmental sensitivity between enzymes within the carbon fixation pathways, as well as energy-generating ammonium oxidations, influence the ecophysiology and species distribution of nitrifying organisms.

Agricultural systems modify nitrogen pools through N fertilizers, and legume regimes (7). However, due to variations in biochemical characteristics of AOB, AOA, NOB, and comammox genera, predicting the response of these microorganisms to agronomic practices is complicated. Shifts in the abundance, diversity, composition, and functional potential of nitrifying microbes have been shown to correlate with soil quality parameters, like temperature (8), soil organic carbon (SOC)/ organic matter (OM) (9), pH (10), cation exchange capacity [CEC; (11)], NH_4^+/NO_3^- (12, 13). Many of these parameters, like SOC/OM, accumulate gradually, as soil forming processes are slow and depend complex geophysical and biotic processes. When management strategies, like monocultures or fertilization, manipulate carbon and nitrogen inputs, soil structure and abiotic characteristics of soil which govern biotic interactions change (14). Therefore, assessing not only nitrifier response to major selective factors, like OM and pH, but also micronutrients, may provide insight regarding nitrifier species responses to long-term disturbances.

Nitrifiers are a particularly sensitive to both large-scale ecological filters and subtle deviations in substrates and micronutrients. Bacterial AMO has the ability to oxidize numerous non-growth substrates such as methane, methanol, benzene, and phenols (15–17). Moreover, ammonium oxidation is sensitive to numerous metal cations, like Zn, Cu, Mg, and Cd. Metal cations also drive soil formation, promoting aggregation through cationic bridging (18). Fertilizer, particularly manure, can be a dominant source of soil metal fluxes, which through time, promote soil stabilization (19, 20). However, they are easily over-applied and toxicity of cationic metals perturbed microbial communities (21, 22). Monitoring the long-term shifts in nitrification and its chemical and physical drivers can illuminate human-driven changes in N-dynamics over a deep temporal scale.

While previous research has identified management-driven changes to the soil microbial community as a whole within the Morrow Plots (23), that study did not evaluate biogeochemical transformations or the microbial functional groups responsible for them. Given the importance of nitrification for environmental quality, the sensitivity of nitrifiers to edaphic factors, and the limited opportunities to study this process in long-term agricultural experiments (particularly one so historic), the goal of this study was to assess how long-term fertilizer and rotational management impact nitrification potential activity (NP), as well as community structure in the Morrow Plots. The Morrow Plots experiment is an agricultural mesocosm for observing management-driven effects on nitrification and functionally important soil microbiota over very long time scales. The Morrow Plots are a particularly advantageous study site as Illinois is situated in a region with a high density of industrial agriculture and is a major contributor to nitrogen loading into the Mississippi River. This experiment allows a glimpse into the future of industrial agriculture on nitrogen loss processes, and the potential for regenerative management practices to mitigate nitrogen losses and transition modern American agricultural to minimally detrimental and environmentally sustainable systems.

METHODS

Study Site—The Morrow Plots

The Morrow Plots, located on the campus of the University of Illinois at Urbana-Champaign, are the oldest continuously maintained agricultural research plots in the United States. The plots were established in 1876 to demonstrate the long-term effects of crop rotation, soil nutrient depletion, and the effects of synthetic and natural fertilizers (24, 25). Briefly, this long-term experiment (**Supplementary Figure 1**) consists of three blocks of crop rotation treatments: continuous corn (*Zea mays*) (C), a 2-year corn and soybean rotation (CS), and a 3-year corn-oats-alfalfa rotation (COA). Each crop rotation block is split into eight plots comprising replicated fertilizer treatments: unfertilized (UF); inorganic fertilizers (IN) with nitrogen (as urea), phosphorus (as P_2O_5), potassium (as K_2O), and limestone; and organic fertilization (OR) with dairy manure, limestone, and phosphorus (**Supplementary Figure 1**). Refer to Aref and Wander (25) or Odell et al. (24) for a more detailed description of the Morrow Plots site (24, 25).

Soil Sampling

Soil samples were collected on June 4 and August 25 in 2015—a year when all the plots were planted in corn. Sampling during an all-corn year controls enables the investigation of long-term effects of the crop-rotation treatments on soil microbial communities, by avoiding the short-term effects of plant-microbe interactions from the annual rotations. Each sample consisted of five cores (1.9 cm dia × 12 cm deep). Bulk soil cores from each plot were placed in sealed plastic bags on ice while in the field and transported back to the lab and processed within 2 h of collection. The cores from each plot were composited and homogenized with a 2 mm sieve. Subsamples from each the composited, homogenized soil sample were processed as appropriate for chemical analyses, nitrification assays, and DNA extraction for soil microbiome analysis.

Soil Chemical Analyses

Soil chemical analyses were conducted by Waypoint Analytical (Champaign, IL). Soil NH4-N and NO3-N were quantified using Lachat QuickChem methods 12-107-06-2-F and 12-107-04-1-J, respectively [Soil, Plant and Water Reference Methods for the Western Region (Mod), 2013]. Soil P, K, S, Fe, K, Mg, Ca, Mn, B, and CEC, as well as percent cation saturations, were measured using Mehlich 3 extraction protocols (Handbook on Reference Methods for Soil Analysis-1999, Soil and Plant Analysis Council, Inc.). Percent organic matter (OM) was quantified using the LOI method, the results of which were used to calculate the estimated-N-released in pounds per acre. Buffer pH (SMB buffer pH) and 1:1 soil pH (pH) were quantified for each sampled (Soil, Plant and Water Reference Methods for the Western Region 2013, 4th Edition).

Potential Nitrification Assay

Soil nitrification potential (NP) was quantified colorimetrically using the Griess-Ilosvay's method (26, 27), originally adapted from Berg and Rosswall (28). Briefly, 5 g of homogenized field soil in 50 mL Falcon tubes was shaken for 5 h at room temperatures after adding 1 mM $(NH_4)_2SO_4$, and 1.5 M sodium chlorate. Each sample had a corresponding control sample which was treated identically, but frozen at −20°C for the 5-h incubation. 2M KCl was added after the incubation, and the tubes were manually shaken, then centrifuged for 2 min at 2,000 RPM. The supernatant was filtered using Whatman 42 filter papers. NO_2-N was measured using a Genesys 20 spectrophometer (Thermo Scientific, Rochester, NY) after adding Griess-Ilosavay reagent (sulfanilamide and N-napthylethyldiamine) at 520 nm. NO_2-N concentration was quantified against a $NaNO_2$-N standard curve. Potential nitrification rates were measured as the change in NO_2-N concentration between the aerated and frozen samples, by the soil gram dry weight (% dry matter) per hour.

DNA Extraction and qPCR

DNA Extraction

Total genomic DNA was extracted from soils using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH), and further purified using cetyl trimethyl ammonium bromide (CTAB) extraction to remove contaminating humic acids (29). DNA concentration was adjusted to 30 ng/μl and subjected 16S rRNA V4 region amplicon sequencing and *amoA* qPCR analyses at the University of Illinois Biotechnology Center (Urbana, IL).

Fluidigm qPCR

Bacterial amoA (*BamoA*) and archaeal amoA (*AmoA*) genes were quantified with fluidigm qPCR, using the amoA-1F (5′-GGGGTTTCTACTGGTGGT-3′), amoA-2R (5′ -CCCCTCKGSAAAGCCTTCTTC−3′) for *BamoA*, and the CrenamoA23f (5′-ATGGTCTGGCTWAGACG-3′) and CrenamoA616r (5′-GCCATCCATCTGTATGTCCA-3′) primers for *AamoA* (30, 31). To increase the amount of template DNA prior to Fluidigm qPCR, a preamplification (specific target amplification; STA) reaction was performed in 5 μl reaction mixtures containing 2× Taqman PreAmp Master Mix (Applied Biosysterms), 0.5 μM of each primer, and 1.25 μl of the DNA template. The STA reaction was performed on an MJ Research Tetrad thermal cycler with the following cycling program: 95°C for 10 min followed by 14 cycles of 95°C for 15 s and 58°C for 4 min. Standards for each gene were mixed and 5-fold diluted from 1×10^5 to 3.2×10^1 copies/μl, and amplified by the STA reaction together with the soil genomic DNA to provide standard curves for Fluidigm qPCR. The STA products were treated by exonuclease to remove excessive primers. For Fluidigm qPCR, 5 μl of sample premix was prepared containing 2× SsoFast Evagreen Supermix with Low Rox (BioRad), 20× DNA Binding Dye Sample Loading Reagent (Fluidigm), and 2.25 μl exonuclease treated products. Five μl of assay mix was prepared containing 2× Assay Loading Reagent (Fluidigm), 1× DNA Suspension Buffer (Teknova), and 50 μM each mixed forward and reverse primer. The sample premix and assay mix were loaded on a 96.96 chip (Fluidigm), and the target genes were amplified on the Fluidigm Biomark HD Real Time PCR system using the following cycling program: 70°C for 40 min, 58°C for 30 s, 95°C for 1 min followed by 30 cycles of 96°C for 5 s, 58°C for 20 s, and followed by dissociation curve. All the samples and standards were analyzed in 12 replicates with

molecular grade water as no template control. The C_T values (cycle threshold) were determined using Fluidigm Real-Time PCR Analysis software version 4.1.3. The copy number of genes per μl was determined for each soil sample by comparison to the standard curve in the assay, and then normalized to ng of DNA.

16S rRNA Gene Amplicon Sequencing

Illumina sequencing was used to target the prokayotic 16S rRNA V4 region for nitrifier community analyses (Illumina, San Diego, CA). Sequencing amplicons were prepared by PCR using a Fluidigm Access Array IFC chip, which allowed simultaneous amplification of each target gene (Fluidigm, San Francisco, CA). Initial reactions were carried out according to a 2-step protocol using Fluidigm-recommended reagent concentrations, and an annealing temperature of 55°C. The first PCR was performed in a 100-μL reaction volume using 2 ng DNA template, and this PCR amplified the target DNA region using the 16S rRNA V4 primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) augmented with Fluidigm-specific amplification primer pads CS1 (5′-ACACTGACGACATGGTTCTACA-3′) and CS2 (5′-TACGGTAGCAGAGACTTGGTCT-3′), producing amplicons that consisted of (2) CS1 Fluidigm primer pad, (3) 16S rRNA forward primer 515F, (4) 16S rRNA V4 amplicon (5) 16S rRNA reverse PCR primer 806R, and (6) CS2 Fluidigm primer pad. A secondary 30-μL PCR used 1 μL of 1:100 diluted product from the first PCR as template, and PCR primers with CS1 and CS2 sequences and Illumina-specific sequencing linkers P5 (5′-AATGATACGGCGACCACCGAGATCT-3′) and P7 (5′-CAAGCAGAAGACGGCATACGAGAT-3′), along with a 10-bp sample-specific barcode sequence, so the final construct consisted of (1) Illumina linker P5, (2) CS1, (3) 515F primer, (4) 16S rRNA V4 amplicon, (5) 806R primer, (6) CS2, (7) sample-specific 10-bp barcode, and (8) the Illumina linker P7. Final amplicons were gel-purified, quantified (Qubit; Invitrogen, Carlsbad CA, USA), combined to the same concentration, and then sequenced from both directions on an Illumina HiSeq 2,500 2 × 250 bp Rapid Run. Fluidigm amplification and Illumina sequencing was conducted at the Roy J. Carver Biotechnology Center (Urbana, IL, USA).

Barcodes were used to assign each sequence to its original sample. After de-multiplexing, paired-end sequences generated for 16S rRNA were merged using software FLASH (Fast Length Adjustment of SHort reads) (32). Quality filtering of fastq files was performed using software in the FASTX-Toolkit (33), which removed sequences with more than 10% bases with quality score lower than 30 and sequences containing ambiguous bases "N" from downstream processing. Filtered sequences were clustered into operational taxonomic units (OTUs) using USEARCH64 and a 97% similarity threshold (33). USEARCH was used to (1) de-replicate sequences and remove singletons; (2) remove chimeras contained in the sequences using GOLD (34) as a reference database; (3) form OTU clusters from sequences that were 97% similar and represent each OTU by representative sequences. The cluster file was converted into an OTU table using functions available in MacQIIME (35). Representative sequences for 16S rRNA OTUs were assigned taxonomic attribution in QIIME with the uclust algorithm (36) using the August 2013 Greengenes database (37) as a reference. Amplicon sequence data for 16S rRNA genes is available for download on the NCBI SRA database at accession number: PRJNA789310 (https://www.ncbi.nlm.nih.gov/sra/PRJNA789310). Nitrifier community diverse was then assessed by subsetting the OTU table based on nitrifier Order: specifically, *Nitrosomonadales* (AOB), Nitrososphaerales (AOA), Nitrospirales (NOB).

Statistical Analyses

All statistical analyses were performed in R Studio statistical software (Version 4.1.2, 2021) (38). All figures were produced using *ggplot2* v. 3.3.5 (39). Two-way ANOVA's, with interaction effects, were conducted to understand the impact of fertility and rotation on potential nitrification rates, qPCR abundances, alpha-diversity metrics (Observed Richness, Chao1, Shannon Diversity index). Potential rates and qPCR abundances were natural log transformed to ensure normality. Shapiro-Wilk test was used to identify deviations in residual variances of all models and calculated with the shapiro.test() function of the *stats* package v. 4.1.1.; $W > 0.9$ was used to indicate normally distributed residual variances. Levene's Test for homogeneity of variances across fertility and rotation groups was calculated using LeveneTest() function of the *car* package v. 3.0-11. Means were separated using Tukey's honestly significant difference test using the HSD.test() function from the *agricolae* package v. 1.3-5. One-way regressions were used to identify significant chemical drivers influencing potential nitrification rates. Shapiro-Wilk's test was used to ensure no violations of regression assumptions, and the regression *P*-values were adjusted using a false-discovery rate test. Outliers of ANOVA and regression models were only removed to assume normality, due to low sample size.

Microbial community data was analyzed using *phyloseq* (40) and *vegan* package v. 2.57 (41). Richness parameters were calculated using *phyloseq* package v. 1.36 (40). The complete OTU table was subset by Nitrifier taxa (Order: *Nitrosomonadales*, *Nitrososphaerales*, *Nitrospirales*). Nitrifier beta-diversity was calculated using a Non-metric Multiscale Dimensional Analysis (NMDS) on the Bray-Curtis dissimilarity matrix using the metaMDS() function of *vegan* package v. 2.57. Chemical variables were fit onto the NMDS matrix to identify significant drivers of matrix structure using the envfit() function from the *vegan* package v. 2.57. PERMANOVA analysis was conducted on the dissimilatory matrix to identify the influence of fertility and rotation on matrix structure using the adonis() function of the *vegan* package v. 2.57.

RESULTS

Long-Term Fertilization and Rotation Impact on Potential Nitrification

The influence of fertility and rotational management strategies on potential nitrification rates and nitrifying microbial communities was assessed. Nitrification data is reported in **Supplementary Table 1**. Two-way ANOVA analyses (**Table 1**) concluded that potential nitrification was significantly influenced by fertility [2-Way ANOVA: $F_{(2, 37)} = 29.8442$, $P < 0.0001$],

TABLE 1 | Two-way analysis of variance (ANOVA) table for model assessing the influence of long-term fertility (MLP, IN, UF) and rotation (CC, CS, COA) on natural log transformed nitrification potential (NP) activity in the Morrow Plots.

	ln(NP) ~ fertility × rotation			
Variables	**SS**	**df**	**F-value**	***P*-value**
(Intercept)	181.733	1	3,551.0966	<0.001
Fertility	3.055	2	29.8442	<0.001
Rotation	0.338	2	3.3028	0.047886
Fertility × rotation	1.151	4	5.6240	0.001221
Residuals	1.894	37		

Type III sums of squares was used. NP was natural log transformed to prevent violations of ANOVA assumptions.

rotation [$F_{(2, 37)} = 3.3028$, $P = 0.0478$], and the interaction of both [2-Way ANOVA: $F_{(4, 37)} = 5.624$, $P = 0.001221$]. The highest nitrification potential was in the MLP-COA plots, followed by IN-CC and IN-COA (**Figure 1**). Regression analyses identified the chemical and physical drivers significantly influencing the transformed nitrification rates (**Figure 2**). NH_4^+ ($R^2 = 0.1347$, $P = 0.0218$), NO_3^- ($R^2 = 0.2453$, $P = 0.0023$), OM ($R^2 = 0.2155$, $P = 0.0034$), Est-N-Released ($R^2 = 0.2155$, $P = 0.0034$), Ca^{+2} ($R^2 = 0.1106$, $P = 0.03801$), Mg^{+2} ($R^2 = 0.1575$, $P = 0.0131$), Na^+ ($R^2 = 0.1574$, $P = 0.0131$), B ($R^2 = 0.2445$, $P = 0.0023$), and CEC ($R^2 = 0.3084$, $P = 0.0009$) positively influenced potential nitrification rates, individually (**Figure 2**; **Table 2**). Copper (Cu^{+2}) content negatively impacted potential nitrification rates ($R^2 = 0.0957$, $P = 0.0516$).

The influence of fertility and rotation on nitrifier microbial communities' abundance was assessed using qPCR of *BamoA* and *AamoA* genes (**Figure 3**; **Table 3**). Similar to the potential nitrification rates, *BamoA* gene copy number was significantly influence by fertilizer [2-Way ANOVA: $F_{(2, 36)} = 56.4657$, $P < 0.0001$], and the interaction of fertility and rotation [2-Way ANOVA: $F_{(4, 35)} = 9.1299$, $P < 0.0001$], but not by rotation [$F_{(2, 36)} = 0.3767$, $P = 0.6888$]. The highest *BamoA* gene copy numbers were within the IN-CC, IN-COA, and MLP-COA treatments (**Figure 3A**). *BamoA* abundances significantly influenced potential rates (**Supplementary Figure 7**), based on a one-way linear regression between natural log-transformed *BamoA* copy number/ng DNA and nitrification potential activity. *AamoA* did not vary by fertility or rotation (**Figure 3B**), and did not influence potential nitrification rates ($P > 0.05$).

Long-Term Fertility and Rotation Impact on Nitrifying Microbial Communities

16S rRNA V4 amplicon sequencing revealed shifts in the nitrifier community alpha and beta diversity. Observed nitrifier richness was significantly influenced by fertilizer [$F_{(2, 36)} = 3.6893$, $P = 0.03487$], and the interaction of fertilizer and rotation [$F_{(2, 36)} = 3.2706$, $P = 0.02188$]. Chao1 richness was not influenced by fertility, rotation, or the interaction of the two. Shannon Diversity Index was significantly impacted by fertility [2-Way ANOVA: $F_{(2, 36)} = 13.97$, $P < 0.0001$]. A pairwise comparison revealed no significant differences between inorganic and manure treatment Shannon values, regardless of rotation. The primary differences in Shannon index values were between inorganic and unfertilized rotations, as well as manure and unfertilized rotations (**Supplementary Figure 6**). While Chao1 index was the only richness index not influenced by fertility and rotation, all three indices influenced potential nitrification rates. Observed richness was nearly significant in positively influencing potential nitrification rates [$F_{(1, 43)} = 3.957$, $P = 0.05206$]. Chao1 [$F_{(1, 43)} = 6.806$, $P = 0.01245$], and Shannon [$F_{(1, 43)} = 14.7883$, $P = 0.0003926$] positively influenced nitrification potential.

Effects of management on beta-diversity was analyzed using non-metric multidimensional scaling (NMDS) of the Bray-Curtis dissimilatory matrix (**Figure 4**), paired with a PERMANOVA analyses (**Table 4**). The NMDS ordination (Stress = 0.1319) was significantly influenced by fertility [PERMANOVA: $R^2_{(2, 36)} = 0.344$, $P = 0.001$], rotation [$R^2_{(2, 36)} = 0.11019$, $P = 0.001$] and the interaction of both [$R^2_{(4, 36)} = 0.13939$, $P = 0.001$]. Chemical and physical variables were fit onto the ordination to identify the variables significantly influencing the ordination structure. In agreement with the multiple regressions against the potential nitrification rates, NH_4^+ ($R^2 = 0.1418$, $P = 0.038$), NO_3^- ($R^2 = 0.1474$, $P = 0.034$), OM ($R^2 = 0.3037$, $P = 0.001$), Est-N-Release ($R^2 = 0.3037$, $P = 0.001$), Ca^{+2} ($R^2 = 0.4483$, $P = 0.001$), Mg^{+2} ($R^2 = 0.5245$, $P = 0.001$), B ($R^2 = 0.6175$, $P = 0.001$), Na^+ ($R^2 = 0.3164$, $P = 0.001$), and CEC ($R^2 = 0.3725$, $P = 0.001$) influenced the NMDS ordination (**Table 5**). However, community composition was additionally influenced by soil pH ($R^2 = 0.2110$, $P = 0.007$), S ($R^2 = 0.1729$, $P = 0.022$), Mg-Saturation ($R^2 = 0.1677$, $P = 0.021$), Na-Saturation ($R^2 = 0.2411$, $P = 0.004$), and H-saturation ($R^2 = 0.1743$, $P = 0.015$). Within the bulk soil nitrifier community, AOA within the phylum *Crenarcheota* had the largest abundances (**Figure 5**), yet regression analysis identified no relationship between total abundance of Order *Nitrososphaerales* and NP (**Supplementary Figure 3**; $P > 0.05$). Of the bacterial nitrifiers, the genus *Nitrospira* (NOB) had highest relative abundances within all manure plots, compared to the other fertility treatments (**Supplementary Figure 5**). Linear regression identified Order *Nitrospirales* as positively influencing NP (**Supplementary Figure 4**). *Nitrosovibrio* (AOB) had the highest relative abundances within the inorganic fertilizer treatments, with remarkably lower abundances in the manure and unfertilized plots (**Supplementary Figure 5**). Order *Nitrosomonadales* also significantly influenced NP, but non-linearly, following a non-linear regression (**Supplementary Figure 2**).

DISCUSSION

This study explored the edaphic and management drivers of nitrification potential (NP) and nitrifier community structure in the Morrow Plots long-term agricultural experiment. Fertilizer and rotation significantly influence NP, as well as nitrifier community structure and evenness. The most striking observation was the enriched NP in the MLP-COA treatment, when compared to IN-CS and IN-COA treatments. The IN

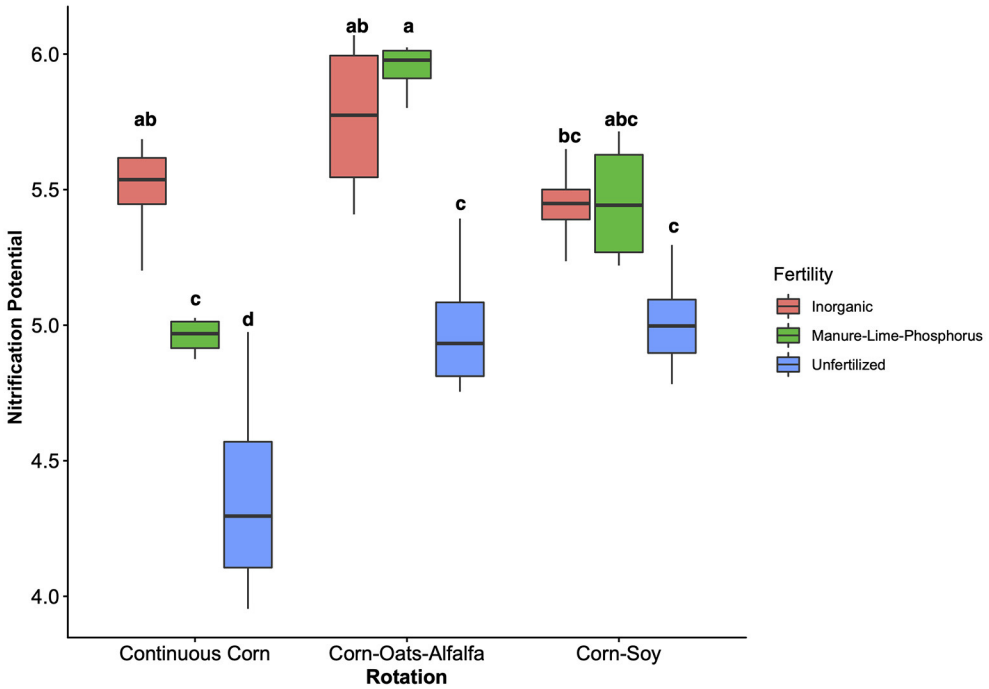


FIGURE 1 | Nitrification potential by management treatment (fertility and rotation). Nitrification potential is measured in μg NO_2-N g^{-1} DM^{-1} hr^{-1} and natural log transformed to assume normality. Tukey's HSD was used for separations of group means, and group membership is indicated via lettering on top of each bar. Model coefficients and *P*-values are reported in **Table 1**. Fertilization and rotational treatments are as follows: manure-lime-phosphorus (MLP), inorganic urea fertilizer (IN), unfertilized (UF); corn-oat-alfalfa (COA), corn-soy (CS), continuous corn (CC).

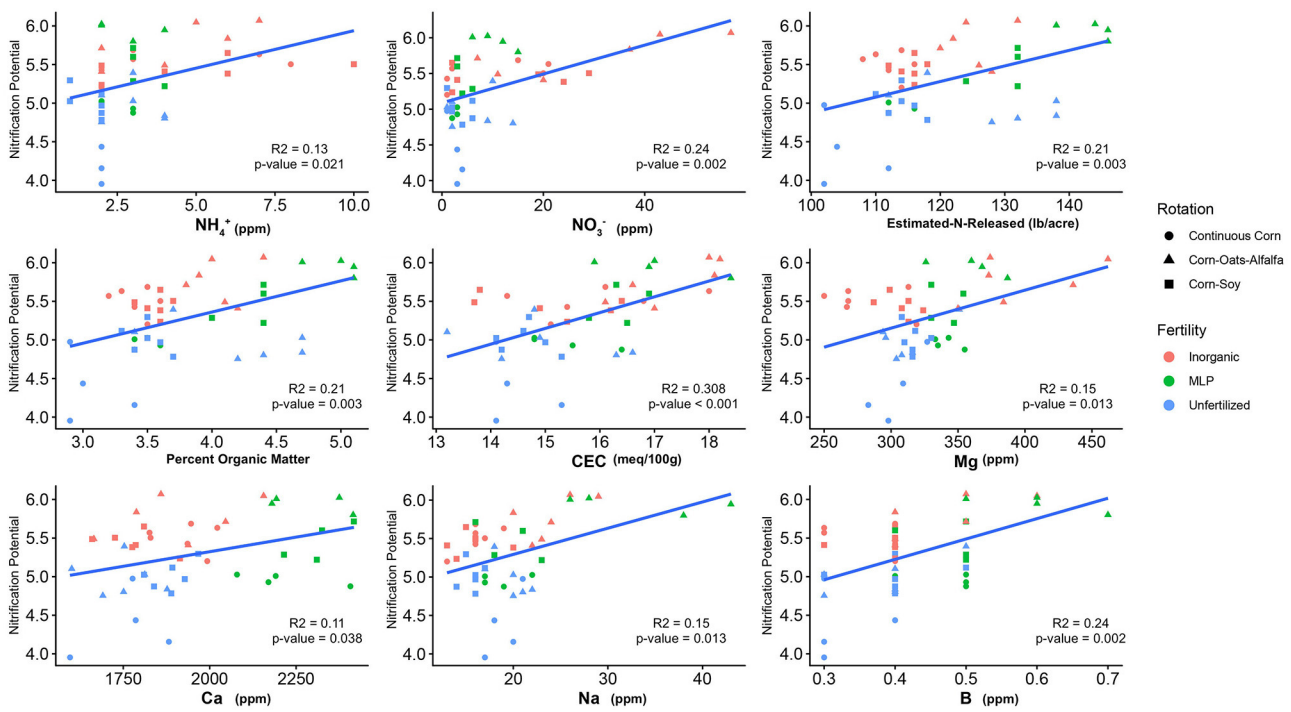


FIGURE 2 | Relationship between nitrification potential and soil edaphic variables. Only edaphic factors which significantly influenced nitrification potential are reported. Model coefficients and *P*-values are reported in **Table 2**.

TABLE 2 | Results of one-way linear regressions assessing the influence of individual edaphic variables on natural-log transformed nitrification potential (NP) activity.

Edaphic factor	Adjusted R^2	*p*-value	FDR *p*-value
NH_4^+	0.1347	0.00699	0.02187
NO_3^-	0.2454	0.00027	0.00235
OM	0.2155	0.00068	0.00341
Est-N-Release	0.2155	0.00068	0.00341
Ca	0.1106	0.01368	0.03801
Mg	0.1575	0.00368	0.01314
B	0.2445	0.00028	0.00235
Na	0.1575	0.00368	0.01315
CEC	0.3085	< 0.0001	0.00092
Cu	0.0957	0.02064	0.05161

Only statistically significant variables are reported below. Due to large number of one-way regressions (25 total, one for each edaphic variable), p-values were adjusted using the p.adjust() function of the stats package version 4.1.1, with a false-discovery rate (FDR) adjustment. Note that after FDR adjustment, Cu is nearly significant (p-value = 0.051). All edaphic variables not listed had a non-significant influence on NP.

fertilizer in the Morrow Plots is urea based; urea is hydrolyzed to NH_3 and CO_2, acting as a source of both energy (ammonium oxidation) and biomass (carbon fixation) yielding substrates (42). However, these results suggest the complexity and diversity of soil physiochemistry under regenerative management promotes nitrifier growth and activity in dynamic, and potentially stimulatory, ways.

Nitrogen losses through soil nitrification have been a major focus in agronomy and microbial ecology for over a century (43–45). Yet, many of the long-term drivers of nitrification remain a major topic of exploration, due to the spatiotemporal variation in NP and nitrifier communities, and the influence of soil type, but also due to the difficulty studying this fastidious functional group. Many studies report AOB as more responsive to anthropogenic perturbations (46, 47). AOB are also reported as disproportionately contributing to soil nitrification activity (12, 47). Identifying the selective agents that determine the tradeoffs between AOB and AOA communities is of major importance for understanding controls on nitrification (48). AOA have higher substrate affinity for NH_4^+ (49), and it is hypothesized that this higher substrate affinity allows AOA to persist at lower NH_4^+ concentrations in oligotrophic environments, resisting the aggressive agriculture-induced variations in NH_4^+ content (50, 51). This may be species-dependent, however as a novel AOA taxon has recently been discovered to withstand NH_4^+-rich environments (52). Additionally, nitrifiers have a wide variety of substrate affinities (53). In this study, Crenarcheota was the largest phylum of nitrifiers, yet *AamoA* gene abundance and AOA (Order *Nitrosophaerales*) total abundance did not influence NP. Additionally, archaeal a*moA* copy number was not significantly influenced by long-term fertilizer or crop rotation treatments. Future research should prioritize identifying and validating the contributions of AOA to agricultural nitrogen biogeochemistry. Due to their lack of response to the long-term agricultural treatments in this study, the remainder of the discussion will focus on AOB and NOB.

Exploring the Impact of Manure on Nitrification

Numerous studies have explored the long-term influence of agronomic management on nitrification and nitrifier communities (12, 54, 55). Large-scale ecological filters, such as organic matter (%OM), pH, and NH_4^+ are among the most well-documented drivers of nitrification rates (56, 57). Our study unsurprisingly identified NH_4^+ as a significant factor influencing NP and nitrifier community composition. The primary step of nitrification is the oxidation of ammonia by AMO, yielding two electrons and reducing O_2 to H_2O, and is the rate-limiting step of nitrification. Many studies have identified NH_4^+ content in soils as a dominant driver of nitrification and nitrifier niche differentiation (2, 46, 48, 58, 59). As stated above, variations in enzymatic affinity to NH_4^+ among AOB, NOB, and comammox microorganisms influence their success in oligotrophic or copiotrophic environments (2, 53). However, NH_4^+ availability is strongly influenced by agricultural management, human-driven manipulations of soil physiochemistry, such as pH and CEC, as well as microbial resource competition. These interconnected processes require more precise methods to analyze and model predictable relationships that can inform soil management, but it is clear that NH_4^+ is a strong predictor of NP in agricultural soils.

Carbon quantity and quality are a fundamental difference induced by management practices (e.g., fertilizer regime and crop rotation) between industrial and regenerative agriculture. Carbon inputs from manure and crop residues promote soil matrix stabilization through increased soil surface area and water holding capacity. This, in turn, maximizes the buffering capacity of the soil matrix, stabilizing pH fluctuations which may alter abiotic and biotic components. pH fluctuations dictate the ratio of NH_3/NH_4^+ (60), which is the primary reason that nitrifiers are consistently reported as sensitive to pH fluctuations. Soil matrix pH levels, in turn, influence AOB community distributions (pH: $R^2 = 0.2110$, $P = 0.007$; H-saturation: $R^2 = 0.1743$, $P = 0.015$). Interestingly, neither soil-pH, nor buffer-pH, significantly influenced NP, suggesting pH drives nitrifier species distributions more than function.

Organic matter incorporation through regenerative practices would also promote heterotrophy and microbial biomass accumulation (61). Labile carbon from manure amendments stimulates microbial biomass and enzyme activity (62). Microbial biomass may promote N-immobilization and could reduce NP (63). Yet, N-mineralization may be stimulated due to the high urea content and lower C:N ratio in manures when compared to the unfertilized treatments (64, 65). However, as the IN treatments are directly fertilized with urea, it is unlikely urease activity, alone, contributed to the high NP in the MLP treatments. Certain *Nitrospira* (NOB/Comammox) possess genes encoding cyanase and urease enzymes (66). The dynamics of cyanate availability have only recently been explored (67), and research suggests that soil microorganisms rapidly consume cyanate, when compared to urea hydrolysis (67). AOB

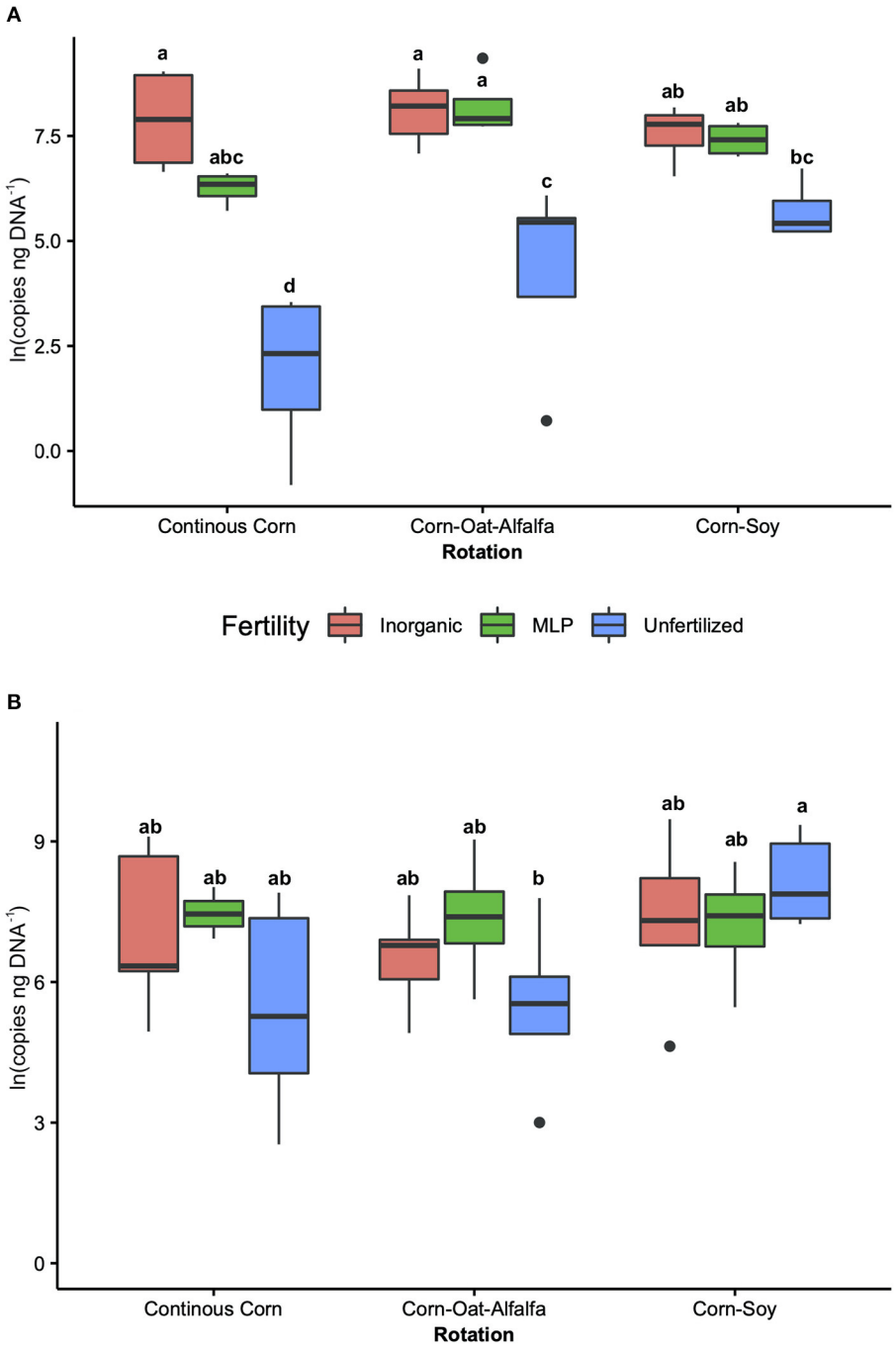


FIGURE 3 | Bacterial (*BamoA*) and archaeal (*AamoA*) shown in **A** and **B**, respectively, compared among long-term management treatments (fertility and rotation). Abundance, determined by *amoA*-specific qPCR, is reported as gene copy number/ng DNA. Tukey's HSD was used for separations of group means, and group membership is indicated via lettering on top of each bar. 2-way ANOVA results are reported in **Table 3**. Fertilization and rotational treatments are as follows: manure-lime-phosphorus (MLP), inorganic urea fertilizer (IN), unfertilized (UF); corn-oat-alfalfa (COA), corn-soy (CS), continuous corn (CC).

can utilize liberated CO_2 and NH_3, a term called "reciprocal feeding" between AOB and *Nitrospira* (66). *Nitrospira* can form symbioses with AOB within biofilms, occupying microsites called nitrification aggregates (68). While reciprocal feeding was not measured in this study, it may contribute to the comparable NP between the MLP-COA and MLP-CS, and the IN-COA and IN-CS treatment, as well as the higher relative abundance of *Nitrospira* in the MLP-COA treatment (69). This

TABLE 3 | Two-way Analysis of Variance (ANOVA) for model assessing the influence of fertility and rotation on quantitative polymerase chain reaction (qPCR) ammonium oxidation gene abundances.

	Bacteria amoA				Archaeal amoA			
Variables	**SS**	**df**	**F-value**	***P***	**SS**	**df**	**F-value**	***P***
Intercept	310.194	1	272.1811	<0.001	202.905	1	57.4623	<0.001
Fertility	128.704	2	56.4657	<0.001	17.074	2	2.4177	0.1035
Rotation	0.859	2	0.3767	0.688	6.699	2	0.9486	0.3968
Fertility × rotation	41.620	4	9.1299	<0.001	16.906	4	1.1969	0.3289
Residuals	41.028	36			127.120	36		

qPCR gene copy number/ng DNA for Bacterial amoA (BamoA) and Archaeal amoA (AmoA) were natural log transformed to assume normality. Outliers were removed to assume normality.

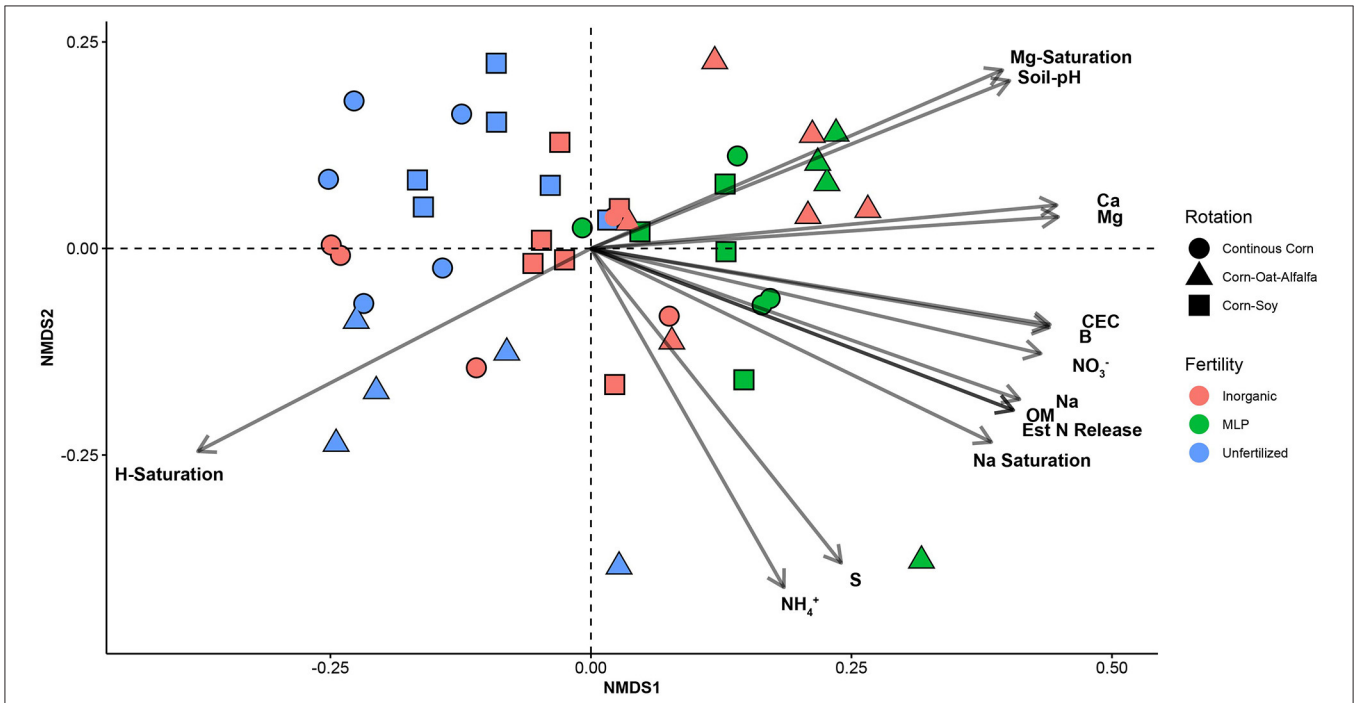


FIGURE 4 | Non-metric multidimensional scaling (NMDS) ordination constructed from a Bray-Curtis dissimilarity matrix of the soil nitrifier community, based on 16S rRNA sequence reads assigned to nitrifier taxa. Edaphic factors were correlated to NMDS1 and NMDS2 using the envfit() function of the *vegan* package in R. Only significant factors are displayed for visual clarity.

study also identified a direct relationship between *Nitrospira* abundance and NP (**Supplementary Figure 4**), determined using 16S rRNA gene sequencing, implicating *Nitrospira* in ammonium oxidation potential.

Ammonium oxidizers require CO_2 for carbon fixation (4, 70). Promotion of not only ammonium oxidation, but also carbon fixation, would benefit nitrifiers, maintaining nitrifier biomass and overall soil nitrification potential (71). Heterotrophic respiration and the release of CO_2 supply nitrifiers with carbon for growth. Additionally, NOB can utilize both the Calvin-Benson cycle and the reductive TCA cycle for carbon fixation (5, 72); the enzymes of the reductive TCA cycle are more sensitive to oxygen, forcing certain NOB to occupy microaerophilic sites in soil (72). This may further promote NOB occupation of biofilms within microsites; shifts in water retention and labile carbon inputs stimulate biofilm formation through exopolysaccharide production (73). Nitrification potential has been reported to be greatest in the clay fraction of soil, suggesting the physiochemical properties of microaggregates (74) compared to macroaggregates, benefit nitrification (75). In biofilm reactors, the nitrification rate was particularly high even at low pH, suggesting that biofilms are ideal environments for autotrophic nitrification (76). Moreover, biomass aggregation was associated with stress avoidance in *Nitrosomonas mobilis* Ms1 and in late stages of aggregation was associated with an upregulation in biosynthesis genes (77). *Nitrosomonadaceae* and *Nitrospiraceae* have recently been characterized as important exopolysaccharide producers under alfalfa regimes cultivated on reclaimed soils (78). As Vuko et al. (78) did not compare alfalfa cultivation to other legumes, it is uncertain if alfalfa has a unique capacity for

TABLE 4 | Permutational analysis of variance (PERMANOVA) table.

Variables	df	SS	MS	F-Value	R^2	*P*-value
Fertility	2	0.54336	0.271679	15.2742	0.34447	0.001
Rotation	2	0.17382	0.086909	4.8861	0.11019	0.001
Fertility × rotation	4	0.21987	0.054968	3.0904	0.13939	0.001
Residuals	36	0.64033	0.017787		0.40594	
Total	44	1.57737			1.00000	

PERMANOVA was conducted using the adonis() function of vegan package version 2.5-7 on nitrifier community Bray-Curtis Dissimilarity matrix to identify significant effect of Fertility and Rotation variables on nitrifier community structure.

TABLE 5 | Influence of edaphic factors on non-metric multidimensional scaling (NMDS) ordination constructed using the 16S rRNA-based nitrifier Bray-Curtis dissimilarity matrix.

Edaphic factor	R	*P*	NMDS1	NMDS2
Soil-pH	0.2110256	0.007	0.8926288	0.45079236
NO3	0.1474599	0.034	0.9592079	−0.28270176
NH4	0.1418022	0.038	0.4115523	−0.91138614
OM	0.3037273	0.001	0.9004432	−0.43497355
Est-N-release	0.3037273	0.001	0.9004432	−0.43497355
Ca	0.4483928	0.001	0.9932289	0.11617369
Mg	0.5245139	0.001	0.9964094	0.08466622
S	0.1729010	0.022	0.5334847	−0.84580971
B	0.6175841	0.001	0.9771081	−0.21274357
Na	0.3614104	0.001	0.9138552	−0.40604032
CEC	0.3725405	0.001	0.9787671	−0.20497542
Mg-saturation	0.1677519	0.021	0.8775176	0.47954446
Na-saturation	0.2411897	0.004	0.8535050	−0.52108468
H-saturation	0.1743390	0.015	0.8382630	−0.54526618

The envfit() function of vegan package version 2.5-7 was used to correlate edaphic variables to NMDS points and ordination structure. Only significant environmental variables are included in the table.

supporting nitrifier biofilm production. Since biofilms contribute to soil structure, as well as microbial microhabitats, future research should expand on the findings of Vuko et al. (78) to investigate long-term management shifts in biofilms and importance for AOB and NOB in agricultural soils.

Micronutrients Influence on Nitrification and Nitrifiers

Long-term field experiments offer unique insight and opportunities to evaluate drivers of soil microorganisms and their activities. Over time, soil physiochemical properties which seem irrelevant to nitrification may emerge as important drivers of nitrifier distribution and function. Nitrifiers are particularly fastidious and are sensitive to micronutrients and non-growth substrates (79), making them a particularly difficult functional guild to study. Organic matter-induced changes in CEC would promote the accumulation of cations, which over time, could influence nitrification (80). In this study, NH_4^+ ($R^2 = 0.1347$, $P = 0.0218$), Ca^{+2} ($R^2 = 0.1106$, $P = 0.03801$), Mg^{+2} ($R^2 = 0.1575$, $P = 0.0131$), and Na^+ ($R^2 = 0.1574$, $P = 0.0131$) positively influenced NP. Prior research identified NH_4^+ as a primary driver of nitrification in soil (8); this is unsurprising, considering ammonium oxidation is the primary and rate-limiting step of nitrification (8). However, few studies assess the impact of additional micronutrients within soils, although engineered systems, such as wastewater treatment plants (WWTP), have explored micronutrient drivers of NP for decades. Therefore, this section will discuss the role of micronutrients on nitrification. It is important to note that the mode of action is not identified during this study, particularly because the biological activity of metal cations is dependent on the form in which these cations exist in the soil matrix, i.e., occluded, exchangeable, organic bound, etc. (81), which is heavily influenced by physiochemical controls such as pH (82). Moreover, many of these cations influence soil aggregation (18), and biofilm formation (83), so it is not clear if they directly act on nitrifier cellular machinery or indirectly through altering their microhabitats. Ultimately, this section serves to explore literature that supports the findings of this study, and to pose avenues of exploration for future research.

Calcium positively influenced NP in this study. The manure treatments were neutralized with the addition of lime ($CaCO_3$/limestone). Nitrifiers can use $CaCO_3$ as a biomass substrate for adherence, as well as a buffer for pH during reactor cultivation (84). The interaction between nitrifiers and calcium promotes high ammonia-removal rates (85). It is also reported that liming promotes CH_4-oxidation, the effect of which is dependent on soil type and acidification (86). Methanotrophs are phylogenetically (87) and enzymatically (15, 88) related to ammonium oxidizers, causing substrate infidelity between ammonium monooxygenase and particulate-methane monooxygenase (88). It is therefore possible that liming also promotes ammonium oxidation, due to similar mechanisms as the liming-induced methane-oxidation stimulation (86), but the mechanism remains inconclusive.

Nitrosovibrio is a genus of AOB isolated from oligotrophic environments, such as building sandstone, and is associated with biodegradation of natural building materials (89). They represent a very small percent of the bulk soil microbial community (ranging from 0.021 to 0.265% relative abundance), but are enriched within all the inorganically fertilized treatments—most particularly the IN-CC (0.262%) and IN-CS (0.265%). The soil within the Morrow Plots is a Flanagan Silt loam formed over calcareous glacial till (25). Presence of a small percentage of *Nitrosovibrio* may indicate soil acidification and promotion of soil erosion, as their presence in building material is associated

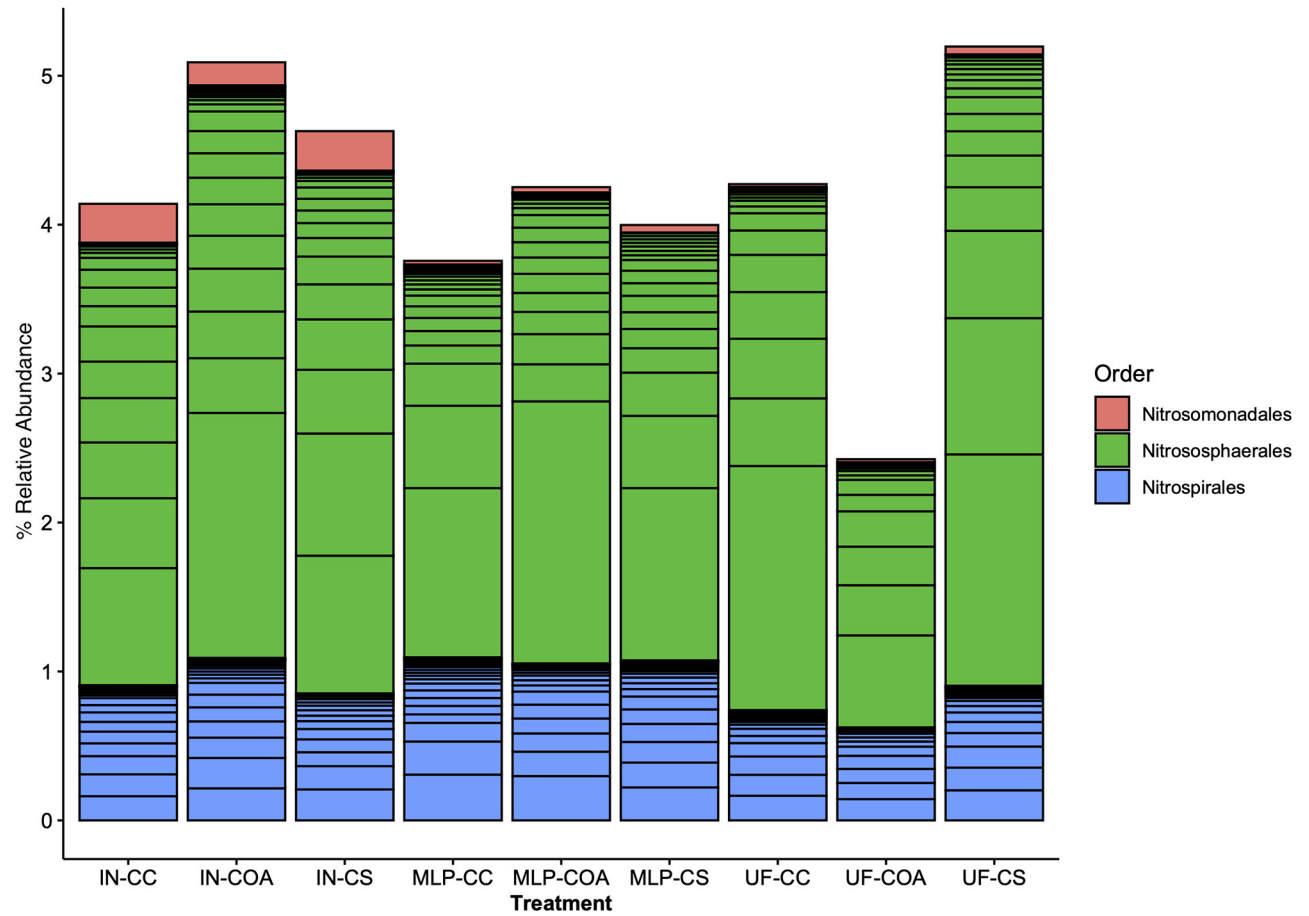


FIGURE 5 | Nitrifier composition compared among long-term agronomic management treatments, represented as percent relative abundance of the total bulk soil 16S rRNA-based community. The bulk soil bacterial 16S rRNA community was subset by nitrifier taxa (AOA, AOB, NOB). Results are represented here with color representing order-level taxonomic classifications: *Nitrosomonadales* (AOB), *Nitrososphaerales* (AOA), *Nitrospirales* (NOB). Fertilization and rotational treatments are as follows: manure-lime-phosphorus (MLP), inorganic urea fertilizer (IN), unfertilized (UF); corn-oat-alfalfa (COA), corn-soy (CS), continuous corn (CC).

with acidification and salt stress of calcareous material. Shi et al. (90) identify soil salt content as a major driver of *Nitrosovibrio* abundance (90). In addition to calcium, Na^{+} was a significant driver of nitrifier community structure ($R^2 = 0.3164$, $P = 0.001$), in agreement with Shi et al. (90), but did not influence NP, suggesting that salt stress enriches specific nitrifying genera.

Magnesium (Mg^{2+}) is present in soils in an exchangeable and mobile form (91). Magnesium influences the coordination chemistry of nucleoside triphosphates and is obligatory for maximum activity of succinyl-CoA synthetase (SCS) activity in *Nitrosomonas europeae* pure culture (92). Succinyl-CoA synthetase (SCS) produces one ATP *via* substrate-level phosphorylation during the TCA cycle (92). In the same study, Cu^{2+} had a strong capacity for SCS inhibition (92). The results of this study agree with Kondo et al. (92). After *P*-values were corrected with a false-discovery test, Cu^{2+} was nearly significant in negatively influencing nitrification potential ($P = 0.051$). While research regarding the inhibitory effect of copper on nitrification has yielded variable results (93), Mertens et al. (94) saw a positive correlation between nitrification inhibition and Cu^{2+}concentrations in soils (94). Cu^{2+} has also been shown to decrease soil urease activity (95, 96), and influence AOB community structure (97). More recently, manure application was shown to increase heavy metal concentrations in soils, with Cu and Cd negatively correlating to net nitrification rates (20). Therefore, it is possible that the high cation exchange capacity in the MLP-COA promotes Cu^{2+}accumulation and negatively influenced nitrifiers, whereas Mg^{2+} positively influence nitrifier growth. It is also important to note that Cu restores the specific growth rate of AOA inhibited by organic carbon substrates in WWTP (98). The differential effect of Cu on AOB and AOA is potentially due to AOA possessing Cu-dominant catalytic centers within electron transport enzymes (99). This implies the negative relationship between Cu and NP in our study is specific to AOB. However, the mode of action cannot be determined with this study.

Sulfur contributes to the activity of numerous redox metalloproteins by complexing with metal ions in the catalytic site (100). For example, numerous nitrogen-cycling metalloenzymes, like assimilatory nitrate reductase, periplasmic nitrate reductase, and the nitrogenase enzyme, have molybdenum

catalytic sites complexed to cysteine ligands (101, 102). While sulfur would be required for the replication of N-cycling redox enzymes, sulfur within the Morrow Plots could contribute to niche differentiation between AOB and AOA. In agreement, sulfur influenced beta-diversity, but not NP ($R^2 = 0.1729$, $P = 0.022$). A recent analysis identified a correlation between sulfatase activity and AOA community abundances, potentially due to their ability to adapt to hypoxic ecosystems, like those seen in marine ecosystems (103). Moreover, in reactors, AOB ammonium oxidization is particularly sensitive to hydrogen sulfide (103, 104). Sulfur was also identified as a significant factor influencing *BamoA* terminal restriction fragment analyses in tropical soils, along with Cu, Na, and B (97). Therefore, agricultural manipulation of sulfur may drive the differentiation of nitrifier community structures.

Finally, our results demonstrate that boron (B) significantly influenced both NP and nitrifier beta-diversity. Boron is an essential micronutrient that is required for cell wall synthesis and proper cellular replication in plants (105), and is important for cyanobacteria heterocyst stability. In fact, B plays a crucial role in legume nodulation, and is important for nodule membrane and cell wall structure, nodule infection, and the development of the symbiosome during legume-rhizobia symbioses (106, 107). In alfalfa, it is particularly important for reproductive phenology and seed quality and yield (108). Regenerative fertilization management approaches often accumulate B through time (109), but B availability can interact with calcium from liming to reduce B assimilation into plant biomass (110). Adsorption of B increases with soil pH (111), reducing B availability with liming due to neutralization of soil pH and complexation with calcium ions (105). The comparatively larger pool of B in the MLP-COA (**Table 2**) could be due to the combination of liming and pH buffering, promoting B accumulation in this treatment. Boron accumulation may influence microbial respiration and N-liberating activity, as B has been shown to increase urease and dehydrogenase activity in soil (112), as well as nitrate reductase activity (113). Boron also has been shown to influence nitrification by increasing nitrifying bacterial populations when applied with molybdenum (Mo), and had a strong effect on nitrification activity when applied without Mo (113). Additional research is required to dissect the synergistic effects of alfalfa rotations, liming, and B accumulation on nitrification and nitrifying microbial communities.

Study Limitations

The most significant limitation of this study is the lack of spatiotemporal resolution. The samples during this preliminary study were collected in June and August, but due to the low number of samples (and lack of in-field replication that reflects modern statistical methods), we could not assess the intra-annual variation in nitrification potential, nitrifier communities, or the edaphic drivers. Biotic factors, such as microbial enzyme potential and community structure, vary significantly spatiotemporally (8, 114, 115). Particularly, N-mineralization (116) and urease enzyme activity (117) increase with temperature, resulting in late-season pulses in these enzyme activities. These two processes contribute to ammonium availability, potentially impacting nitrification potential through time. Moreover, nitrification potential differs among soil particle fractions and depths (118) as well as temperature (119). This highlights the importance of assessing the long-term effect of abiotic variables, in addition to single-season effects, as fertilization and rotational practices significantly alter the physical structure of soil (18). Future sample collection should include a finer scale temporal resolution to understand the interaction of various N-cycling enzyme activity and nitrifier communities.

It is important to note that nitrification potential is not the same metric as field nitrification (120). This is important to distinguish because it is unclear if sustainable practices such as manure fertilization or tri-rotational regimes promote field nitrifier-induced N loss (12). The manipulation of carbon, nitrogen, and the promotion of aggregation (121) influences anaerobic microsites and anaerobic respiration strategies, e.g., denitrification. The presence of crop varieties such as legume species (122) also influences the factors driving field N-loss (56). However, due to the oxygen requirement of nitrification, it is uncertain if the high nitrification *potential* in the MLP-COA corresponds to increased nitrogen loss from these treatments (13, 63, 123, 124). Both denitrification and nitrifier denitrification (125, 126) contribute to global N_2O emissions (127). Recent studies have shed light on dissimilatory nitrate reduction to ammonium (DNRA) as a competitor for available NO_3^-, particularly under rewetted soils high in labile carbon inputs and water-filled pore space (128, 129). It is therefore uncertain whether the high nitrification potential in the MLP-COA treatment increases N_2O emissions through nitrifier denitrification and NO_3^- respiration by denitrifiers, or stimulates DNRA due to an increase in heterotrophic respiration and lowering of soil redox potential (128). These questions require precise analytical methods, such as ^{15}N pool dilutions or soil transcriptomics analyses.

CONCLUSION

This study identified a significant influence of long-term rotation and fertilization on nitrification potential in the Morrow Plots. Surprisingly, the most regenerative management treatment (MLP-COA) possessed the greatest capacity for nitrification. While this study did not employ precise methods like ^{15}N-isotope tracer analyses to pinpoint the N-cycling processes supporting the high nitrification potential, it does point to the influence of numerous abiotic macro- and micronutrients on both nitrification potential and nitrifier community structure. As agronomic management practices greatly alter soil matrix structure through time, the resulting variation in physiochemical parameters may slowly shift nitrifier communities. Moreover, chemical constituents which impact both ammonium oxidation and carbon fixation enzymes could partially explain the distinct nitrifier communities and their resulting activities. Understanding these slow-acting distal drivers of soil nitrification and how they vary through time is critical for predicting

the long-term outcome of agronomic practices on soil health and sustainability.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA789310.

AUTHOR CONTRIBUTIONS

SR: writing and data analysis. AK: writing—review and editing. CS: study design, sampling, and laboratory assays. YM: qPCR and amplicon sequencing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Cooperative State Research, Education, and Extension Service, US Department of Agriculture, under Project Number ILLU 875-374. This work was supported by a UIUC Graduate College Fellowship to SR. CS was supported by the National Science Foundation through the Science, Engineering, and Education for Sustainability Post-Doctoral Fellowship, Award # 1314064. YM was supported by funding from the National Institute of Food and Agriculture, U.S. Department of Agriculture, under Award Number 2015-67019-23584.

ACKNOWLEDGMENTS

We would like to acknowledge Robert Dunker and previous managers and the University of Illinois Agricultural Experiment Station for long-term support and maintenance of the Morrow Plots. We are very grateful to Alonso Favela, Dora Cohen, Maya Scott, Natalie Stevenson, and Dongfang Li for technical assistance with sample collection and laboratory assays.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsoil.2022.838497/full#supplementary-material

REFERENCES

1. Bienes R, Marques MJ, Sastre B, García-Día AZ, Esparza I, Antón O, et al. Tracking changes on soil structure and organic carbon sequestration after 30 years of different tillage and management practices. *Agronomy*. (2021) 11. doi: 10.3390/agronomy11020291
2. Lehtovirta-Morley LE. Ammonia oxidation: ecology, physiology, biochemistry and why they must all come together. *FEMS Microbiol Lett*. (2018) 365:1–9. doi: 10.1093/femsle/fny058
3. Arp DJ, Sayavedra-Soto LA, Hommes NG. Molecular biology and biochemistry of ammonia oxidation by Nitrosomonas europaea. *Arch Microbiol*. (2002) 178:250–5. doi: 10.1007/s00203-002-0452-0
4. Pratscher J, Dumont MG, Conrad R. Ammonia oxidation coupled to CO2 fixation by archaea and bacteria in an agricultural soil. *Proc Natl Acad Sci USA*. (2011) 108:4170–5. doi: 10.1073/pnas.1010981108
5. Alfreider A, Grimus V, Luger M, Ekblad A, Salcher MM, Summerer M. Autotrophic carbon fixation strategies used by nitrifying prokaryotes in freshwater lakes. *FEMS Microbiol Ecol*. (2018) 94:1–12. doi: 10.1093/femsec/fiy163
6. Ren M, Zhang Z, Wang X, Zhou Z, Chen D, Zeng H, et al. Diversity and contributions to nitrogen cycling and carbon fixation of soil salinity shaped microbial communities in Tarim Basin. *Front Microbiol*. (2018) 9:431. doi: 10.3389/fmicb.2018.00431
7. Gelfand I, Robertson GP. Mitigation of greenhouse gases in agricultural ecosystems. In: Hamilton SK, Doll JE, Robertson GP, editors. *The Ecology of Agricultural Landscapes: Long-Term Research on the Path to Sustainability*. New York, NY: Oxford University Press (2015). p. 310–39. Available online at: https://books.google.com/books?hl=en&lr=&id=gPFxBgAAQBAJ&oi=fnd&pg=PA310&dq=info:b60_93ehv_UJ:scholar.google.com&ots=MNm385kwgW&sig=ZVnYvVBc8ngcHNz-T5QN-E1FlTs#v=onepage&q&f=false
8. Ouyang Y, Norton JM, Stark JM. Ammonium availability and temperature control contributions of ammonia oxidizing bacteria and archaea to nitrification in an agricultural soil. *Soil Biol Biochem*. (2017) 113:161–72. doi: 10.1016/j.soilbio.2017.06.010
9. Chen Z, Luo X, Hu R, Wu M, Wu J, Wei W. (2010). Impact of long-term fertilization on the composition of denitrifier communities based on nitrite reductase analyses in a paddy soil. *Microb Ecol*. (2010) 60:850–61. doi: 10.1007/s00248-010-9700-z
10. Barton L, Gleeson DB, Maccarone LD, Zúñiga LP, Murphy DV. Is liming soil a strategy for mitigating nitrous oxide emissions from semi-arid soils? *Soil Biol Biochem*. (2013) 62:28–35. doi: 10.1016/j.soilbio.2013.02.014
11. Robertson GP, Groffman PM. Nitrogen transformations. *Soil Microbiol Ecol Biochem*. (2015) 421–46. doi: 10.1016/B978-0-12-415955-6.00014-1
12. Wang F, Chen S, Wang Y, Zhang Y, Hu C, Liu B. Long-term nitrogen fertilization elevates the activity and abundance of nitrifying and denitrifying microbial communities in an upland soil: implications for nitrogen loss from intensive agricultural systems. *Front Microbiol*. (2018) 9:2424. doi: 10.3389/fmicb.2018.02424
13. Meng L, Ding W, Cai Z. Long-term application of organic manure and nitrogen fertilizer on N2O emissions, soil quality and crop production in a sandy loam soil. *Soil Biol Biochem*. (2005) 37:2037–45. doi: 10.1016/j.soilbio.2005.03.007
14. Gross A, Glaser B. Meta-analysis on how manure application changes soil organic carbon storage. *Sci Rep*. (2021) 11:1–13. doi: 10.1038/s41598-021-82739-7
15. Bedard C, Knowles R. Physiology, biochemistry, and specific inhibitors of CH4, NH4, and CO oxidation by methanotrophs and nitrifiers. *Microbiology*. (1989) 53:68–84. doi: 10.1128/mr.53.1.68-84.1989
16. Lauchnor EG, Semprini L. Inhibition of phenol on the rates of ammonia oxidation by Nitrosomonas europaea grown under batch, continuous fed, and biofilm conditions. *Water Res*. (2013) 47:4692–700. doi: 10.1016/j.watres.2013.04.052
17. Hyman MR, Page CL, Arp DJ. Oxidation of methyl fluoride and dimethyl ether by ammonia monooxygenase in Nitrosomonas europaea. *Appl Environ Microbiol*. (1994) 60:3033–5. doi: 10.1128/aem.60.8.3033-3035.1994
18. Bronick CJ, Lal R. Soil structure and management: a review. *Geoderma*. (2005) 124:3–22. doi: 10.1016/j.geoderma.2004.03.005
19. Nath M, Bhatt D, Bhatt MD, Prasad R, Tuteja N. Microbe-mediated enhancement of nitrogen and phosphorus content for crop improvement. In: Prasad R, Gill SS, Tuteja N, editors. *Crop Improvement Through Microbial Biotechnology*. Elsevier (2018). p. 293–304. doi: 10.1016/B978-0-444-63987-5.00014-1
20. Ali MM, Khanom A, Nahar K, Ali MY, Azad MAK, Rahman MM. Effect of manure application on net nitrification rates, heavy metal concentrations and nitrifying archaea/bacteria in soils. *Bull Environ Contam Toxicol*. (2021) 106:707–13. doi: 10.1007/s00128-021-03112-y

21. Gupta AK, Singh RP, Singh A, Ibrahim MH. Effects of heavy metal and metalloid contamination on the soil microbial response: an overview. *Microb Ecol Trop Soils.* (2011) 44:303–18.
22. Roane TM, Pepper IL, Gentry TJ. *Microorganisms and Metal Pollutants*. 2nd ed. London; California; Massachusetts: Elsevier Inc. (2014).
23. Soman C, Li D, Wander MM, Kent AD. Long-term fertilizer and crop-rotation treatments differentially affect soil bacterial community structure. *Plant Soil*. (2017) 413:145-59. doi: 10.1007/s11104-016-3083-y
24. Odell RT, Walker W, Boone L, Oldham M. The Morrow Plots: a century of learning. Bulletin-Agricultural Experiment Station, College of Agriculture, University of Illinois at Urbana-Champaign. *Agric Exp Station Coll Agric Univ Illinois Urbana Champaign Urbana.* (1982) 775:775. doi: 10.5962/bhl.title.16691
25. Aref S, Wander MM. Long-term trends of corn yield and soil organic matter in different crop sequences and soil fertility treatments on the morrow plots. *Adv Agron.* (1997) 62:153–97. doi: 10.1016/S0065-2113(08)60568-4
26. Kandeler E. Potential nitrification. In: *Methods in Soil Biology*, editors Schinner F, Kandeler E, Ohlinger R, dans Margesin R. Berlin: Spinger-Verlag Berlin Heidelberg (1995). p. 146–9.
27. Schinner F, Öhlinger R, Kandeler E, Margesin RTATT. *Methods in Soil Biology*. NV-1 onl. Berlin, Heidelberg: Springer Berlin Heidelberg (1996).
28. Berg P, Rosswall T. Ammonium oxidizer numbers, potential and actual oxidation rates in two swedish arable soils. *Biol Fertil Soils.* (1985) 1:131–40. doi: 10.1007/BF00301780
29. Sambrook J, Russell D. *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press (2001).
30. Rotthauwe JH, Witzel KP, Liesack W. The ammonia monooxygenase structural gene amoa as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl Environ Microbiol.* (1997) 63:4704–12. doi: 10.1128/aem.63.12.4704-4712.1997
31. Nicol GW, Leininger S, Schleper C, Prosser JI. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. *Environ Microbiol*. (2008) 10:2966–78. doi: 10.1111/j.1462-2920.2008.01701.x
32. Magoč T, Salzberg SL. FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*. (2011) 27:2957–63. doi: 10.1093/bioinformatics/btr507
33. Gordon A, Hannon G. FASTX-Toolkit (2010). Available online at: http://hannonlab.cshl.edu/fastx_toolkit/index.html
34. Reddy TBK, Thomas AD, Stamatis D, Bertsch J, Isbandi M, Jansson J, et al. (2015). The Genomes OnLine Database (GOLD) v.5: A metadata management system based on a four level (meta)genome project classification. *Nucleic Acids Res*. (2015) 43:D1099–106. doi: 10.1093/nar/gku950
35. Kuczynski J, Stombaugh J, Walters WA, González A, Caporaso JG, Knight R. Using QIIME to analyze 16S rRna gene sequences from microbial communities. *Curr Protoc Bioinforma*. (2011) 1–20. doi: 10.1002/0471250953.bi1007s36
36. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*. (2010) 26:2460–1. doi: 10.1093/bioinformatics/btq461
37. McDonald D, Price MN, Goodrich J, Nawrocki EP, Desantis TZ, Probst A, et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J*. (2012) 6:610–8. doi: 10.1038/ismej.2011.139
38. R Core Team. *R: A Language and Environment for Statistical Computing*. R Found. Stat. Comput. Vienna, Austria 3 (2021). Available online at: https://www.r-project.org/
39. Wickham H. *ggplot2: Elegant Graphics for Data Analysis* (2016).
40. McMurdie PJ, Holmes S. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE.* (2013) 8:e61217. doi: 10.1371/journal.pone.0061217
41. Oksanen AJ, Blanchet FG, Friendly M, Kindt R, Legendre P, Mcglinn D, et al. *Vegan: Community Ecology Package. R Package Version 2.5-7*. CRAN (2020). p. 2395–2396.
42. Antonious GF, Turley ET, Dawood MH. Monitoring soil enzymes activity before and after animal manure application. *Agric.* (2020) 10:1–12. doi: 10.3390/agriculture10050166
43. Warington R. IV. - On nitrification. *J Chem Soc Trans.* (1878) 33:44–51. doi: 10.1039/CT8783300044
44. Lees H. Effect of copper-enzyme poisons on soil nitrification. *Nature.* (1946) 4003:97. doi: 10.1038/158097a0
45. Meiklejohn J. Minimum phosphate and magnesium requirements of nitrifying bacteria. *Nature.* (1952) 170:1131. doi: 10.1038/1701131a0
46. Carey CJ, Dove NC, Beman JM, Hart SC, Aronson EL. Meta-analysis reveals ammonia-oxidizing bacteria respond more strongly to nitrogen addition than ammonia-oxidizing archaea. *Soil Biol Biochem.* (2016) 99:158–66. doi: 10.1016/j.soilbio.2016.05.014
47. Wang J, Wang J, Rhodes G, He JZ, Ge Y. Adaptive responses of comammox Nitrospira and canonical ammonia oxidizers to long-term fertilizations: implications for the relative contributions of different ammonia oxidizers to soil nitrogen cycling. *Sci Total Environ.* (2019) 668:224–33. doi: 10.1016/j.scitotenv.2019.02.427
48. Hatzenpichler R. Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. *Appl Environ Microbiol.* (2012) 78:7501–10. doi: 10.1128/AEM.01960-12
49. Martens-Habbena W, Berube PM, Urakawa H, De La Torre JR, Stahl DA. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nature.* (2009) 461:976–9. doi: 10.1038/nature08465
50. Di HJ, Cameron KC, Shen JP, Winefield CS, O'Callaghan M, Bowatte S, et al. Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. *FEMS Microbiol Ecol.* (2010) 72:386–94. doi: 10.1111/j.1574-6941.2010.00861.x
51. Straka LL, Meinhardt KA, Bollmann A, Stahl DA, Winkler MKH. Affinity informs environmental cooperation between ammonia-oxidizing archaea (AOA) and anaerobic ammonia-oxidizing (Anammox) bacteria. *ISME J.* (2019) 13:1997–2004. doi: 10.1038/s41396-019-0408-x
52. Lehtovirta-Morley LE, Ross J, Hink L, Weber EB, Gubry-Rangin C, Thion C, et al. Isolation of "Candidatus Nitrosocosmicus franklandus", a novel ureolytic soil archaeal ammonia oxidiser with tolerance to high ammonia concentration. *FEMS Microbiol Ecol.* (2016) 92:1–10. doi: 10.1093/femsec/fiw057
53. Jung MY, Sedlacek CJ, Kits KD, Mueller AJ, Rhee SK, Hink L, et al. Ammonia-oxidizing archaea possess a wide range of cellular ammonia affinities. *ISME J.* (2021) 16:272–83. doi: 10.1038/s41396-021-01064-z
54. Enwall K, Nyberg K, Bertilsson S, Cederlund H, Stenström J, Hallin S. Long-term impact of fertilization on activity and composition of bacterial communities and metabolic guilds in agricultural soil. *Soil Biol Biochem.* (2007) 39:106–15. doi: 10.1016/j.soilbio.2006.06.015
55. Chu H, Fujii T, Morimoto S, Lin X, Yagi K. Population size and specific nitrification potential of soil ammonia-oxidizing bacteria under long-term fertilizer management. *Soil Biol Biochem.* (2008) 40:1960–3. doi: 10.1016/j.soilbio.2008.01.006
56. Roberston G. Nitrification and denitrification in humid tropical ecosystems: potential controls on nitrogen retention. *Miner Nutr Trop For Savanna Ecosyst.* (1989) 473:55–69.
57. Tao R, Wakelin SA, Liang Y, Chu G. Response of ammonia-oxidizing archaea and bacteria in calcareous soil to mineral and organic fertilizer application and their relative contribution to nitrification. *Soil Biol Biochem.* (2017) 114:20–30. doi: 10.1016/j.soilbio.2017.06.027
58. Ke X, Angel R, Lu Y, Conrad R. Niche differentiation of ammonia oxidizers and nitrite oxidizers in rice paddy soil. *Environ Microbiol.* (2013) 15:2275–92. doi: 10.1111/1462-2920.12098
59. Guo J, Ling N, Chen H, Zhu C, Kong Y, Wang M, et al. Distinct drivers of activity, abundance, diversity and composition of ammonia-oxidizers: evidence from a long-term field experiment. *Soil Biol Biochem.* (2017) 115:403–14. doi: 10.1016/j.soilbio.2017.09.007
60. Suzuki I, Dular U, Kwok SC. Ammonia or ammonium ion as substrate for oxidation by Nitrosomonas europaea cells and extracts. *J Bacteriol.* (1974) 120:556–8. doi: 10.1128/jb.120.1.556-558.1974
61. Chu H, Lin X, Fujii T, Morimoto S, Yagi K, Hu J, et al. Soil microbial biomass, dehydrogenase activity, bacterial community structure in response

to long-term fertilizer management. *Soil Biol Biochem.* (2007) 39:2971–6. doi: 10.1016/j.soilbio.2007.05.031
62. Stark C, Condron LM, Stewart A, Di HJ, O'Callaghan M. Influence of organic and mineral amendments on microbial soil properties and processes. *Appl Soil Ecol.* (2007) 35:79–93. doi: 10.1016/j.apsoil.2006.05.001
63. Gao S, Zhou G, Liao Y, Lu Y, Nie J, Cao W. Contributions of ammonia-oxidising bacteria and archaea to nitrification under long-term application of green manure in alkaline paddy soil. *Geoderma.* (2020) 374:114419. doi: 10.1016/j.geoderma.2020.114419
64. Luxhøi J, Elsgaard L, Thomsen IK, Jensen LS. Effects of long-term annual inputs of straw and organic manure on plant N uptake and soil N fluxes. *Soil Use Manag.* (2007) 23:368–73. doi: 10.1111/j.1475-2743.2007.00126.x
65. Azeez JO, Van Averbeke W. Nitrogen mineralization potential of three animal manures applied on a sandy clay loam soil. *Bioresour Technol.* (2010) 101:5645–51. doi: 10.1016/j.biortech.2010.01.119
66. Koch H, Lücker S, Albertsen M, Kitzinger K, Herbold C, Spieck E, et al. Expanded metabolic versatility of ubiquitous nitrite-oxidizing bacteria from the genus Nitrospira. *Proc Natl Acad Sci USA.* (2015) 112:11371–6. doi: 10.1073/pnas.1506533112
67. Mooshammer M, Wanek W, Jones SH, Richter A, Wagner M. Cyanate is a low abundance but actively cycled nitrogen compound in soil. *Commun Earth Environ.* (2021) 2:1–10. doi: 10.1038/s43247-021-00235-2
68. Daims H, Lücker S, Wagner M. A new perspective on microbes formerly known as nitrite-oxidizing bacteria. *Trends Microbiol.* (2016) 24:699–712. doi: 10.1016/j.tim.2016.05.004
69. Li X, Han S, Wan W, Zheng L, Chen W, Huang Q. Manure fertilizes alter the nitrite oxidizer and comammox community composition and increase nitrification rates. *Soil Tillage Res.* (2020) 204:104701. doi: 10.1016/j.still.2020.104701
70. Wendeborn S. The chemistry, biology, and modulation of ammonium nitrification in soil. *Angew Chemie Int Ed.* (2020) 59:2182–202. doi: 10.1002/anie.201903014
71. Smith JM, Mosier AC, Francis CA. Spatiotemporal relationships between the abundance, distribution, and potential activities of ammonia-oxidizing and denitrifying microorganisms in intertidal sediments. *Microb Ecol.* (2015) 69:13–24. doi: 10.1007/s00248-014-0450-1
72. Lücker S, Wagner M, Maixner F, Pelletier E, Koch H, Vacherie B, et al. A Nitrospira metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. *Proc Natl Acad Sci USA.* (2010) 107:13479–84. doi: 10.1073/pnas.1003860107
73. Davey ME, O'toole GA. Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev.* (2000) 64:847–67. doi: 10.1128/MMBR.64.4.847-867.2000
74. Li P-P, Han Y-L, He J-Z, Zhang S-Q, Zhang L-M. Soil aggregate size and long-term fertilization effects on the function and community of ammonia oxidizers. *Geoderma.* (2019) 338:107–17. doi: 10.1016/j.geoderma.2018.11.033
75. Zhang Q, Liang G, Myrold DD, Zhou W. Variable responses of ammonia oxidizers across soil particle-size fractions affect nitrification in a long-term fertilizer experiment. *Soil Biol Biochem.* (2017) 105:25–36. doi: 10.1016/j.soilbio.2016.11.005
76. Tarre S, Green M. 2004). High-rate nitrification at low pH in suspended- and attached-biomass reactors. *Appl Environ Microbiol.* (2004) 70:6481–7. doi: 10.1128/AEM.70.11.6481-6487.2004
77. Isshiki R, Fujitani H, Tsuneda S. Transcriptome analysis of the ammonia-oxidizing bacterium Nitrosomonas mobilis Ms1 reveals division of labor between aggregates and free-living cells. *Microbes Environ.* (2020) 35:1–9. doi: 10.1264/jsme2.ME19148
78. Vuko M, Cania B, Vogel C, Kublik S, Schloter M, Schulz S. Shifts in reclamation management strategies shape the role of exopolysaccharide and lipopolysaccharide-producing bacteria during soil formation. *Microb Biotechnol.* (2020) 13:584–98. doi: 10.1111/1751-7915.13532
79. Fujitani H, Nomachi M, Takahashi Y, Hasebe Y, Eguchi M, Tsuneda S. Successful enrichment of low-abundant comammox Nitrospira from nitrifying granules under ammonia-limited conditions. *FEMS Microbiol Lett.* (2020) 367:1–8. doi: 10.1093/femsle/fnaa025
80. Smith JH. (1964). Relationships between soil cation-exchange capacity and the toxicity of ammonia to the nitrification process. *Soil Sci Soc Am J.* (1964) 28:640-4. doi: 10.2136/sssaj1964.03615995002800050019x
81. Gadd GM, Griffiths AJ. Microorganisms and heavy metal toxicity. *Microb Ecol.* (1977) 4:303–17. doi: 10.1007/BF02013274
82. Hooda PS. *Trace Elements in Soil Systems*. West Sussex: Wiley (2019).
83. Ansari FA, Jafri H, Ahmad I, Abulreesh HH. Factors affecting biofilm formation in *in vitro* and in the rhizosphere. *Biofilms Plant Soil Heal.* (2017) 275–90. doi: 10.1002/9781119246329.ch15
84. Green M, Ruskol Y, Tarre S, Loewenthal RE. Nitrification utilizing CaCO3 as the buffering agent. *Environ Technol.* (2002) 23:303–8. doi: 10.1080/09593332508618410
85. Sun M, Wang H, Zhang H. Effect of Ca2+ and Fe3+ addition on nitrification-denitrification process in a membrane bioreactor. *Adv Mater Res.* (2013) 610–3:422–7. doi: 10.4028/www.scientific.net/AMR.610-613.422
86. Kunhikrishnan A, Thangarajan R, Bolan NS, Xu Y,Mandal S, Gleeson DB, et al. Functional relationships of soil acidification, liming, and greenhouse gas flux. In: *Advances in Agronomy*. London; California, CA; Massachussets, MA; Oxford: Academic Press Inc. (2016). p. 1–71.
87. Holmes AJ, Costello A, Lidstrom ME, Murrell JC. Evidence that participate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. *FEMS Microbiol Lett.* (1995) 132:203–8. doi: 10.1111/j.1574-6968.1995.tb07834.x
88. Suzuki I, Kwok SC, Dular U. Competitive inhibition of ammonia oxidation in *Nitrosomonas europaea* by methane, carbon monoxide or methanol. *FEBS Lett.* (1976) 72:117–20. doi: 10.1016/0014-5793(76)80825-3
89. Meincke M, Krieg E, Bock E. (1989). Nitrosovibrio spp., the dominant ammonia-oxidizing bacteria in building sandstone. *Appl Environ Microbiol.* (1989) 55:2108–10. doi: 10.1128/aem.55.8.2108-2110.1989
90. Shi Y, Liu X, Zhang Q, Gao P, Ren J. Biochar and organic fertilizer changed the ammonia-oxidizing bacteria and archaea community structure of saline–alkali soil in the North China Plain. *J Soils Sediments.* (2019) 20:12–23. doi: 10.1007/s11368-019-02364-w
91. Gransee A, Führs H. Magnesium mobility in soils as a challenge for soil and plant analysis, magnesium fertilization and root uptake under adverse growth conditions. *Plant Soil.* (2013) 368:5–21. doi: 10.1007/s11104-012-1567-y
92. Kondo H, Ohmori T, Shibata H, Sase K, Takahashi R, Tokuyama T. Thermostable succinyl-Coenzyme A synthetase from Nitrosomonas europaea ATCC 25978: purification and properties. *J Ferment Bioeng.* (1995) 79:499–502. doi: 10.1016/0922-338X(95)91270-F
93. Premi PR, Cornfield AH. Effects of addition of copper, manganese, zinc and chromium compounds on ammonification and nitrification during incubation of soil. *Plant Soil.* (1969) 31:345–52. doi: 10.1007/BF01373578
94. Mertens J, Wakelin SA, Broos K, Mclaughlin MJ, Smolders E. Extent of copper tolerance and consequences for functional stability of the ammonia-oxidizing community in long-term copper-contaminated soils. *Environ Toxicol Chem.* (2010) 29:27–37. doi: 10.1002/etc.16
95. Doelman P, Haanstra L. Short- and long-term effects of heavy metals on urease activity in soils. *Biol Fertil Soils.* (1986) 2:213–8. doi: 10.1007/BF00260846
96. Hemida SK, Omar SA, Abdel-Mallek AY. Microbial populations and enzyme activity in soil treated with heavy metals. *Water Air Soil Pollut.* (1997) 95:13–22. doi: 10.1007/BF02406152
97. De Gannes V, Eudoxie G, Hickey WJ. Impacts of edaphic factors on communities of ammonia-oxidizing archaea, ammonia-oxidizing bacteria and nitrification in tropical soils. *PLoS ONE.* (2014) 9:e89568. doi: 10.1371/journal.pone.0089568
98. Gwak JH, Jung MY, Hong H, Kim JG, Quan ZX, Reinfelder JR, et al. Archaeal nitrification is constrained by copper complexation with organic matter in municipal wastewater treatment plants. *ISME J.* (2020) 14:335–46. doi: 10.1038/s41396-019-0538-1
99. Reyes C, Hodgskiss LH, Kerou M, Pribasnig T, Abby SS, Bayer B, et al. Genome wide transcriptomic analysis of the soil ammonia oxidizing archaeon Nitrososphaera viennensis upon exposure to copper limitation. *ISME J.* (2020) 14:2659–74. doi: 10.1038/s41396-020-0715-2
100. Sparacino-Watkins C, Stolz JF, Basu P. Nitrate and periplasmic nitrate reductases. *Chem Soc Rev.* (2014) 43:676–706. doi: 10.1039/C3CS60249D

101. Gani B. Enzyme chemistry. *Annu Rep Prog Chem Sect B Org Chem.* (1986) 83:303–30. doi: 10.1039/oc9868300303
102. Liu J, Chakraborty S, Hosseinzadeh P, Yu Y, Tian S, Petrik I, et al. Metalloproteins containing cytochrome, iron-sulfur, or copper redox centers. *Chem Rev.* (2014) 114:4366–9. doi: 10.1021/cr400479b
103. Zhang Q, Zhou W, Liang G, Sun J, Wang X, He P. Distribution of soil nutrients, extracellular enzyme activities and microbial communities across particle-size fractions in a long-term fertilizer experiment. *Appl Soil Ecol.* (2015) 94:59–71. doi: 10.1016/j.apsoil.2015.05.005
104. Sears K, Alleman JE, Barnard JL, Oleszkiewicz JA. Impacts of reduced sulfur components on active and resting ammonia oxidizers. *J Ind Microbiol Biotechnol.* (2004) 31:369–78. doi: 10.1007/s10295-004-0157-2
105. Arunkumar B, Thippeshappa G, Anjali M, Prashanth K. Boron : a critical micronutrient for crop growth and productivity. *J Pharmacogn Phytochem.* (2018) 7:2738–41.
106. Redondo-Nieto M, Rivilla R, El-Hamdaoui A, Bonilla I, Bolaños L. Boron deficiency affects early infection events in the pea-Rhizobium symbiotic interaction. *Aust J Plant Physiol.* (2001) 28:819–23. doi: 10.1071/PP01020
107. Bolaños L, Lukaszewski K, Bonilla I, Blevins D. Why boron? *Plant Physiol Biochem.* (2004) 42:907–12. doi: 10.1016/j.plaphy.2004.11.002
108. Chen L, Xia F, Wang M, Wang W, Mao P. Metabolomic analyses of alfalfa (*Medicago sativa* L. cv 'Aohan') reproductive organs under boron deficiency and surplus conditions. *Ecotoxicol Environ Saf.* (2020) 202:111011. doi: 10.1016/j.ecoenv.2020.111011
109. Bulluck LR, Brosius M, Evanylo GK, Ristaino JB. Organic and synthetic fertility amendments influence soil microbial, physical and chemical properties on organic and conventional farms. *Appl Soil Ecol.* (2002) 19:147–60. doi: 10.1016/S0929-1393(01)00187-1
110. Tsadilas CD, Kassioti T, Mitsios IK. Influence of liming and nitrogen forms on boron uptake by tobacco. *Commun Soil Sci Plant Anal.* (2005) 701–8. doi: 10.1081/CSS-200043348
111. Dhaliwal SS, Naresh RK, Mandal A, Singh R, Dhaliwal MK. Dynamics and transformations of micronutrients in agricultural soils as influenced by organic matter build-up: a review. *Environ Sustain Indic.* (2019) 1–2:100007. doi: 10.1016/j.indic.2019.100007
112. Bilen S, Bilen M, Bardhan S. The effects of boron management on soil microbial population and enzyme activities. *African J Biotechnol.* (2011) 10:5311–9. doi: 10.4314/AJB.V10I27
113. Sun T, Wang YP, Wang ZY, Liu P, Xu GD. The effects of molybdenum and boron on the rhizosphere microorganisms and soil enzyme activities of soybean. *Acta Physiol Plant.* (2013) 35:763–70. doi: 10.1007/s11738-012-1116-6
114. Lauber CL, Ramirez KS, Aanderud Z, Lennon J, Fierer N. Temporal variability in soil microbial communities across land-use types. *ISME J.* (2013) 7:1641–50. doi: 10.1038/ismej.2013.50
115. Jeong J, Bolan N, Kim C. Heterotrophic soil respiration affected by compound fertilizer types in red pine (*Pinus densiflora* S. et Z) stands of Korea. *Forests.* (2016) 7:309. doi: 10.3390/f7120309
116. Cabrera ML, Kissel DE, Vigil M. Nitrogen mineralization from organic residues: researchh opportunities. *J Environ Qual.* (2005) 34:75–9. doi: 10.2134/jeq2005.0075
117. Xu JG, Heeraman DA, Wang Y. Fertilizer and temperature effects on urea hydrolysis in undisturbed soil. *Biol Fertil Soils.* (1993) 16:63–5. doi: 10.1007/BF00336517
118. Lu X, Nicol GW, Neufeld JD. Differential responses of soil ammonia-oxidizing archaea and bacteria to temperature and depth under two different land uses. *Soil Biol Biochem.* (2018) 120:272–82. doi: 10.1016/j.soilbio.2018.02.017
119. Taylor AE, Myrold DD, Bottomley PJ. Temperature affects the kinetics of nitrite oxidation and nitrification coupling in four agricultural soils. *Soil Biol Biochem.* (2019) 136:107523. doi: 10.1016/j.soilbio.2019.107523
120. Dick RP. A review: long-term effects of agricultural systems on soil biochemical and microbial parameters. *Agric Ecosyst Environ.* (1992) 40:25–36. doi: 10.1016/0167-8809(92)90081-L
121. Kiani M, Hernandez-Ramirez G, Quideau S, Smith E, Janzen H, Larney FJ, et al. Quantifying sensitive soil quality indicators across contrasting long-term land management systems: crop rotations and nutrient regimes. *Agric Ecosyst Environ.* (2017) 248:123–35. doi: 10.1016/j.agee.2017.07.018
122. Rochette P, Angers DA, Bélanger G, Chantigny MH, Prévost D, Lévesque G. Emissions of N2O from Alfalfa and Soybean Crops in Eastern Canada. *Soil Sci Soc Am J.* (2004) 68:493–506. doi: 10.2136/sssaj2004.4930
123. Menéndez S, López-Bellido RJ, Benítez-Vega J, González-Murua C, López-Bellido L, Estavillo JM. Long-term effect of tillage, crop rotation and N fertilization to wheat on gaseous emissions under rainfed Mediterranean conditions. *Eur J Agron.* (2008) 28:559–69. doi: 10.1016/j.eja.2007.12.005
124. Duan Y, Xu M, Gao S, Liu H, Huang S, Wang B. Long-term incorporation of manure with chemical fertilizers reduced total nitrogen loss in rain-fed cropping systems. *Sci Rep.* (2016) 6:33611. doi: 10.1038/srep33611
125. Wrage N, Velthof GL, Van Beusichem ML, Oenema O. Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biol Biochem.* (2001) 33:1723–32. doi: 10.1016/S0038-0717(01)00096-7
126. Wrage-Mönnig N, Horn MA, Well R, Müller C, Velthof G, Oenema O. The role of nitrifier denitrification in the production of nitrous oxide revisited. *Soil Biol Biochem.* (2018) 123:A3–A16. doi: 10.1016/j.soilbio.2018.03.020
127. Prosser JI, Hink L, Gubry-Rangin C, Nicol GW, Gubry-Rangin C, Nicol GW. Nitrous oxide production by ammonia oxidizers: Physiological diversity, niche differentiation and potential mitigation strategies. *Glob Chang Biol.* (2020) 26:103–18. doi: 10.1111/gcb.14877
128. Friedl J, De Rosa D, Rowlings DW, Grace PR, Müller C, Scheer C. Dissimilatory nitrate reduction to ammonium (DNRA), not denitrification dominates nitrate reduction in subtropical pasture soils upon rewetting. *Soil Biol Biochem.* (2018) 125:340–9. doi: 10.1016/j.soilbio.2018.07.024
129. Pandey CB, Kumar U, Kaviraj M, Minick KJ, Mishra AK, Singh JS. DNRA: A short-circuit in biological N-cycling to conserve nitrogen in terrestrial ecosystems. *Sci Total Environ.* (2020) 738:139710. doi: 10.1016/j.scitotenv.2020.139710

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Soil Science

ORIGINAL RESEARCH
published: 17 March 2022
doi: 10.3389/fsoil.2022.832600

Season and No-Till Rice Crop Intensification Affect Soil Microbial Populations Involved in CH_4 and N_2O Emissions

*Ana Fernández-Scavino [1], Daniela Oreggioni [1], Andrea Martínez-Pereyra [1], Silvana Tarlera [1], José A. Terra [2] and Pilar Irisarri [3]**

[1] *Área Microbiología, Departamento de Biociencias, Facultad de Química, Universidad de la República, Montevideo, Uruguay,* [2] *Instituto Nacional de Investigación Agropecuaria (INIA), Programa Producción de Arroz, Estación Experimental INIA Treinta y Tres, Treinta y Tres, Uruguay,* [3] *Laboratorio de Microbiología, Departmento de Biología Vegetal, Facultad de Agronomía, Universidad de la República, Montevideo, Uruguay*

OPEN ACCESS

Edited by:
Nigel Victor Gale,
University of Toronto, Canada

Reviewed by:
Hyo Jung Lee,
Kunsan National University, South Korea
Md Mozammel Haque,
Bangladesh Rice Research Institute, Bangladesh

***Correspondence:**
Pilar Irisarri
irisarri@fagro.edu.uy

Specialty section:
This article was submitted to Soil Biology, Ecosystems and Biodiversity, a section of the journal Frontiers in Soil Science

Received: *10 December 2021*
Accepted: *01 February 2022*
Published: *17 March 2022*

Citation:
Fernández-Scavino A, Oreggioni D, Martínez-Pereyra A, Tarlera S, Terra JA and Irisarri P (2022) Season and No-Till Rice Crop Intensification Affect Soil Microbial Populations Involved in CH_4 and N_2O Emissions. Front. Soil Sci. 2:832600. doi: 10.3389/fsoil.2022.832600

Rice is an important source of methane (CH_4) and other crops may be sources of nitrous oxide (N_2O), both of which are powerful greenhouse gases. In Uruguay, irrigated rice rotates with perennial pastures and allows high productivity and low environmental impact. A long-term experiment with contrasting rice rotation intensification alternatives, including rice–soybean and continuous rice, was recently carried out in an Argialboll located in a temperate region of South America. To know if rotation systems influence soil microbial activity involved in CH_4 and N_2O emissions, the abundance and potential rate for gas production or consumption of microbial populations were measured during the rice crop season. CH_4 was only emitted when rice was flooded and N_2O emission was not detected. All rotational soils showed the highest rate for methanogenesis at tillering (30 days after rice emergence), while for methanotrophy, the maximum rate was reached at flowering. The abundance of related genes also followed a seasonal pattern with highest densities of *mcr*A genes being observed at rice flowering whereas *pmo*A genes were more abundant in dry soils after rice harvest, regardless of the rotation system. Differences were found mainly at tillering when soils with two consecutive summers under rice showed higher amounts of *mcr*A and *pmo*A gene copies. The potential denitrification rate was highest at the tillering stage, but the abundance of *nir*K and *nir*S genes was highest in winter. Regarding ammonium oxidation, bacterial *amo*A abundance was higher in winter while the archaeal *amo*A gene was similar throughout the year. A strong influence of the rice growth stage was registered for most of the parameters measured in rice paddy soils in this no-till rice intensification experiment. However, differences among rotations begin to be observed mainly at tillering when the abundance of populations of the methane and nitrous oxide cycles seemed to respond to the rice intensification.

Keywords: rice rotations systems, microbial abundance, methane, nitrous oxide, intensification

INTRODUCTION

Uruguay has one of the highest rice yield potentials in the world, with farmers averaging more than 8,000 kg ha^{-1} (1, 2). Recently, Pittellkow et al. (3) concluded that the increase in rice yield during 20 years in Uruguay was obtained with high resource-use eco-efficiencies and low environmental impact. This sustainable increase in productivity was related, among other factors, to the predominant production system where flooded rice rotates the use of soil with diverse pastures advocated for beef and wool production during 2–4 years.

In rice–pasture systems, the soil is exposed in different periods to both oxic and anoxic conditions. These changes in soil redox conditions can favor different microbiological processes.

Under anaerobic conditions, CH_4 is the main greenhouse gas (GHG) emitted as the end product of the degradation of organic matter (4) and is also the principal contributor to global warming potential in rice systems (5). The CH_4 emission from flooded rice soils results from the balance between CH_4 production in the anoxic deep layers and its oxidation to CO_2 by methanotrophic bacteria during upward diffusion through oxic soil/water layers (6). Another important GHG produced by agricultural activities is nitrous oxide (N_2O) (7). In contrast to CH_4 emissions, N_2O emissions from paddy rice fields are a result of both aerobic and anaerobic conditions, which include nitrification–denitrification processes, respectively. In nitrification, N_2O is a side product, and the process can also provide NO_3^- to denitrification, while in denitrification, N_2O can be an intermediate or an end product.

Water management and previous land use under aerobic conditions are strategies proposed to mitigate CH_4 emissions from rice cultivation (8, 9). In a previous 3-year study, we showed that an alternative system of controlled deficit irrigation allowing for wetting and drying (AWDI) delayed and decreased the seasonal emission of CH_4 in comparison with the usual Uruguayan irrigation system of continuous flooding between V4 stage and physiological maturity (10). Different crop rotations of flooded rice with upland crops such as rice–maize (11), wheat–rice (12), and soybean–rice (13) are common agricultural practices across tropical and subtropical Asia where rice is the most important crop. In rice–soybean rotations, 1 year of summer soil submersion is followed by another year of summer drained conditions where soybean is cultivated. Even more extended periods of aerated soil conditions are present in the typical Uruguayan management of rice fields where 1–3 years of rice cultivation during summer is followed by 2–4 years of grazed pastures with cattle, giving rotation systems of several phases. Previous land management with short- or long-term aerobic conditions can strongly affect the relative importance of CH_4 contribution to GHG in comparison to N_2O as well as the onset of CH_4 emissions in rice paddies. Several authors have studied the effect of water management on GHG emissions measuring gas fluxes in paddy fields (9, 10, 14). Less is known on the specific impact of previous land use on microbial communities present in the soil and the consequent C and N cycling processes and, in return, its effect on GHG fluxes.

A preferred approach for studying functional groups of microorganisms involved in CH_4 and N_2O emissions is the use of specific genes encoding enzymes involved in these processes. The final key step of methane production by methanogenic archaea is catalyzed by methyl-coenzyme M reductase whose α-subunit encoded by the highly conserved *mcrA* gene is commonly used for analysis of methanogenic communities in rice fields (15). On the other hand, the generated CH_4 can be further oxidized by the methanotrophs present in soil. The *pmoA* gene has been used as the indicator for the methanotrophs in rice ecosystems (16).

Under anaerobic conditions, N_2O is mainly produced by denitrification through a series of reduction steps catalyzed by specific enzymes of functional genes (17, 18), which can be further reduced to N_2 *via* nitrous oxide reductase (*nosZ*). In this study, we focused on the abundance of genes encoded by nitrite reductase (*nirS*/*nirK*), which catalyzes the limiting step of denitrification reducing NO_2^- to NO, and on the abundance of the *nosZ* gene. Aerobically, NH_4^+ can be oxidized to NO_2^- by ammonia-oxidizing archaea and bacteria (AOA and AOB, respectively), through ammonia monooxygenase encoded by *amoA* gene. The combined use of these genes can be useful as an indicator of the potential for one or both processes to occur. The quantification of ribosomal and functional genes as well as the microcosm assays to determine microbial potential activities are useful to assess microbial biogeochemical processes involved in GHG emissions in soils (15, 19, 20).

We measured CH_4 and N_2O emissions and soil microbial activity from three contrasting rice rotation systems (perennial pasture–rice; soybean–rice, and continuous rice) during the rice crop growing cycle to assess specific microbial community responses to the rice intensification. The systems were no-till, excluding soil tillage, and preventing crop or pasture residue from being incorporated into the soil. Rice was sown in the field full of residue from the previous crop. Additionally, measurements were performed in winter, under non-flooded conditions, to assess the effect of the previous summer crop on the microbial soil carbon and nitrogen transformations. Quantitative polymerase chain reaction (qPCR) targeting CH_4 and N_2O-related functional genes was used to assess the abundance of microbial populations potentially involved in GHG emissions. In addition, soil incubation experiments were performed to assay potential rates of CH_4 production and consumption, denitrification, and nitrification. The work aimed to compare the effect of rice intensification in the microbial C and N transformations involved in CH_4 and N_2O emissions at different stages of the rice growing cycle in a long-term experiment that has been recently installed.

MATERIALS AND METHODS

Site Description and Experimental Design

An 8-ha field-scale experiment was installed in 2012 in a 30-year rice–pasture rotation field located in Treinta y Tres-Uruguay (33° 16′ 23″ S; 54° 10′ 24″ O; 22 MASL) at the INIA (National Institute of Agricultural Research) "Paso de la Laguna" Research Unit. The dominant soil at the site is an Argialboll with a slope <0.5% (21). Soil physicochemical properties (0–15 cm) when the experiment was installed were as follows: TOC 14.2 g kg^{-1}, TN 1.4 g kg^{-1}, P Bray 7.0 μg g^{-1}, and pH 5.7. Mean annual temperature at the

site is 22.3°C in summer and 11.5°C in winter. Rainfall is evenly distributed throughout the year, with a total annual mean of 1,360 ± 315 mm; annual total potential evapotranspiration is 1,138 ± 177 mm.

The experiment consists of six rice rotation systems under no till with contrasting soil use intensity determined by the proportion of crops and pastures in the rotation (22). In this work, we analyzed three rotation treatments: continuous rice (*Oryza sativa*) (cR; rice every summer); rice–soybean (*Glycine max*), a 2-year rotation cycle with soybean alternating with rice in summer; and rice–ryegrass (*Lolium multiflorum* Lam.) in winter—rice, 3 years of perennial pasture of tall fescue (*Festuca arundinacea*), white clover (*Trifolium repens*), and birdsfoot trefoil (*Lotus corniculatus*), a 5-year rotation cycle with 2 consecutive years of rice followed by 3 years of pastures. Cover crops of annual ryegrass (*Lolium multiflorum*) or *Trifolium alexandrinum L.* were installed in fall–winter between cash crops in all rotations; pastures were rotationally grazed with lambs during the year. The experimental design consisted in a randomized complete block with three replications and all phases of rotations present simultaneously (23). Each plot measures 20 × 60 m and was isolated from other plots by levees to control rice flood. Rice was no-till drill seeded in the last week of October 2015, emerged on November 10, and was flooded ∼4 weeks after emergence at rice V4–V5 stages.

The nitrogen was broadcasted as urea and fractionated 60% at V4 (dry soil) and 40% at R0 (flooded soil) growing stages. In soybean–rice and pasture–rice rotations, nitrogen dose (40 kg N ha^{-1} $year^{-1}$) was based on soil nitrogen mineralization potential, while in continuous rice, it was based on crop demand (148 kg N ha^{-1} $year^{-1}$) (22). Other relevant information for these no-till systems is the amount of dry matter left on the soil after crops and winter covers, which is highest for rice residues (9 Mg ha^{-1}), followed by perennial pasture (7 Mg ha^{-1}), soybean (3.8 Mg ha^{-1}), and finally winter covers (between 2 and 3 Mg ha^{-1}). Other crop management practices, including fertilization, pest and weeds control, and rice cultivars seeded, were chosen for each specific rotation treatment to optimize rice productivity. More details of the experiment are reported in Macedo et al. (22).

Sampling

Soil samples were taken from each plot in the three replicated blocks. Six phases of three rotation were sampled (**Table 1**): continuous rice (cR), the two phases of the rice–soybean rotation (rice phase Rs and soybean phase rS), and three phases of the rice–pasture rotation: first year of rice (Rrppp), second year of rice (rRppp), and third year of pasture (rrppP). Samples were taken between December 2015 and July 2016 and were labeled according to the rice crop cycle: 30 days after emergence (dae) corresponding to rice tillering (vegetative stage, end of seedling stage), 98 dae corresponding to rice flowering, 124 dae corresponding to rice ripening, and at the following winter after harvest (259 days after rice emergence). All samples taken in winter and samples from rS and rrppP corresponded to non-flooded, dry soil.

Greenhouse Gases (GHG) Measurement

Gas sampling for flooded rice was conducted using the closed-chamber technique (24, 25). The chambers consisted of an aluminum base measuring 60 × 60 × 20 cm (length × width × height) and an aluminum top of the same size. The bases were driven 5 cm deep into the soil before permanent flooding and remained in the soil during the entire growing season. Each base had an open bottom and sealable channels on the sides to allow irrigation water to flow freely, which were sealed during air sampling events. Each base covered three rice plant rows. Additional 20-cm aluminum extensors were stacked on the bases as the rice plants grew taller, and the chamber volume was considered for GHG emission calculations. Each chamber was equipped with (i) a gas sampling port, (ii) a stainless-steel thermometer, and (iii) three battery-operated fans to circulate and homogenize gases within the chamber. Headspace gas samples were obtained with airtight 20-ml propylene syringes and were immediately transferred to pre-evacuated 12-ml glass Exetainer® vials (Labco Ltd., Buckinghamshire, UK). Gas samples from dry soil were taken using smaller chambers with steel bases, 40 cm in diameter and 20 cm in height, that were left in place and inserted 10 cm into the soil. The lid was fitted with a sampling port with a three-way valve and placed on top of the box at the beginning of each gas sampling day when the effective height of each chamber was recorded.

Gas flux measurements were taken between 10:00 and 11:30 a.m., as recommended by Minamikawa et al. (24). Chamber temperature, floodwater depth, and headspace height were recorded and used to calculate gas flux rates from the soil surface to the chamber atmosphere assuming a linear increase in gas concentration over time as described previously (10). Methane and nitrous oxide were analyzed on a GC-FID-mECD 7890 Agilent gas chromatograph with a HayeSep Q 80/100 mesh 1/8 column.

Soil Sampling and Chemical Analysis

Ten random soil core samples from 0 to 10 cm soil layer were collected from each replicated plot, homogenized by hand, composited, and mixed thoroughly. Fresh soils were used for the following analysis: pH (1:1 soil/water extract), moisture content, and microbial activities (denitrifying, nitrifying, methanotrophic, and methanogenic). Air-dried and sieved (2 mm mesh) soils were used for NO_3^--N, NH_4^+-N and soluble organic carbon analysis. Subsamples of fresh soils were stored at −80°C until DNA extraction for molecular analysis.

Soils were extracted in duplicate with 2 M KCl (soil:solution ratio of 1:10) and analyzed for NH_4^+-N using the standard indophenol blue method and for NO_3^--N using the Cu-Cd reduction method followed by the colorimetric modified Griess-Ilosvay method (26).

Soil organic carbon was determined before rice sowing by the method of wet oxidation (27) followed by quantification by spectrophotometry at 600 nm (28). Soil moisture was determined by placing it in an oven at 105°C until constant weight.

TABLE 1 | Crop rotation system, annual and seasonal schedules (SS, spring–summer; AW, autumn–winter).

Rotation treatment*	Year									
	1st		2nd		3rd		4th		5th	
	SS	AW	SS	AW	SS	AW	SS	AW	SS	AW
Cr	Rice	p_S								
rrppp	Rice	p_S	Rice				$-\!-\!-p_L-\!-\!-$			
Rs	Rice	p_S	Soybean	p_S						

*cr, Continuous rice; rrppp, Rice (2 years)–long-term pasture (3 years); rs, Rice (1 year)–soybean (1 year); p_S, short-term pasture; p_L, long-term pasture (perennial pasture).

Substrate Production or Consumption Rates in Soil Laboratory Incubations

All assays were performed with soil from the three replicated plots.

Potential Rate of Methane Production (MPP) and Oxidation (MOP)

The rate of CH_4 production was measured by incubating three replicates of 5 g of fresh soil from the three replicated plots and autoclaved anoxic distilled water in 25-ml pressure vials under a headspace of N_2 at 25°C for 2 months. At regular time intervals, the headspace was analyzed for CH_4 accumulated by GC as described before.

To measure CH_4 consumption, 5 g of fresh soil was incubated with 20 ml of sterile distilled water into 120-ml glass vials. The vials were capped with a cotton plug and incubated at 25°C in the dark for 3 days to deplete any soil organic substrates. At the end of this period, bottles were capped with butyl stoppers and aluminum seals and pure methane was added to obtain 7% CH_4 (v/v). The slurries were incubated in the dark at 25°C on a gyratory shaker (120 r.p.m.). The methane concentration in the headspace was monitored by GC-FID analysis (GC-2014, Gas Chromatograph, Shimadzu). Maximum methane oxidation rates were calculated by linear regression analysis of the methane consumption over time.

Potential Denitrification (PDA) and Nitrification Activity Rate (PNA)

Denitrification rates were measured with the acetylene blockage technique (29). Five grams of fresh soil was added into a 60-ml glass vial followed by 10 ml of sterile distilled water. Vials were flushed with filtered O_2-free N_2 while the following amendments were aseptically added according to a complete NO_3^- reduction stoichiometry (final concentration): sodium succinate 0.86 mM, potassium acetate 1.5 mM, methanol 2 mM, and KNO_3 4.5 mM. Vials were capped with butyl stoppers and aluminum seals and acetylene was added (10% of the headspace) to every vial, and vials were incubated in the dark at 25°C on a gyratory shaker (120 r.p.m.). Samples from the headspace of every vial were removed at several time points for N_2O measurement by gas chromatography (GC-2014, Gas Chromatograph, Shimadzu); gas chromatography conditions and calculations of denitrification rates were done as described by Tarlera and Denner (30).

Nitrification rate was determined by measuring nitrite accumulation rate over time according to Kalender (31) with some modifications. Vials with 5 g of fresh soil with 15 ml of $(NH_4)_2SO_4$ 1.33 mM, 0.1 ml of sodium chlorate 1.5 mM (to inhibit nitrite oxidation), and 5 ml sterile distilled water were incubated at 28°C on an orbital shaker (180 r.p.m.) for 24 h. Replicate soil suspensions were kept at −20°C as controls. Nitrite concentration extracted with 2 M KCl was measured colorimetrically (Griess-Ilosvay reaction).

Soil DNA Extraction and Quantitative Real-Time PCR Analysis

All assays were performed with soil from the three replicated plots. DNA was extracted from the soil using PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA).

Abundance of Bacteria, Archaea, and certain groups of prokaryotes was performed using real-time PCR (qPCR). Specific primers were used to quantify gene copy numbers of Bacteria or Archaea (16S rRNA), methanogens (*mcr*A), methanotrophs (*pmo*A), denitrifiers (*nir*S and *nir*K), complete denitrifiers (*nos*Z), and nitrifiers of the domain Bacteria and Archaea (*amo*A) (Primer's information is detailed in **Supplementary Table 1**). Amplifications were performed on a Rotor-Gene® 6000, model 5-Plex (CORBETT Research Sydney) using SYBR Green I for detection. The samples were amplified in 10-µl reaction volumes containing 1 µl of concentrated or 10-fold diluted template DNA, 0.5 µM of each primer (except *pmo*A primer, 1 µM), and 5 µl of Rotor-Gene SYBR Green PCR Mastermix (QIAGEN®, Hilden, Germany). The thermal cycle consisted of an initial step at 95°C for 5 min for all genes followed by 40 cycles of 95°C for 5 s and 60°C for 10 s for *mcr*A and *pmo*A; 30 cycles of 95°C for 5 s and 55°C for 10 s for bacterial 16S rRNA; 35 cycles of 95°C for 5 s and 60°C for 10 s for *nir*S, *nir*K, and *nos*Z. Fluorescence was recorded in a single step at 80°C for 1 vs. for all genes except for *pmo*A, which was done at 82°C for 1 s. A melting curve was obtained after each amplification by increasing temperature from 60 to 94°C at a rate of 1°C s^{-1} in order to verify the specificity of amplification. Standard curves were obtained using gradient dilutions of standard plasmids containing archaeal 16S rRNA and *mcr*A genes and bacterial 16S rRNA, *pmo*A, *nir*S, *nir*K, and *nos*Z genes with known copy numbers.

Statistical Analysis

Each parameter was tested for normality of distribution and homogeneity of variances using Shapiro-Wilk's test and Levene's test, respectively. Gene abundance data were log-transformed and PDA data were ln-transformed to obtain normal

distributions. Comparisons were conducted using ANOVA with a factorial model (phases of rotation treatments, sampling dates, and the interaction of both) followed by Tukey's HSD test.

The PNA and pH were analyzed using the non-parametric Kruskal–Wallis test followed by a Wilcoxon Rank Sum test to determine significant differences between phases of the rotation treatments.

All these statistical analyses were performed in R version 4.1.0 with R studio version 1.4.1717.

In addition, principal component analysis (PCA) was performed and Pearson correlation coefficients were determined to test relationships between the variables related to methane and nitrous oxide cycles, separately for each cycle, using the "prcomp" function in the "vegan" package in R software.

All statistical significance was considered at $p < 0.05$.

RESULTS

Soil Properties and Rice Yield

Organic carbon (%) was similar for all soils sampled: cR (2.45 ± 0.28), Rrppp (2.41 ± 0.27), rRppp (2.38 ± 0.24), rrppP (1.94 ± 0.32), Rs (2.70 ± 0.44), and rS (2.21 ± 0.56).

Soil pH, ammonia, and nitrate were measured during the crop growing season in all treatments and redox potential was measured only in soils cultivated with rice (**Table 2**). Soil pH was not affected by the growing season or the rotation system. The soil redox potential decreased from rice tillering (30 dae) to flowering (98 dae) remaining reductive during ripening (124 dae), then soil became oxic in winter, after harvest draining. No significant redox potential differences between rice phases were observed along the sampling time. Ammonia and nitrate followed a seasonal pattern, with the mean of all soils showing the highest value for ammonia at 98 dae and the lowest at 124 dae, whereas nitrate was maximum at 30 dae and minimum at 98 dae.

Rice productivity was high, between 8,766 and 10,286 kg ha^{-1}, but no significant yield differences between rotations were found (**Supplementary Table 2**).

Dynamics of Bacteria and Archaea

The abundance and dynamics of the bacterial and archaeal populations were evaluated along the crop growing season in all rotations through the quantification of the 16Sr RNA gene. The abundance of Bacteria (**Figure 1A**) and Archaea (**Figure 1B**) was greatly affected by the season in all soils. Bacterial density was significantly higher in winter, when none of the soils were flooded, than in other seasons. Bacterial density was significantly higher in winter, when none of the soils were flooded, than in other seasons having a mean value for all treatments of 1.48×10^9 copies g^{-1} dry soil, which was at least 0.52 logs higher than the mean density in soils at any previous sampling date (**Figure 1A**). The same trend was observed for archaeal abundance since the mean value for all treatments was 1.15×10^7 copies g^{-1} dry soil in winter, a value that was significantly higher than the mean value at 30 dae, the beginning of the rice crop season, which was 4.57×10^6 copies g^{-1} dry soil. The increase in archaeal populations toward winter was less pronounced than for bacteria since in-between sampling dates (95 and 124 dae), intermediate archaeal densities were observed (**Figure 1B**). These results indicate that microbial biomass, Bacteria, and Archaea, increased significantly for all soils from the beginning of the rice growing season to winter.

Soils from different rotations' phases also showed significant differences in the abundance of bacterial and archaeal populations during the growing season. Consistent differences were observed within phases of the rotation rice–pasture, where soils of the second year of rice (rRppp) exhibited significantly higher density of bacteria, and archaea, than soils in the first year of rice (Rrppp). The archaeal density seems to be more responsive to previous crop since soils with rice following rice (cR and rRppp) showed significantly higher abundance of archaea than soils that had no rice in the previous summer (rrppP and rS) or soils that had rice and were preceded by 3 years of pasture (Rrppp).

Influence of the Rotation System on Microbial Parameters of the Methane Cycle

To know the effect of the rotation over the potential for methane emission, the abundance and activity of the microbial populations directly involved in methane production and consumption and the methane flux were evaluated at four sampling dates.

Methane Flux

Field measurements of methane flux were performed for all treatments during the annual crop cycle, but methane emission was only detected in flooded rice. The methane production rate was maximum at flowering (98 dae) and ranged between 14,102 g CH_4 ha^{-1} day^{-1} for Rrppp and 20,951 g CH_4 ha^{-1} day^{-1} for Rs with no significant differences between treatments (**Figure 2**). Methane production rate decayed significantly at ripening (124 dae), showing similar flux values for all treatments. Significant differences between treatments were observed only at 30 dae, when soils with rice as previous crop showed higher methane flux (1,699 and 1,435 g CH_4 ha^{-1} day^{-1} for cR and rRppp, respectively) than the soil with pastures in the previous summer (Rrppp).

Activity of Methane-Producing and Methane-Consuming Prokaryotes

Soil incubations in microcosm assays were set up to determine the methane production potential (MPP) and the methane oxidation potential (MOP) in the four phases of the rotations that had rice during the summer season. The MPP was strongly influenced by the season showing the highest mean value for all soils (35.3 nmol CH_4 h^{-1} g^{-1} dry soil) at 30 dae at the beginning of the rice crop season (**Figure 3A**). Then, the MPP decayed significantly at flowering (19.5 nmol CH_4 h^{-1} g^{-1} dry soil), ripening (21.8 nmol CH_4 h^{-1} g^{-1} dry soil), and at the winter after the rice harvest (20.4 nmol CH_4 h^{-1} g^{-1} dry soil).

On the other hand, some minor differences between rotations' rice phases were observed along the whole sampling time. Soil from the rice–pasture rotation having the first rice after pastures (Rrppp) showed significantly higher MPP than soil that had rice every summer (cR).

TABLE 2 | Soil properties for each rotation phase at dates corresponding to 30, 98, and 124 dae (days after rice emergence) and in the winter post-harvest.

Rotation phase	pH				*Eh* (mV)[(1)(2)]				NH_4^+ - N (mg kg^{-1})[(2)]				NO_3^- - N (mg kg^{-1})[(2)(3)]			
	30	98	124	w	30^A	98^B	124^B	w	30^{AB}	98^A	124^B	w^A	30^A	98^C	124^{BC}	w^B
cR	6.2	6.0	6.2	5.9	−203	−497	−441	nd	25.2	32.5	11.8	23.9	4.9	1.8	2.0	5.6
Rrppp	5.6	5.8	6.0	5.6	−338	−484	−433	nd	19.7	25.7	15.0	28.2	8.2	0.4	1.5	2.3
rRppp	5.8	5.8	5.9	5.7	−201	−497	−383	nd	22.3	26.5	13.3	21.9	5.4	0.5	2.4	3.6
rrppP	5.6	5.5	5.7	5.7	nd	nd	nd	nd	25,2	36.5	9.3	41.1	1.3	6.8	6.1	4.1
Rs	5.7	5.8	5.8	5.7	−323	−488	−436	nd	29.2	37.4	11.3	40.8	9.3	nd	2.0	3.2
rS	5.4	5.5	5.7	5.6	nd	nd	nd	nd	22,0	31,1	7.9	26.5	7.0	4.8	5.1	5.5

*(1) nd: not determined; Eh was only measured for flooded paddy soils. (2) Different capital letters indicate significant differences between mean values of soils of all rotations phases for different sampling dates ($p \leq 0.05$). (3) Different lowercase letters indicate significant differences between mean values of soils nitrate content of the different rotation phases for all the sampling dates: cR**[ab]**; Rrppp**[b]**; rRppp**[b]**; rrrppP**[b]**; Rs**[ab]** and rS**[a]**.*

The methanotrophic activity also revealed a profile influenced by the season (**Figure 3B**), with consistently high MOP rates for the four soils at flowering that decayed toward winter. The highest rate was observed at 98 dae with a mean value from the four soils of 1,409 nmol CH_4 consumed h^{-1} g^{-1} dry soil. At the beginning of the rice crop season (30 dae), soils in rotation with pastures exhibited very low methanotrophic activity, but other soils (cR and Rs) showed such dispersion among the values of replicated plots that made it doubtful to assess a behavior for all soils at this stage. No significant differences were observed between soils from different treatments along the season.

Abundance of Methanogenic and Methanotrophic Populations

The abundance of methanogens and methanotrophs was assessed through the quantification of the specific genes *mcr*A (methanogens) and *pmo*A (methanotrophs) in soils with rice during the season studied. Season influenced the abundance of methanogenic archaea, showing a consistent decrease in all soils from flowering to winter. The abundance of *mcrA* gene was significantly higher at flowering than at other sampling times. The mean value for the four soils was 1.55×10^5 copies g^{-1} dry soil at 98 dae, whereas at ripening and winter, the mean values were 1.05×10^5 copies g^{-1} dry soil (**Figure 4A**). The abundance of methanogens was quite heterogeneous among different soils at the beginning of the rice crop season (30 dae), but methanogens increased in all soils by flowering. At tillering, methanogens represented between 1.43 and 3.98% of total Archaea, with the lowest proportion for Rrppp soils and the highest proportion for cR. Soils from the continuous rice system (cR) showed the highest density of *mcrA* genes having a mean value of 2.09×10^5 copies g^{-1} dry soil along the four sampling dates ($p < 0.05$).

The abundance of the *pmoA* gene revealed a consistent increase of methanotrophic bacteria along the rice crop season for the four soils, reaching the highest density at winter (**Figure 4B**). The abundance of *pmoA* increased significantly from a mean value of 2.24×10^5 copies g^{-1} dry soil at rice flowering (98 dae) to 6.02×10^5 copies g^{-1} dry soil in winter. Similar to methanogens, methanotrophs showed at tillering different densities for different soils, but their abundance increased consistently at rice flowering in all soils. At tillering, methanotrophs represent between 0.004 and 0.029% of total Bacteria, with the lowest proportion for Rrppp soils and the highest proportion for cR. Contrastingly to methanogens, the abundance of methanotrophs continued to increase after flowering. An interesting trend was observed for soils from different rotations along the season. Soils with rice in the previous crop season (cR and rRppp) showed significantly higher density of *pmo*A genes than soils that had an upland crop (soybean in Rs) or perennial pasture (Rrppp) previously.

To evaluate the microbiological parameters of the C cycle contributing to soil variability in rice rotations, a PCA was plotted (**Figure 5**). PC1 and PC2 explained 71.1% of the overall variation for the four sampling dates, revealing that soil samples from different rotations showed higher dispersion at tillering (30 dae) tending to group together when the rice cropping cycle evolve until the winter post rice harvest. Whereas, no correlation was observed between activities and gene abundance, the abundance of *mcr*A and *pmo*A genes evidenced a weakly positive correlation ($r^2 = 0.445$, $p = 0.002$). These results confirmed the strong influence of the phenology of rice crop in the microbial-specific populations of the methane cycle. To analyze the effect of the rotations, PCA was performed for physicochemical and microbiological soil parameters at two sampling dates separately. At tillering, PC1 and PC2 together explained 67.9% of the overall variation (**Figure 6A**). This biplot shows the separation mainly on the PC1 of rice-cropping systems having no rice in the previous summer (Rs and Rrppp) compared to systems that had two consecutive summers with rice (cR and rRppp), which displayed the highest value of *mcr*A and *pmo*A gene copies. The abundance of *mcr*A and *pmo*A genes were positively correlated ($r^2 = 0.729$, $p = 0.007$). In winter, PC1 and PC2 together explained 65.8% of the overall variation (**Figure 6B**). This biplot shows high environmental heterogeneity among replicates and the separation mainly on the PC1 of Rs compared to other rotation systems. The abundance of *mcr*A and *pmo*A genes was also positively correlated ($r^2 = 0.668$, $p = 0.0176$) and seems to explain the clustering of the other three soils.

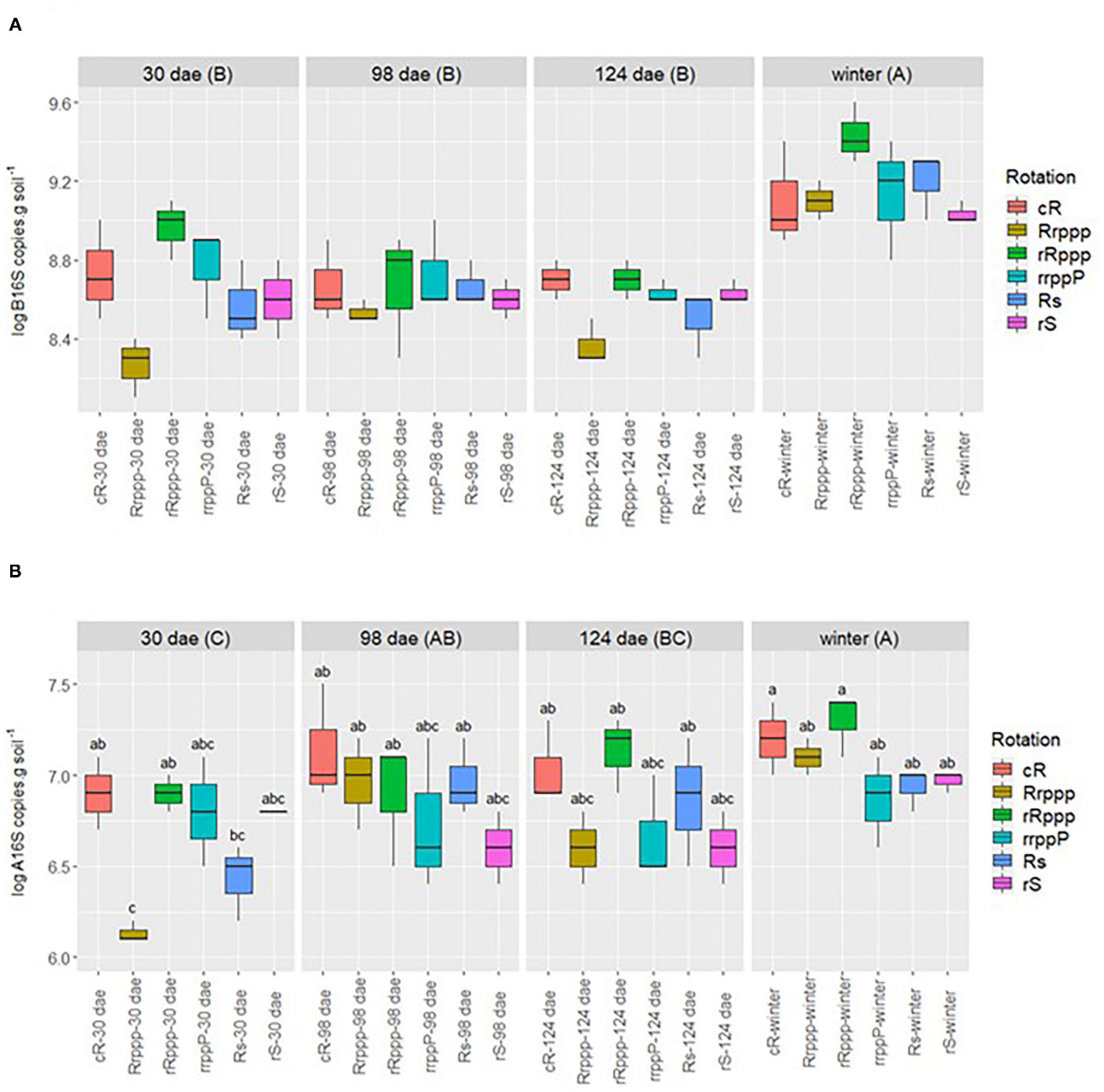


FIGURE 1 | Dynamics of 16S rRNA gene copies of Bacteria **(A)** and Archaea **(B)** by g dry soil for all treatments. Sampling dates: days after rice emergence (dae) for all rotations' phases (with or without rice) and winter (259 days after rice emergence). Uppercase letters indicate significant differences between sampling dates for all phases. Lowercase letters next to each sample name indicate significant differences for rotation phases along the year. The differences ($p < 0.05$) between phases for log B 16S rRNA along all the sampling time were continuous rice rotation (cR^{ab}); rice–pasture rotation at the first ($Rrppp^{c}$); and second ($rRppp^{a}$) year of rice, or at the third year ($rrppP^{ab}$) of pasture; rice–soybean rotation having rice (Rs^{abc}) or soybean (rS^{bc}) at the crop season. The differences ($p < 0.05$) between phases for log A 16S rRNA along all the sampling time were as follows: cR^{a}, $Rrppp^{b}$, $rRppp^{a}$, $rrppP^{b}$. Rs^{ab}, or rS^{b}. Significant differences of the interaction phases*date are presented in the figure with lowercase letters.

Influence of the Rotation System on Microbial Parameters of the Nitrous Oxide Cycle

Nitrous oxide emission fluxes were not detected for any of the crops or pasture in the dates studied.

Denitrification Related Genes and Potential Activity

Potential denitrification activity of all soils was significantly higher at rice tillering, 30 dae (average 120 μg $N\text{-}N_2O$ g $soil^{-1}$ h^{-1}) than at the other rice stages or in winter and decreased along the year (**Figure 7**). When considering only the four soils having

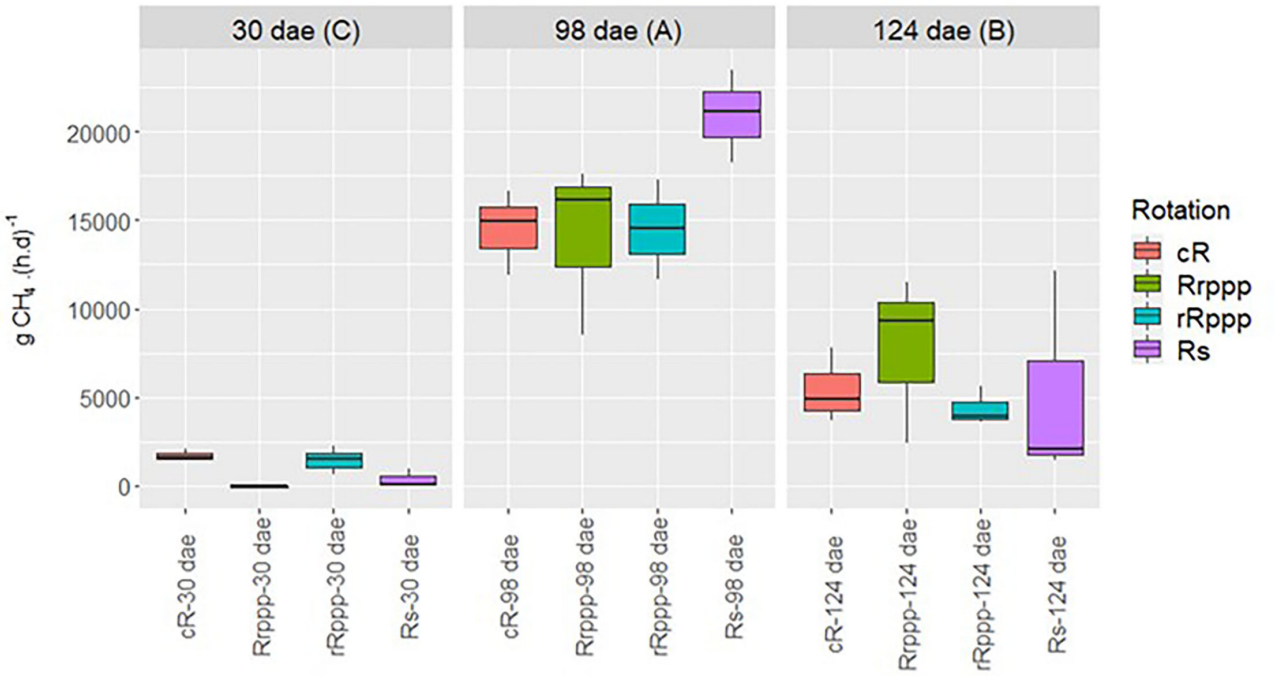


FIGURE 2 | Dynamics of methane flux (g CH_4 ha^{-1} day^{-1}) for rotations' phases with flooded rice. Uppercase letters indicate significant differences between sampling dates for all rice phases. Sampling dates: days after rice emergence (dae) for all treatments. Rotation phases: continuous rice rotation (cR); rice–pasture rotation at the first (Rrppp) or second (rRppp) year of rice; rice–soybean rotation having rice (Rs) at the crop season.

rice, rRppp showed higher denitrification activity than Rs, 88 and 51 μg N-N_2O g $soil^{-1}$ h^{-1}, respectively.

The behavior of some of the functional communities related to N_2O emission by denitrification (*nir*K, *nir*S, and *nos*Z) differed for each gene. The *nir*K abundance was always greater than *nir*S, ∼1 order of magnitude, and the copy number of both genes was highest in winter and lowest at the rice flowering stage (98 dae, **Table 3**). The *nir*K abundance was higher for both rRppp and rrppP than for Rs and Rrppp (between 2.5 and 1.3 × 10^7 *nir*K copies g^{-1}). In a different way, the abundance of *nir*S was greater for rRppp (2.2 × 10^6) than for Rs, rrppP, and Rrppp, (1.5 × 10^6). The highest abundance of *nir*S genes was also achieved in winter and the lowest was attained at 98 dae with significant differences among dates (**Table 3**).

The copy number of *nos*Z was not significantly different for all phases and rotations or sampling dates. However, when analyzing only the tillering stage (30 dae), the second rice after pasture (rRppp) had also higher *nos*Z abundance than the first rice after pasture (Rrppp), 2.3 vs. 1.9 × 10^6. The highest PDA was preceded for highest denitrification gene abundance in the same phase of the rotation.

Ammonia Oxidation Potential Activity and amoA Genes Abundance

On the other hand, potential nitrification activity data were not normally distributed, and a non-parametric ANOVA showed that cR ranked lower than Rrppp, rS, and rrppP, these latter two had no rice during the year. At tillering rice stage, ammonia oxidation was lower for rRppp and cR (the two treatments that have had rice the previous season) than for rS and rrppP, average 2 and 23 mg N-NO_2^- g^{-1} h^{-1}, respectively (**Supplementary Figure 1**).

Archaeal ammonia oxidizers were always higher than bacteria (log *amo*A copies g^{-1} soil 6.1 and 5.2 on average, respectively). Bacteria ammonia oxidizer number for rice soils was only different between dates, being higher for winter than for 98 or 124 dae (**Figure 8A**). However, the archaeal *amo*A copy number was only different among rotations' phases, where the second rice for the classical rotation (rRppp) presented higher values than the first rice of this rotation (Rrppp) along the year, 2.3 vs. 0.7 × 10^6 (**Figure 8B**).

The PCA for microbiological parameters of the N cycle shows that 63.8% of the variability in rice soils was explained by the two main components (**Figure 9**). A trend in a seasonal grouping of the samples can be observed along the PC1 associated with the increase of all genes at 124 dae and winter. A high positive correlation between *nir*K and *nir*S genes (r^2 = 0.796, p = 0.001) was observed, with lower positive correlation coefficients for the complete denitrification set of genes *nir*K and *nos*Z (r^2 = 0.322, p = 0.026) or *nir*S and *nos*Z (r^2 = 0.354, p = 0.014). The density of ammonia oxidation genes from bacteria and archaea was also positively correlated (r^2 = 0.556, p = 0.001). To analyze the effect of the rotations on the N cycle, PCA was performed for physicochemical and microbiological soil parameters at two sampling dates separately. At the beginning of the rice crop season (30 dae; tillering rice stage) both principal components (PC1 and PC2) explained

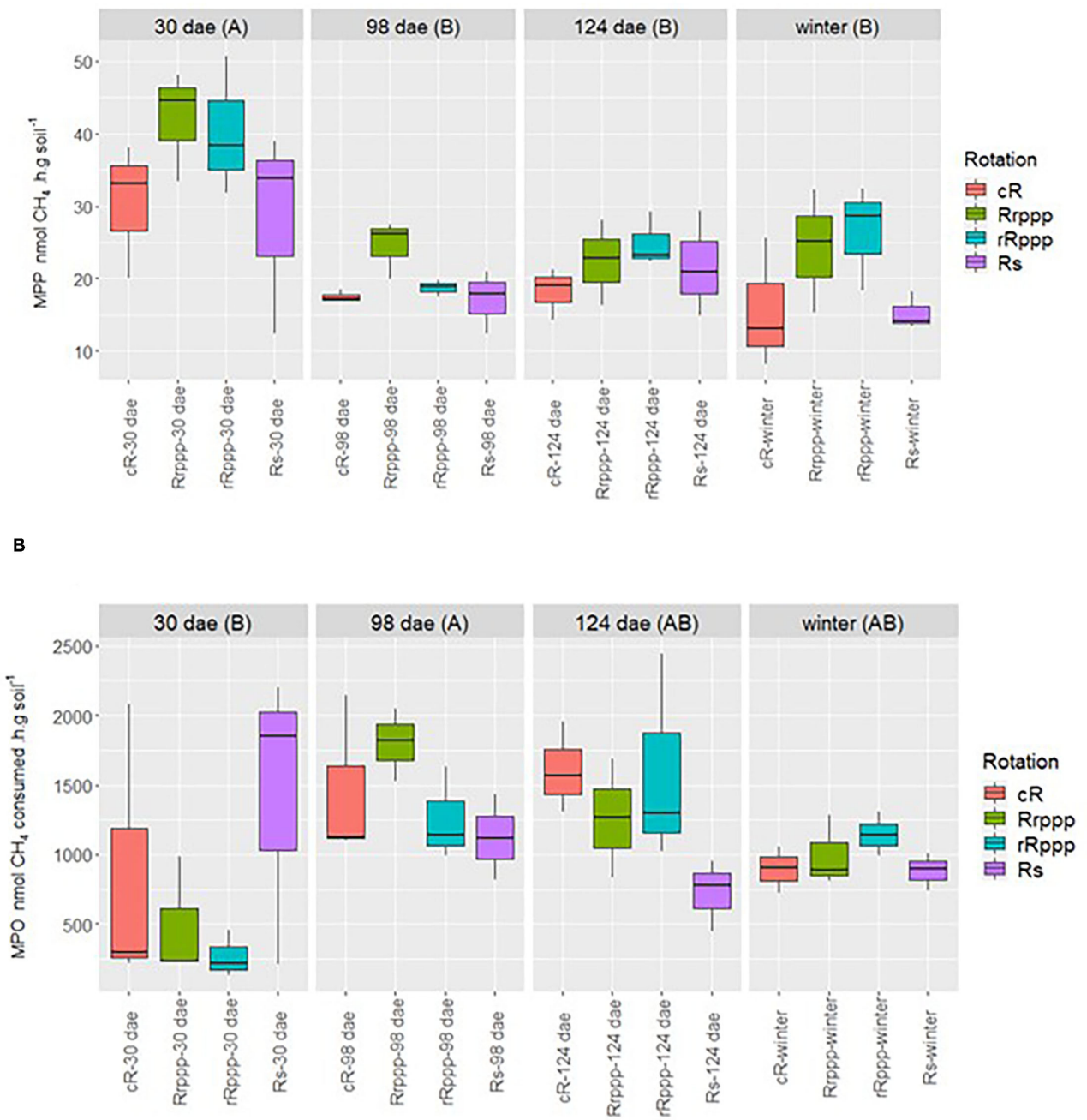


FIGURE 3 | Dynamics of **(A)** methane production potential (MPP) and **(B)** methane oxidation potential (MOP) in soils that had rice in summer. Uppercase letters indicate significant differences between sampling dates for all rice phases. Sampling dates: days after rice emergence (dae) for all treatments. Lowercase letters next to each sample name indicate significant differences for these rotation's phases along the year. Rice phases of rotations: continuous rice rotation (cR); rice–pasture rotation at the first (Rrppp) or second (rRppp) year of rice; rice–soybean rotation having rice (Rs) at the crop season. For MPP: cR^{b}; $Rrppp^{a}$; $rRppp^{ab}$; and Rs^{ab}. There were no significant differences between rice phases for MOP.

almost 70% of the overall variation of physicochemical and microbiological soil parameters of N cycle contributing the most to soil rotation variability (**Figure 10A**). This biplot shows the separation mainly on the PC1 of rice-cropping systems having no rice in the previous summer (Rs and Rrppp) compared to systems that had two consecutive summers with rice (cR and rRppp),

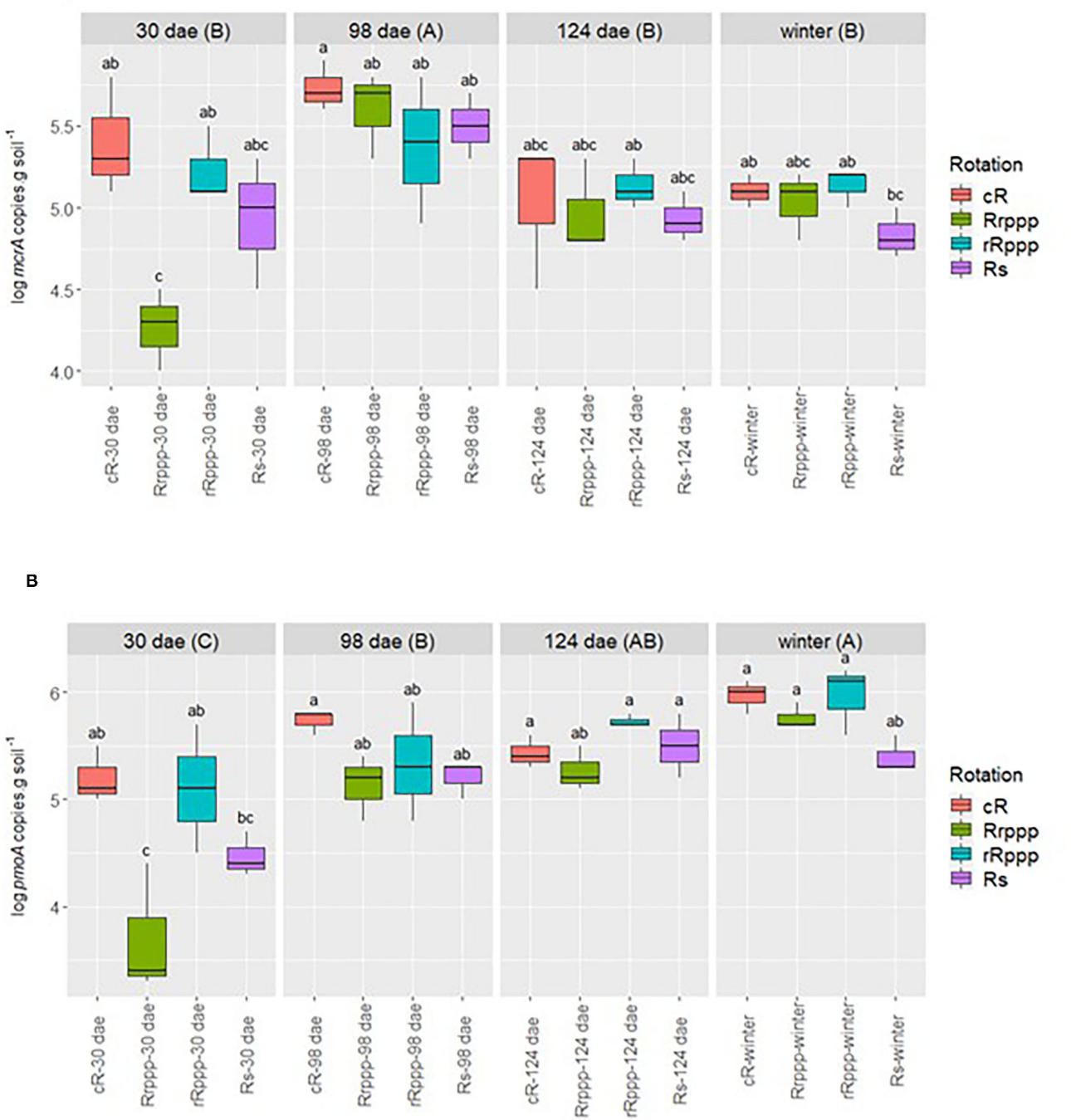


FIGURE 4 | Dynamics of **(A)** *mcr*A and **(B)** *pmo*A genes in soils having rice in summer. Uppercase letters indicate significant differences between sampling dates for all treatments. Sampling dates: days after rice emergence (dae) for all treatments. Treatments: continuous rice rotation (cR); rice–pasture rotation at the first (Rrppp) or second (rRppp) year of rice; rice–soybean rotation having rice (Rs) at the crop season. The differences ($p < 0.05$) between phases for *mcr*A along all the sampling time were cR^{a}, $Rrppp^{b}$, $rRppp^{ab}$, and Rs^{ab}, and those for *pmo*A were cR^{a}, $Rrppp^{b}$, $rRppp^{a}$, and Rs^{b}. Significant differences of the interaction phase*date are presented in the figure with lowercase letters.

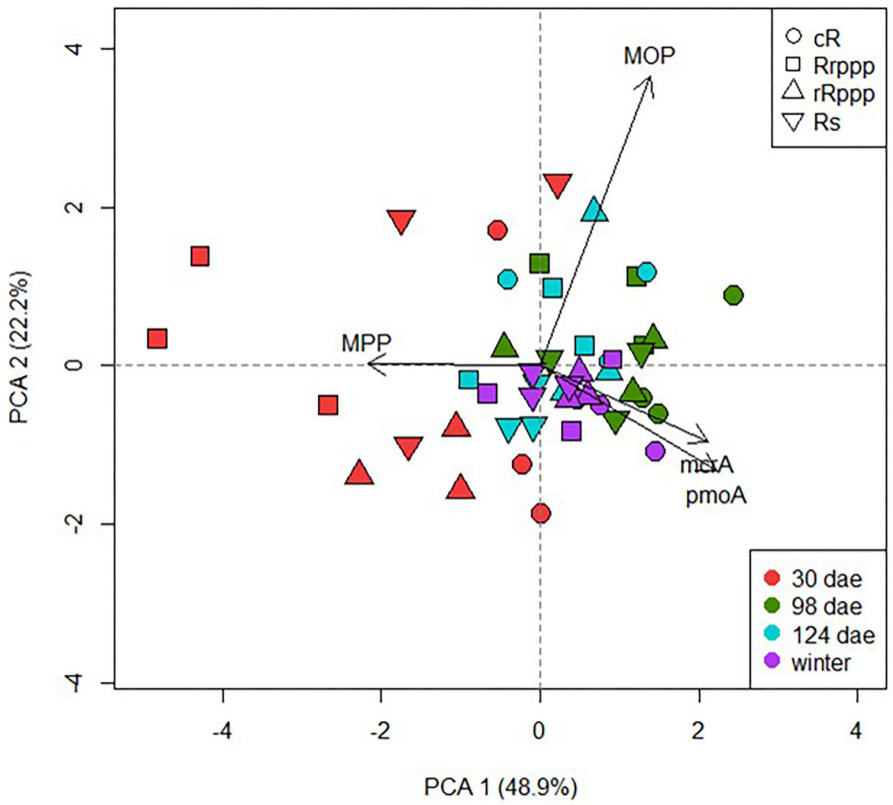


FIGURE 5 | Scatter plot of the two first axis resulting from the principal component analysis displaying the variance explained along the sampling dates among the different rice phases of the rotations: continuous rice rotation (cR); rice–pasture rotation at the first (Rrppp) or second (rRppp) year of rice; rice–soybean rotation under rice (Rs). Based on microbiological parameters (MPP, methane production potential; MOP, methane oxidation potential; mcrA gene copies, and pmoA gene copies).

which displayed the lowest value of soil nitrate content at this stage. The gene *nir*K abundance was correlated with the other denitrification genes, *nir*S and *nos*Z, and with the abundance of *amo*A genes, either bacterial or archaeal. The *nos*Z copy number correlated with redox potential ($p = 0.034$). Soil nitrate content correlated negatively with *nir*S and *nos*Z abundance and positively with PNA and *amo*A abundance. At winter sampling, PC1 and PC2 together explained only 60.6% of the overall variation (**Figure 10B**). This biplot shows high environmental heterogeneity among replicates and the separation mainly on the PC1 of cR, the most intensive rice system, compared to other rotation systems. PDA, PNA, and *nir*K copies were positively correlated at winter sampling.

DISCUSSION

The rice production system commonly used in Uruguay consists in a cycle of 2–3 consecutive years of rice cropping followed by 2–4 years of perennial pastures of grasses and legumes seeded immediately after rice harvest and grazed by cattle and sheep. This system allows a sustainable increase in productivity, minimizing the use of agrochemicals and contributing to greater resource-use efficiencies (1, 32), although there are still opportunities for sustainable intensification (3). The implementation of more intensive rotation systems, either by increasing the proportion of rice or other crops like soybean and reducing the pasture phase, has become an alternative for farmers to increase productivity and economic options.

The transition from more intensive to less intensive rice rotation system influences microbial community structure (8, 33–35), but the information about the opposite conversion is scarce. In the present work, we studied the response of microbial communities to the intensification, in early steps of this conversion (4 years after intensification was implemented). We examined the abundance and activity of bacteria and archaea linked to GHG emission across three rice growth stages and at one time after rice harvest in soils from rice rotations with different levels of intensification.

The abundance of Bacteria and Archaea was greatly affected by the season in all soils from the three rotational systems studied. It has been observed that the stage of rice growth from seedling to rice maturing had a significant effect on soil abundance of bacterial 16S rRNA gene copies (12) but not in archaea (36). Breidenbach and Conrad (37) reported that bacterial and archaeal 16S rDNA copy numbers were highest during rice growth at reproductive stage. The practice of leaving the straw

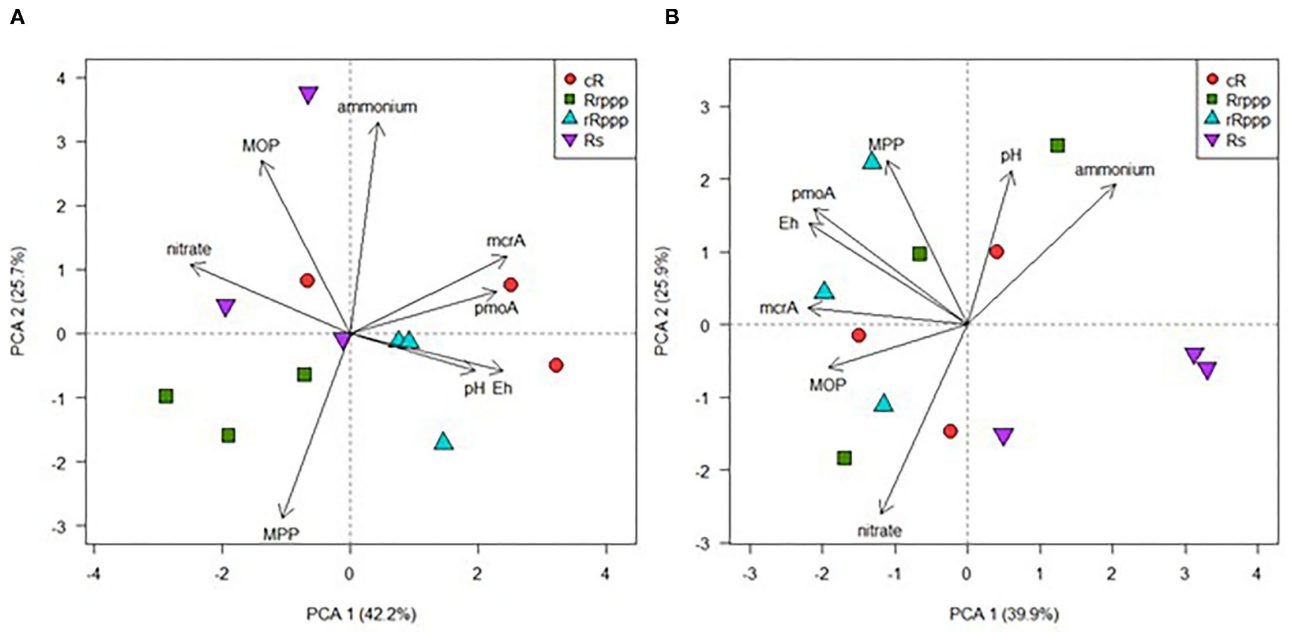


FIGURE 6 | Scatter plot of the two first axis resulting from the principal component analysis displaying the variance explained at 30 dae or tillering **(A)** and at winter **(B)** among the different rice phases of the rotations: continuous rice rotation (cR); rice–pasture rotation at the first (Rrppp) or second (rRppp) year of rice; rice–soybean rotation under rice (Rs). Based on soil properties: physicochemical (nitrate and ammonium concentrations, redox potential, and pH) or microbiological parameters (MPP, methane production potential; MOP, methane oxidation potential; *mcr*A gene copies, and *pmo*A gene copies).

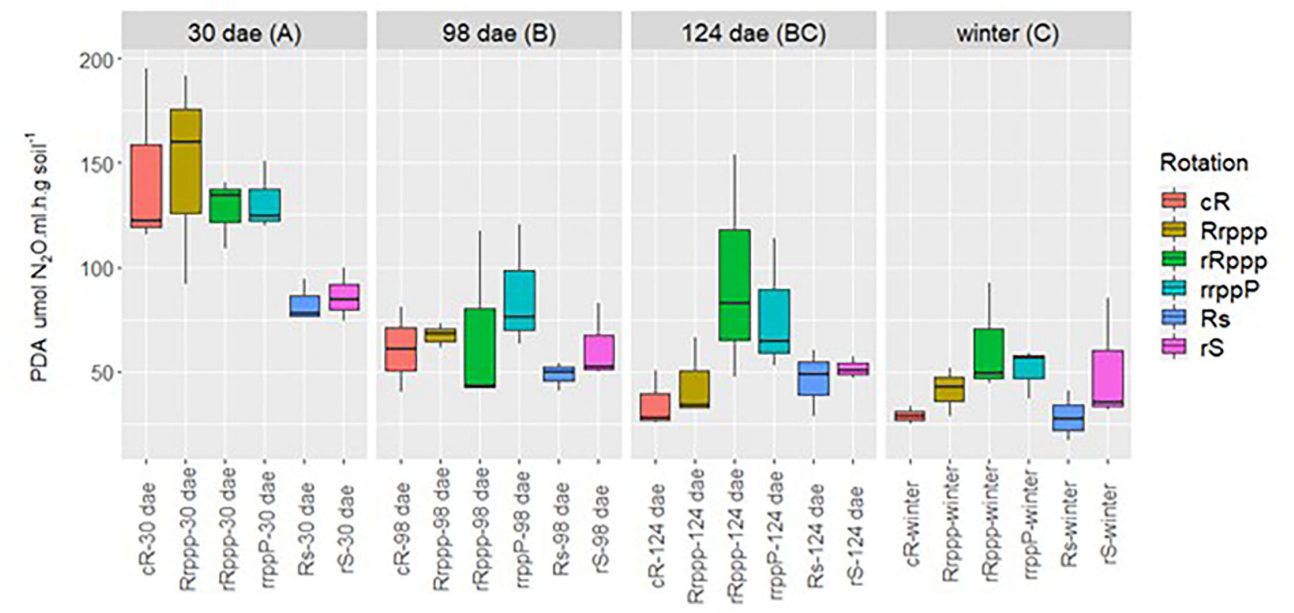


FIGURE 7 | Dynamics of potential denitrification activity (PDA) by g dry soil along the sampling time for all soils. Sampling dates: days after rice emergence (dae) for all treatments (with or without rice) and winter (259 days after rice emergence). Uppercase letters indicate significant differences between sampling dates for all rotations' phases. Lowercase letters next to each sample name indicate significant differences for these phases along the year. Rotations' phases: continuous rice rotation (cR^{ab}); rice–pasture rotation at the first ($Rrppp^{ab}$) and second ($rRppp^{a}$) year of rice, or at the third year ($rrppP^{a}$) of pasture; rice–soybean rotation having rice (Rs^{b}) or soybean (rS^{ab}) at the crop season sampled.

TABLE 3 | Abundance of denitrification marker genes for the different crops and rotation systems at 30, 98, and 124 dae (days after rice emergence) and in winter (W).

Rotation phase	Log gene copy number g^{-1} soil*												
	nirK				*nirS*				*nosZ*				
	30[C]	98[C]	124[B]	W[A]	30[C]	98[D]	124[B]	W[A]	30	98	124	w
cR	7.06 ± 0.32	6.87 ± 0.21	7.23 ± 0.26	7.30 ± 0.20	6.02 ± 0.15	5.60 ± 0.20	6.23 ± 0.06	6.38 ± 0.20	6.69 ± 0.17	6.73 ± 0.25	6.71 ± 0.20	6.54 ± 0.12
Rppp	6.75 ± 0.06	6.84 ± 0.23	7.13 ± 0.21	7.35 ± 0.06	5.66 ± 0.15	5.55 ± 0.12	6.25 ± 0.06	6.57 ± 0.06	6.36 ± 0.06	6.71 ± 0.00	6.64 ± 0.38	6.66 ± 0.06
rRppp	7.18 ± 0.15	6.86 ± 0.23	7.31 ± 0.17	7.67 ± 0.29	6.22 ± 0.21	5.58 ± 0.15	6.38 ± 0.20	6.61 ± 0.10	6.79 ± 0.12	6.46 ± 0.26	6.83 ± 0.38	6.68 ± 0.06
rrppP	7.09 ± 0.20	6.98 ± 0.21	7.44 ± 0.21	7.45 ± 0.42	5.91 ± 0.26	5.45 ± 0.25	6.34 ± 0.10	6.50 ± 0.36	6.63 ± 0.30	6.58 ± 0.26	6.85 ± 0.15	6.71 ± 0.35
Rs	6.93 ± 0.21	6.87 ± 0.12	7.08 ± 0.06	7.42 ± 0.00	5.85 ± 0.25	5.42 ± 0.15	6.20 ± 0.26	6.51 ± 0.10	6.56 ± 0.15	6.53 ± 0.15	6.67 ± 0.30	6.58 ± 0.06
rS	7.05 ± 0.06	6.81 ± 0.20	7.39 ± 0.23	7.45 ± 0.06	6.10 ± 0.17	5.61 ± 0.26	6.50 ± 0.10	6.57 ± 0.17	6.49 ± 0.06	6.42 ± 0.00	6.91 ± 0.30	6.63 ± 0.06

*Significant differences were only observed for *nirK* and *nirS* genes among sampling dates and are indicated for each gene with different capital letters ($p \leq 0.05$).

over the soil after crop harvest in Uruguayan no-till systems likely increases the organic matter available for microbial growth post crop harvest. It has been observed that bacteria, archaea, and methanogenic communities strongly responded to rice straw amendment in laboratory soil incubations by increasing their abundance over the straw degradation period (38). Therefore, our results indicate that the biomass of both archaea and bacteria reached similar densities in rice paddy soils after summer crop season than soils kept under pasture and may indicate that the degradation of vegetal residues is a sustainable agricultural strategy to maintain soil microbial biomass.

The intensification of rice cropping seems to influence the archaeal community since soils with at least two summers of rice showed significantly higher abundance of archaeal 16S rRNA genes than soils that had no rice in the former summer or soils that having rice in the previous summer were preceded by 3 years of pasture. A similar trend of increase was observed for *mcr*A gene copy numbers in soils having rice more frequently. Consecutive rice cropping has been reported as a main factor raising the archaeal community in soil, moreover due to the increase of methanogenic archaea (8).

Methane emission was only detected in the four soils having rice when soil was flooded, as it was expected since waterlogged soils sustain anaerobic conditions for C fermentation and methanogenesis (39, 40). The highest flux was measured at rice flowering, with rates that were not significantly different for the rotational treatments, then decayed by ripening (**Figure 2**). Methane flux was minimum at tillering, but significant differences were observed between rotations. Soils under rice in the previous summer showed higher methane flux than soils with pasture or soybean in the previous summer. A similar pattern for methane emission, maximum at flowering, medium at ripening, and minimum at tillering, was observed previously for a more frequent sampling study in Uruguayan systems (10) as well as for other regions where rice is cultivated (41, 42). The maximum CH_4 flux measured was higher than the previous measurements (10) but consistent with fluxes reported for other temperate rice cultivated in the region with crop residues maintained on the soil surface (43) or with transplanted rice without organic matter amendments for two rice varieties cultivated in Japan (44). The differences observed in methane emission among soil rotations at tillering were not endorsed by the potential for methane production (MPP) or consumption (MOP), since rates measured for all soils were not significantly different at this stage of plant growth (**Figure 3**). The MPP was strongly influenced by the season showing the highest mean value at 30 dae for all soils. The organic matter of the stubble from winter cover pastures may result in an increase of fermentable substrates for methanogenesis after soils were flooded and could explain this high potential for methane production. Since methanogenic activity depends on the small range of products of secondary fermentation, it is feasible that at this step, the higher methanogenic activity would be due to the supply of more direct methanogenic substrates rather than to the abundance and activity of methanogenic archaea. The soil organic C determined previously to rice sowing was not different among different rotations, but crop residues from

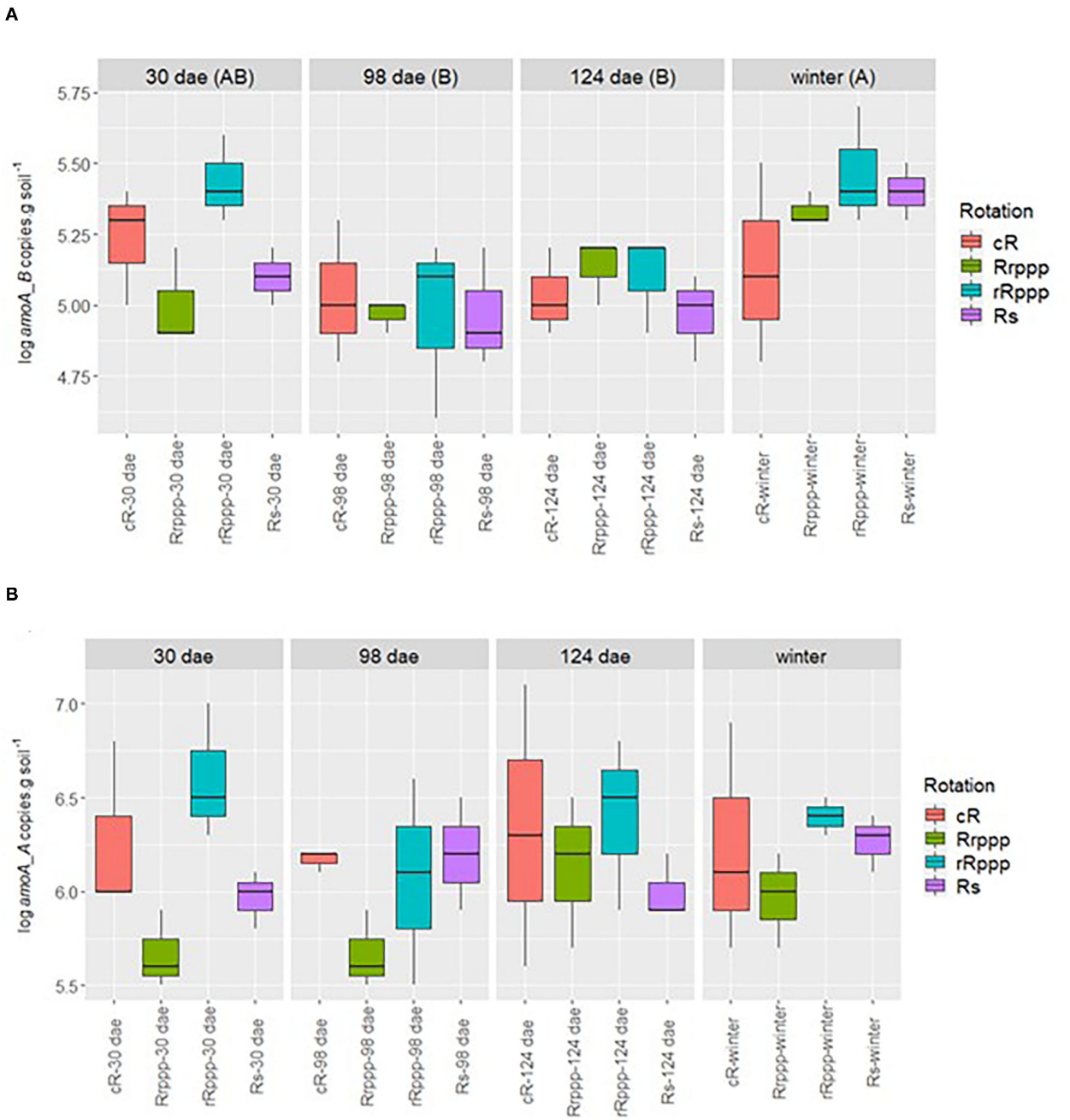


FIGURE 8 | Dynamics of *amo*A gene copies of Bacteria **(A)** and Archaea **(B)** by g dry soil along the sampling time for the soils that had rice during the year. Sampling dates: days after rice emergence (dae) for all treatments (with or without rice) and winter (259 days after rice emergence). Treatments: continuous rice rotation (cR); rice–pasture rotation at the first (Rrppp) or second (rRppp) year of rice; rice–soybean rotation having rice (Rs). Bacteria *amo*A were only significantly different for sampling date. Archaea *amo*A were only significantly different for treatments: cR^{ab}; $Rrppp^{b}$; $rRppp^{a}$; and Rs^{ab}.

different winter cover pastures should be considered in these no-till systems as nutrient source for methanogenic archaea. Methane emissions are stimulated by improving C substrates either directly, by plant straw amendment (38, 45, 46) and N organic fertilization (47), or indirectly, by increasing the availability of labile forms of soil organic carbon in no-tillage systems (48). In addition, the priming effect that straw addition has on soil organic carbon degradation, by causing a release of dissolved organic carbon, also contributes to increase substrates for methane production (49, 50).

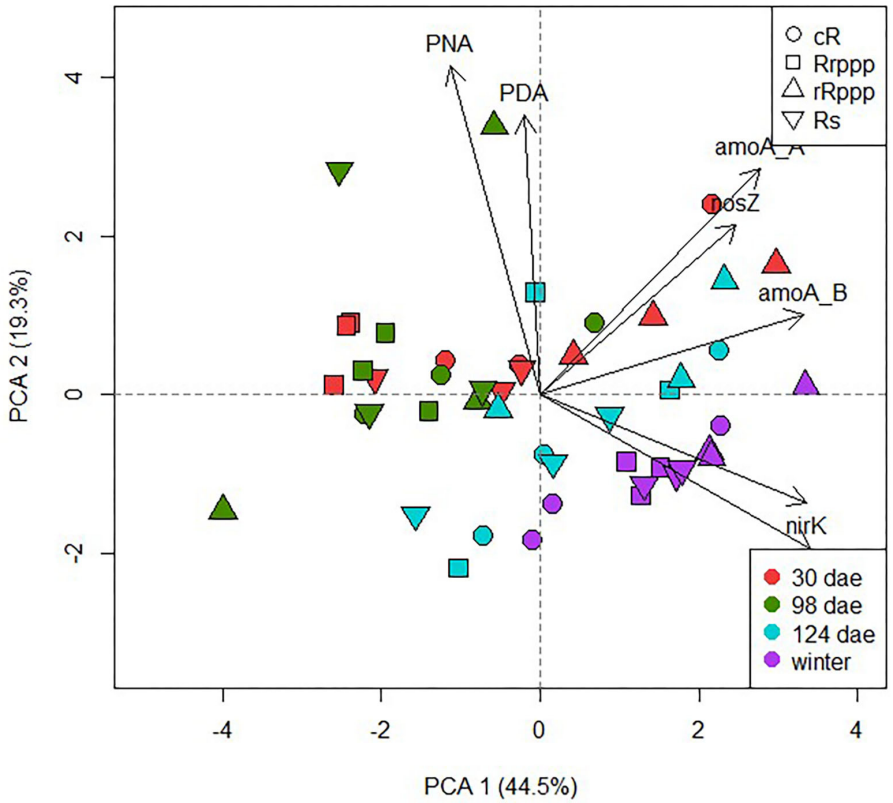


FIGURE 9 | Scatter plot of the two first axis resulting from the principal component analysis displaying the variance explained along the sampling dates among the different rice rotations: continuous rice rotation (cR); rice–pasture rotation at the first (Rrppp) or second (rRppp) year of rice; rice–soybean rotation under rice (Rs). Based on microbiological parameters (PDA, potential denitrification activity; PNA, potential nitrification activity; *nir*K, *nir*S, and *nos*Z gene copies, and *amo*A of bacteria and archaea gene copies).

The main drivers for methane emission are the density and activity of methanogens and methanotrophs, as well as soil properties, rice cultivar, and agronomical practices that affect the C availability for anaerobic fermentation (39, 51). We observed that the highest MPP did not overlap with the highest methane emission measured at flowering. It is suitable to consider that the influence of the plant over microbial community increases with time, mainly by the photosynthesized compounds released to the rhizosphere. Root organic carbon is the main C source for methane emission and increases as the plant grows (49, 52). The exudation rates are lowest at seedling stage, increased until flowering, but decreased at maturity (53). Therefore, the high methane emission observed at flowering is likely due to the increased C released by the plant, whereas the microcosm assays made for the determination of MPP had only C substrates derived from the soil. Furthermore, the highest methane oxidation potential coincided with the higher methane emission (**Figure 3B**), namely, when more methane was available for methanotrophs, then the MOP decreased consistently from flowering to winter of all soils. Lee et al. (52) also reported that methane emission was positively and significantly correlated with activity of methanotrophs and methanogens measured through transcripts of *pmo*A and *mcr*A genes.

The abundance of methanogens was quite heterogeneous among different soils at the beginning of the rice crop season (30 dae), but methanogens increased by flowering and showed similar abundances at the two following sampling dates in all rice rotational soils. This influence of the rice growing season has been reported previously for the abundance of rhizospheric *mcr*A and *pmo*A genes with sequential highest values for *mcr*A genes preceding the highest values for *pmo*A genes (47). In wheat–rice rotational fields, a significant effect of the rice growth season has been observed for the abundance of *mcr*A and *pmo*A genes with higher abundance of these populations at rice maturity (36). The composition of methanogenic archaea instead, was less influenced by the rice growing stage in similar wheat–rice rotational fields (54).

It should be considered that oxygen is a determining factor for the spatial distribution of bacterial and archaeal communities in rice paddy soils, with aerobic methanotrophs being higher near the oxic–anoxic interface whereas methanogens predominate associated to the rhizosphere (55). Therefore, by sampling bulk soil, we might have underestimated the methanogenic

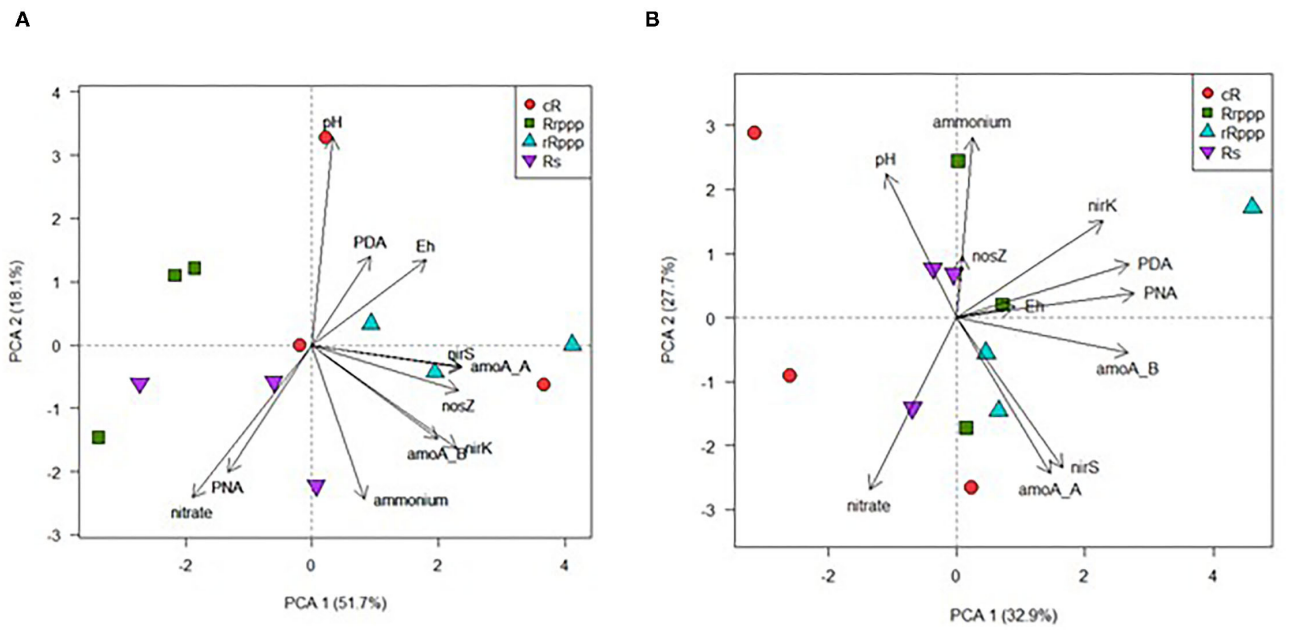


FIGURE 10 | Scatter plot of the two first axis resulting from the principal component analysis displaying the variance explained at 30 dae or tillering **(A)** and at winter **(B)** among the different rice rotations: continuous rice rotation (cR); rice–pasture rotation at the first (Rrppp) or second (rRppp) year of rice; rice–soybean rotation under rice (Rs). Based on soil properties: physicochemical (nitrate and ammonium concentrations, redox potential, and pH) or microbiological (PDA, potential denitrification activity; PNA, potential nitrification activity; *nir*K, *nir*S, and *nos*Z gene copies, and *amo*A of bacteria and archaea gene copies).

population, mainly in young plants with small roots, since at the rice reproductive stage, most of the soil was covered with rice roots.

Contrastingly to methanogens, the growth of methanotrophs continued after flowering, reaching highest abundances in winter in all soils. A similar increase from tillering to maturity was reported for a different rice production system with annual double-rice paddy soil, where rice seedlings were transplanted into flooded soil (56). The increase in the methanotrophs observed after flowering is consistent with the availability of methane, the specific substrate for this population, which is produced under flooding mainly at flowering. Oxygen is a limiting substrate for methanotrophs under flooding conditions. It has been suggested that the incorporation of winter cover crops to flooded soil during rice transplanting decreases the oxygen availability by organic matter consumption, limiting the growth of methanotrophic bacteria (56). The high abundance of methanotrophs observed in winter may be due to higher substrate availability, because of the oxygen increase after rice harvest, combined with the methane that might be still trapped in soil and rice roots.

Although the rice growth stage had pronounced effects on abundance and activity of microbial populations linked to methane production and consumption, we observed slight differences that may be attributed to rice rotations mainly at rice tillering. Liu et al. (13) reported that in rotational fields, where rice alternated with soybean under two kinds of rotational intervals, the abundance of the methanogenic archaeal populations decreased to about one-tenth compared with consecutive paddy rice soil along several sampling seasons and rice growing stages. The authors assert that the upland conversion of flooded paddy soils for 1 year or longer than 1 year affected the methanogenic archaeal community. We only observed minor differences of methanogens related to the rice rotation at tillering, when rice followed by rice (cR and rRppp) had higher amounts of methanogens than rice soils preceded by soybean or pastures (Rs and Rrppp). These slight differences may be because the intensification of rice in our systems has been implemented quite recently. PCA showed that rotational soils with two consecutive summers under rice cropping separated from soils having pasture or soybean previously, with the abundance of *pmo*A and *mcr*A gene copies as main correlated factors explaining this grouping (**Figure 6**). These results suggest that, in our system, where less intensive rice–pasture cropping was recently converted to more intensive systems, the abundance of methane-specific populations was responsive to the increase in rice frequency. This response was observed at rice tillering, when microbial communities experimented the whole rotational previous management (crop in summer + cover pasture in winter), but not at winter after all soils had rice as summer crop. It is appropriate to highlight that rice residues are considerably higher than other plant residues, giving higher amounts of C for microbial decomposition in winter in our no-till systems. The higher amounts of C in soils at tillering that were preceded by rice in summer may improve the methanogenic substrates and therefore populations

linked to methane production and consumption. Zhou et al. (57) reported that MPP and MOP as well as the abundances of *mcr*A and *pmo*A genes increased with the co-incorporation of green manure to the rice straw, suggesting that a rational utilization of leguminous green manure combined with rice straw applications may mitigate methane emissions by reducing the C/N ratio in the residue and consequently reduce the dissolved organic carbon from the residue that may serve as substrate for methanogenesis.

The episodic nature of N_2O emissions (58) did not allow us to detect any N_2O peak when sampling once for each rice growth stage or in winter. However, some peaks of N_2O emission had been measured in the same rice field at other crop seasons in the period until 50 days after rice emergence (10).

Nitrification and denitrification, the main processes associated with N_2O emission in agricultural soils, are influenced by several soil and plant variables, which may be modified through agricultural management practices (e.g., fertilization, crop rotation, tillage, or irrigation) (59). A main reason for N_2O emissions from agricultural soils is the application of inorganic/organic fertilizers when the crops cannot uptake all the applied nitrogen (N) due to the requirements in each growth stage. The fertilization in this case, which adjusts N synthetic rates calculated considering the background soil mineral N and the expected available N from mineralization of cover crop residues, and the low use of N-fertilizer in Uruguayan rice systems (60) may accomplish a high N-use efficiency.

Denitrification seems to be more relevant as a N_2O source than nitrification when studying different agroecosystems (61). PDA that was highest at 30 dae (rice tillering stage) may be influenced by the highest soil nitrate content at this stage and recent flooding, generating redox potential conditions for this process. In this work, PDA was lower in crop soils than in pastures as has been previously reported (62). However, PDA incubation conditions may not favor all denitrifiers that are physiologically very diverse (63). Furthermore, rhizosphere with its oxic/anoxic interphase offers a favorable habitat for coupled nitrification–denitrification (64), but in this work, bulk soil was sampled.

The *nir*K functional community was more abundant than *nir*S as has been previously reported for some paddies (65–67). The abundance of both genes was highest in winter, the only soil sampled without flooding, but also nutrient availability changes may influence both microbial communities (68). As denitrifying microorganisms are very diverse metabolically and denitrification is an alternative growth mechanism, denitrifiers can be active and grow by relying on other electron acceptors. In this respect, Hallin et al. (69) did not find any response from the denitrifier community to agricultural management. NosZ is responsible for N_2O reduction to N_2 and includes two distinct clades (70), though the most common of type I of denitrifiers was quantified here. Besides, not all denitrifiers possess the *nos*Z gene (71), and its proportion may change along the sampling dates. Considering only the rice crop, all these denitrification genes' copy number was higher for the second rice than for the first in the traditional rotation, the less intensive of the rotations considered. In the previous winter, rRppp had ryegrass, whose higher C:N ratio and greater biomass (22), which included the rest of the rice of the previous season, may explain this highest activity.

The potential nitrification activity ranked higher for the rotations' phases without rice and at rice tillering for the phases of the rotations without rice in the previous season, which were the soils with lower ammonia content. Although this activity differs from the actual *in situ* rate due to the broad physiological diversity of ammonia oxidizers (63), its increase in the aerobic phase of the rotations seems reasonable. Bacterial ammonia oxidizers for the soils with rice during the sampling period of this work were higher at winter, the only sampling date without flooding. Moreover, the bacterial ammonia oxidizers did not change with the different rotations while the abundance of *amo*A from archaea increased in rice after ryegrass. Recently, Rütting et al. (72), applying a combination of ^{15}N tracers and selective inhibitors, confirmed that AOB activity increased with high ammonium addition and that of AOA was high for soils with low, continuous NH_4^+ production like in our case. Spatial heterogeneity of these no-till cultivated soils with respect to ammonium and oxygen distribution may explain the differences among the first and second rice in the traditional rotation.

In general, our results confirmed the high resilience of N-microbe guilds to both flooding and drying stress, which also has been previously reported (73). Despite these considerations, the PCA for winter sampling (**Figure 10B**) allowed us to discriminate the cR soil, the most intensive of the rotations, from other soils having rice previously. This rotation has a higher dependence on external inputs, like a much higher application of N-fertilizer, suggesting that this less sustainable rotation system may be different with respect to the N cycle even at this initial implementation of intensification.

Altogether, our results show that the microbial populations involved in GHG were strongly affected by the season. All rotational soils were highly similar mainly when rice was under the reproductive stage and further. In summary, flooding with consequent oxygen deprivation and plant growth phases have a high impact over microbial populations involved in methane emission. The differences imposed by rice intensification systems, at least in these no-till systems where intensification was implemented quite recently, disappear as the rice plant grows and low redox potential is reached. However, a slight effect of the recently implemented intensification could be perceived at rice tillering and at winter postharvest when two consecutive rice summer seasons are separated with respect to the microbiological parameters studied. Consequently, different GHG emission rates would be expected for rotational rice systems after a longer period under different intensification regimes, highlighting that the design of agricultural systems is critical for matching productivity and environmental goals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AF-S, ST, JT, and PI conceived the research. JT planned and managed the long-term field experiment. DO, ST, and AM-P collected the samples and took measures. AM-P made the greater contribution to the statistical analysis of the data. AF-S, ST, and PI drafted the manuscript. All authors revised and made significant contributions to the write-up and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by ANII-Innovagro project FSA_1_2013_1_12447 (Agencia Nacional de Investigación, ANII) and by CSIC-Udelar (Comisión Sectorial de Investigación Científica, Universidad de la República) groups funding project number 976 Microbial regulation of biogeochemical cycles involved in greenhouse gases emission by agricultural soils. The research was also supported by PEDECIBA with funding to AF-S and PI. ANII-Innovagro Program FSA_PP_2018_1_148336 and Project 148579 supported this article publication.

ACKNOWLEDGMENTS

The authors thank workers of INIA Treinta y Tres Experimental Station that contributed to the management of the long-term field experiment, particularly to Ignacio Macedo and Alexander Bordagorri. We are especially grateful to Luciana Pereira, Cecilia Ghiazza, and Gabriela Illarze for their help with sampling and laboratory assays, and to Lucía Ferrando for her help and expertise on molecular techniques.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsoil.2022.832600/full#supplementary-material

REFERENCES

1. Zorrilla G. Uruguayan rice: the secrets of a success story. *RiceToday*. (2015) 14:18–9.
2. Tseng M-C, Roel Á, Macedo I, Marella M, Terra J, Zorrilla G, et al. Field-level factors for closing yield gaps in high-yielding rice systems of Uruguay. *Field Crops Res.* (2021) 264:108097. doi: 10.1016/j.fcr.2021.108097
3. Pittelkow CM, Zorrilla G, Terra J, Riccetto S, Macedo I, Bonilla C, et al. Sustainability of rice intensification in Uruguay from 1993 to 2013. *Glob Food Secur.* (2016) 9:10–18. doi: 10.1016/j.gfs.2016.05.003
4. Carlson KM, Gerber JS, Muller ND, Herrero M, MacDonald GK, Braumen KA, et al. Greenhouse gas emissions intensity of global croplands. *Nat Clim Change.* (2017) 7:63. doi: 10.1038/nclimate3158
5. Linquist B, van Groenigen KJ, Adviento-Borbe MA, Pittelkow C, van Kessel C. An agronomic assessment of greenhouse gas emissions from major cereal crops. *Global Change Biol.* (2012) 18:194–209. doi: 10.1111/j.1365-2486.2011.02502.x
6. Ferrando L, Tarlera S. Activity and diversity of methanotrophs in the soil–water interface and rhizospheric soil from a flooded temperate rice field. *J Appl Microbiol*. (2009) 106:306–16. doi: 10.1111/j.1365-2672.2008.04004.x
7. Bouwman AF, Boumans LJM. Emissions of N_2O and NO from fertilized fields: summary of available measurement data. *Glob Biogeochem Cycle.* (2002) 16:13. doi: 10.1029/2001GB001811
8. Breidenbach B, Blaser MB, Klose M, Conrad R. Crop rotation of flooded rice with upland maize impacts the resident and active methanogenic microbial community. *Environ Microbiol*. (2015) 18:2868–85. doi: 10.1111/1462-2920.13041
9. Lagomarsino A, Agnelli AE, Pastorelli R, Pallara G, Rasse DP, Silvennoinen H. Past water management affected GHG production and microbial community pattern in Italian rice paddy soils. *Soil Biol Biochem*. (2016) 93:17–27. doi: 10.1016/j.soilbio.2015.10.016
10. Tarlera S, Capurro MC, Irisarri P, Fernández Scavino A, Cantou G, Roel A. Yield-scaled global warming potential of two irrigation management systems in a highly productive rice system. *Sci Agric*. (2016) 73:43–50. doi: 10.1590/0103-9016-2015-0050
11. Weller S, Kraus D, Ayag KRP, Wassmann W, Alberto MCR, Butterbach-Bahl K, et al. Methane and nitrous oxide emissions from rice and maize production in diversified rice cropping systems. *Nutr Cycl Agroecosyst.* (2015) 101:37–53. doi: 10.1007/s10705-014-9658-1
12. Wang J, Xue C, Song Y, Wang L, Huang Q, Shen Q. Wheat and rice growth stages and fertilization regimes alter soil bacterial community structure, but not diversity. *Front Microbiol.* (2016) 7:1207. doi: 10.3389/fmicb.2016.01207
13. Liu D, Ishikawa H, Nishida M, Tsuchiya K, Takahashi T, Kimura M, et al. Effect of paddy-upland rotation on methanogenic archaeal community structure in paddy field soil. *Microb. Ecol.* (2015) 69:160–8. doi: 10.1007/s00248-014-0477-3
14. Bertora C, Cucu MA, Lerda C, Peyron M, Bardi L, Gorra R, et al. Dissolved organic carbon cycling, methane emissions and related microbial populations in temperate rice paddies with contrasting straw and water management. *Agric Ecosyst Environ.* (2018) 265:292–306. doi: 10.1016/j.agee.2018.06.004
15. Fernandez Scavino A, Ji Y, Pump J, Klose M, Claus P, Conrad R. Structure and function of the methanogenic microbial communities in Uruguayan soils shifted between pasture and irrigated fields. *Environ Microbiol*. (2013) 15:2588–602. doi: 10.1111/1462-2920.12161
16. Seo J, Gebauer I, Kang H. Abundance of methanogens, methanotrophic bacteria, and denitrifiers in rice paddy soils. Wetlands. (2014) 34:213–23. doi: 10.1007/s13157-013-0477-y
17. Ye RW, Averill BA, Tiedje JM. Denitrification: production and Consumption of nitric oxide. *Appl Environ Microbiol.* (1994) 60:1053–8. doi: 10.1128/aem.60.4.1053-1058.1994
18. Bateman EJ, Baggs EM. Contributions of nitrification and denitrification to N_2O emissions from soils at different water-filled pore space. *Biol Fertil Soils.* (2005) 41:379e388. doi: 10.1007/s00374-005-0858-3
19. Morales SE, Cosart T, Holben WE. Bacterial gene abundances as indicators of greenhouse gas emission in soils. *ISME J*. (2010) 4:799–808. doi: 10.1038/ismej.2010.8
20. Li S, Song L, Gao X, Jin Y, Liu S, Shen Q, et al. Microbial abundances predict methane and nitrous oxide fluxes from a windrow composting system. *Front Microbiol.* (2017) 8:409. doi: 10.3389/fmicb.2017.00409
21. Duran A, Califra A, Molfino JH, Lynn W. *Keys to soil taxonomy for Uruguay*. Washington, DC; USD, Natural Resources Conservation Service (NRCS) (2006).
22. Macedo I, Pravia MV, Castillo J, Terra JA. Soil organic matter in physical fractions after intensification of irrigated rice-pasture rotation systems. *Soil Tillage Res.* (2021) 213:105160. doi: 10.1016/j.still.2021.105160
23. Patterson HD. Theory of cyclic experiments. *J Roy Statis, Soc Ser B*. (1964) 26:1–36. doi: 10.1111/j.2517-6161.1964.tb00535.x
24. Minamikawa K, Yagi K, Tokida T, Sander BO, Wassmann R. Appropriate frequency and time of day to measure methane emissions from an irrigated rice paddy in Japan using the manual closed chamber method. *Greenhouse Gas Measur Manag.* (2012) 2:118–28. doi: 10.1080/20430779.2012.729988
25. Rochette P, Eriksen-Hamel NS. Chamber measurements of soil nitrous oxide flux: are absolute values reliable? *Soil Sci Soc Am J.* (2008) 72:331–42. doi: 10.2136/sssaj2007.0215

26. Tan KH. *Soil Sampling, Preparation and Analysis*. 2nd ed. Boca Raton, FL: CRC Press (2005).
27. Tiesen H, Moir JO. *Soil Sampling and Methods of Analysis, 1st ed*. Carter MR, editor. Boca Raton, FL: Canadian Society of Soil Science, Lewis Publishers (1993).
28. Heanes DL. Determination of total organic-C in soils by an improved chromic acid digestion and spectrophotometric procedure. *Commun Soil Sci Plant Anal.* (1984) 15:1191–213. doi: 10.1080/00103628409367551
29. Mahne I, Tiedje J, Criteria M. Methodology for identifying respiratory denitrifiers. *Appl Environ Microbiol.* (1995) 61:1110–5. doi: 10.1128/aem.61.3.1110-1115.1995
30. Tarlera S, Denner EBM. Sterolibacterium denitrificans gen. nov, sp nov, a novel cholesterol-oxidizing, denitrifying member of the β-Proteobacteria. *Int J Syst Evolut Microbiol.* (2003) 53:1085–91. doi: 10.1099/ijs.0.02039-0
31. Kandeler E. Potential nitrification. In: Schinner F, Öhlinger R, Kandeler E, Margesin R, editors. *Methods in Soil Biology*. Berlin:Springer-Verlag (1996). p. 146–9.21.
32. Deambrosi E. Rice production system in Uruguay and its sustainability. in: *Proceedings of the III International Conference of Temperate Rice*, Punta del Este: INIA (2003).
33. Xuan DT, Guong VT, Rosling A, Alström S, Chai B, Högberg N. Different crop rotation systems as drivers of change in soil bacterial community structure and yield of rice, *Oryza sativa*. *Biol Fertil Soils.* (2012) 48:217–25. doi: 10.1007/s00374-011-0618-5
34. Maarastawi SA, Frindte K, Linnartz M, Knief C. Crop rotation and straw application impact microbial communities in Italian and Philippine soils and the rhizosphere of *Zea mays*. *Front Microbiol.* (2018) 9:1295. doi: 10.3389/fmicb.2018.01295
35. Shen J, Tao Q, Dong Q, Luo Y, Luo J, He Y, et al. Long-term conversion from rice-wheat to rice-vegetable rotations drives variation in soil microbial communities and shifts in nitrogen-cycling through soil profiles. *Geoderma.* (2021) 404:115299. doi: 10.1016/j.geoderma.2021.115299
36. Ji Y, Conrad R, Xu H. Responses of archaeal, bacterial, and functional microbial communities to growth season and nitrogen fertilization in rice fields. *Biol Fertil Soils*. (2020) 56:81–95. doi: 10.1007/s00374-019-01404-4
37. Breidenbach B, Conrad R. Seasonal dynamics of bacterial and archaeal methanogenic communities in flooded rice fields and effect of drainage. *Front Microbiol.* (2014) 5:752. doi: 10.3389/fmicb.2014.00752
38. Ji Y, Liu P, Conrad R. Response of fermenting bacterial and methanogenic archaeal communities in paddy soil to progressing rice straw degradation. *Soil Biol Biochem*. (2018) 124:70–80. doi: 10.1016/j.soilbio.2018.05.029
39. Malyan SK, Bhatia A, Kumar A, Gupta DK, Singh R, Kumar SS, et al. Methane production, oxidation and mitigation: a mechanistic understanding and comprehensive evaluation of influencing factors. *Sci Total Environ.* (2016) 572:874–96. doi: 10.1016/j.scitotenv.2016.07.182
40. Conrad R. Methane production in soil environments-anaerobic biogeochemistry and microbial life between flooding and desiccation. *Microorganisms.* (2020) 8:881. doi: 10.3390/microorganisms8060881
41. Singh S, Kashyap AK, Singh JS. Methane flux in relation to growth and phenology of a high yielding rice variety as affected by fertilization. *Plant Soil*. (1998) 201:157–64.
42. Inubushi K, Cheng W, Aonuma S, Hoque M, Kobayashi K, Miura S, et al. Effects of free-air CO_2 enrichment (FACE) on CH4 emission from a rice paddy field. *Glob Chang Biol.* (2003) 9:1458–64. doi: 10.1046/j.1365-2486.2003.00665.x
43. Zschornack T, Bayer C, Acordi ZJ, Costa BVF, Anghinoni I. Mitigation of methane and nitrous oxide emissions from flood-irrigated rice by no incorporation of winter crop residues into the soil. *Rev Bras Ciênc Solo.* (2011) 35:623–34. doi: 10.1590/S0100-06832011000200031
44. Win EP, Win KK, Bellingrath-Kimura SD, Oo AZ. Influence of rice varieties, organic manure and water management on greenhouse gas emissions from paddy rice soils. *PLoS ONE.* (2021) 16:e0253755. doi: 10.1371/journal.pone.0253755
45. Conrad R, Klose M. Dynamics of the methanogenic archaeal community in anoxic rice soil upon addition of straw. *Eur J Soil Sci.* (2006) 57:476–84. doi: 10.1111/j.1365-2389.2006.00791.x
46. Ma J, Ma E, Xu H, Yagi K, Cai Z. Wheat straw management affects CH_4 and N_2O emissions from rice fields. *Soil Biol Biochem.* (2009) 41:1022–8. doi: 10.1016/j.soilbio.2009.01.024
47. Kong D, Li S, Jin Y, Wu S, Chen J, Hu T, et al. Linking methane emissions to methanogenic and methanotrophic communities under different fertilization strategies in rice paddies. *Geoderma.* (2019) 347:233–43. doi: 10.1016/j.geoderma.2019.04.008
48. Kim SY, Gutierrez J, Kim PJ. Unexpected stimulation of CH_4 emissions under continuous no-tillage system in mono-rice paddy soils during cultivation. *Geoderma.* (2016) 267:34–40. doi: 10.1016/j.geoderma.2015.12.021
49. Yuan Q, Pump J, Conrad R. Partitioning of CH_4 and CO_2 production originating from rice straw, soil and root organic carbon in rice microcosms. *PLoS ONE.* (2012) 7:e49073. doi: 10.1371/journal.pone.0049073
50. Ye R, Horwath WR. Influence of rice straw on priming of soil C for dissolved organic C and CH_4 production. *Plant Soil.* (2017) 417:231–41. doi: 10.1007/s11104-017-3254-5
51. Alpana S, Vishwakarma P, Adhya TK, Inubushi K, Dubey SK. Molecular ecological perspective of methanogenic archaeal community in rice agroecosystem. *Sci Total Environ.* (2017) 596–7:136–46. doi: 10.1016/j.scitotenv.2017.04.011
52. Lee HJ, Jung H, Kim SY, Kim PJ, Madsen EL, Jeon CO. Methane emission and dynamics of methanotrophic and methanogenic communities in a flooded rice field ecosystem. *FEMS Microbiol Ecol.* (2014) 88:195–212. doi: 10.1111/1574-6941.12282
53. Aulakh M. S., Wassmann R, Bueno C, Rennenberg, H. Impact of root exudates of different cultivars and plant development stages of rice (*Oryza sativa* L.) on methane production in a paddy soil. *Plant Soil.* (2001) 230:77–86. doi: 10.1023/A:1004817212321
54. Watanabe T, Kimura M, Asakawa S. Community structure of methanogenic archaea in paddy field soil under double cropping (rice–wheat). *Soil Biol Biochem.* (2006) 38:1264–74. doi: 10.1016/j.soilbio.2005.09.020
55. Lee HJ, Jeong SE, Kimm PL, Madsen EL, Jeon CO. High-resolution depth distribution of Bacteria, Archaea, methanotrophs, and methanogens in the bulk and rhizosphere soils of a flooded rice paddy. *Front Microbiol.* (2015) 6:639. doi: 10.3389/fmicb.2015.00639
56. Liu J-N, Zhu B, Yi L-X, Dai H-C, Xu H-S, Zhang K, et al. Winter cover crops alter methanotrophs community structure in a double-rice paddy soil. *J Integr Agric.* (2016) 15:553–65. doi: 10.1016/S2095-3119(15)61206-0
57. Zhou G, Gao S, Xu C, Dou F, Shimizu K-Y, Cao W. Rational utilization of leguminous green manure to mitigate methane emissions by influencing methanogenic and methanotrophic communities. *Geoderma.* (2020) 361:114071. doi: 10.1016/j.geoderma.2019.114071
58. Weitzman JN, Groffman PM, Adler PR, Dell CJ, Johnson II FE, et al. Drivers of hot spots and hot moments of denitrification in agricultural systems. *J Geophys Res.* (2021) 126:e2020JG006234. doi: 10.1029/2020JG006234
59. Hofstra N, Bouwman AF. Denitrification in agricultural soils: summarizing published data and estimating global annual rates. *Nutr Cycl Agroecosystems.* (2005) 72:267–78. doi: 10.1007/s10705-005-3109-y
60. Castillo J, Kirk GJD,Rivero MJ,Dobermann A, Haefele S. The nitrogen economy of rice-livestock systems in Uruguay. *Global Food Security.* (2021) 30:100566. doi: 10.1016/j.gfs.2021.100566
61. Liang D, Robertson GP. Nitrification is a minor source of nitrous oxide (N_2O) in an agricultural landscape and declines with increasing management intensity. *Glob Change Biol.* (2021) 27:5599–613. doi: 10.1111/gcb.15833
62. Lensi R, Clays-Josserand A, Monrozier LJ. Denitrifiers and denitrifying activity in size fractions of a mollisol under permanent pasture and continuous cultivation. *Soil Biol Biochem.* (1995) 27:61–9. doi: 10.1016/0038-0717(94)00132-K
63. Hazard C, Prosser JI, Graeme WN. Use and abuse of potential rates in soil microbiology. *Soil Biol Biochem*. (2021) 157:108242. doi: 10.1016/j.soilbio.2021.108242
64. Wei X, Zhu Z, Wei L, Wu J, Ge T. Biogeochemical cycles of key elements in the paddy-rice rhizosphere: microbial mechanisms and coupling processes. *Rhizosphere.* (2019) 10:100145. doi: 10.1016/j.rhisph.2019.100145
65. Yoshida M, Ishii S, Otsuka S, Senoo K. Temporal shifts in diversity and quantity of nirS and nirK in a rice paddy field soil. *Soil Biol Biochem*. (2009) 41:2044–51. doi: 10.1016/j.soilbio.2009.07.012

66. Bannert A, Kleineidam K, Wissing L, Muller-Niggemann C, Vogelsang V, Welzl G, et al. Changes in diversity and functional gene abundances of microbial communities involved in nitrogen fixation, nitrification, and denitrification in a tidal wetland versus paddy soils cultivated for different time periods. *Appl Environ Microbiol.* (2011) 77:6109–16. doi: 10.1128/AEM.01751-10
67. Azziz G, Monza J, Etchebehere C, Irisarri P. nirS- and nirK-type denitrifier communities are differentially affected by soil type, rice cultivar and water management. *Euro J Soil Biol.* (2017) 78:20–28. doi: 10.1016/j.ejsobi.2016.11.003
68. Romdhane S, Spor A, Busset H, Falchetto L, Martin J, Bizouard F, et al. Cover crop management practices rather than composition of cover crop mixtur3es affect bacterial communities in no-till agroecosystems. *Front Microbiol.* 10:1618. doi: 10.3389/fmicb.2019.01618
69. Hallin S, Jones C, Schloter M. Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. *ISME J.* (2009) 3:597–605. doi: 10.1038/ismej.2008.128
70. Shan J, Sanford RA, Chee-Sanford J, Ooi SK, Löffler FE, Konstantinidis KT, et al. Beyond denitrification: the role of microbial diversity in controlling nitrous oxide reduction and soil nitrous oxide emissions. *Glob Change Biol.* (2021) 27:1–15. doi: 10.1111/gcb.15545
71. Hallin S, Philippot L, L?offler FE, Sanford RA, Jones CM. Genomics and ecology of novel N_2O-reducing microorganisms. *Trends Microbiol.* (2018) 26:43–55. doi: 10.1016/j.tim.2017.07.003
72. Rütting T, Schleusner P, Hink L, Prosser JI. The contribution of ammonia-oxidizing archaea and bacteria to gross nitrification under different substrate availability. *Soil Biol Biochem.* (2021) 160:108353. doi: 10.1016/j.soilbio.2021.108353
73. Kaurin A, Mihelič R., Kastelec D, Grčman H, Bru D, Philippot L, et al. Resilience of bacteria, archaea, fungi and N-cycling microbial guilds under plough and conservation tillage, to agricultural drought. *Soil Biol Biochem.* (2018) 120:233–45. doi: 10.1016/j.soilbio.2018.02.007

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Soil Science

REVIEW
published: 18 March 2022
doi: 10.3389/fsoil.2022.838595

Opportunities for Microbiome Suppression of Weeds Using Regenerative Agricultural Technologies

*Liang Cheng, Antonio DiTommaso and Jenny Kao-Kniffin**

School of Integrative Plant Science, Cornell University, Ithaca, NY, United States

The goal of regenerative agriculture is to utilize technologies that build healthy soils and improve the environment. Microbial technologies could play a significant role in reducing reliance on synthetic herbicides for weed control. In the United States, the expenditure on herbicides exceeds $5 billion annually and accounts for 58% of the total pesticide use nationally. This overreliance on chemical weed control has exacerbated herbicide resistance in a multitude of weed species, leading to aggressive cultivation practices that contribute to soil erosion and depletion. The proliferation of microbiome research in agriculture has increased our understanding of the complex interactions between plant species and their microbiota. Microbial technologies offer novel weed management strategies that could reduce the need for herbicides. Some of these strategies could also help rebuild soil and improve environmental quality. Specifically, we propose three emerging areas in microbiome science that can enhance weed management: (1) identifying soil microorganisms that inhibit weed growth; (2) discovering microbial natural products that suppress weeds; and (3) developing field management approaches that promote weed suppression by enhancing soil microbiome function.

Keywords: bioherbicide, herbicide resistance, invasive, microbiome, natural product, weeds

OPEN ACCESS

Edited by:
Patricia Dorr De Quadros, University of Waterloo, Canada

Reviewed by:
Muneer Ahmad Malla, Dr. Hari Singh Gour University, India
Garima Singh, Pachhunga University College, India

****Correspondence:***
Jenny Kao-Kniffin
jtk57@cornell.edu

Specialty section:
This article was submitted to Soil Biology, Ecosystems and Biodiversity, a section of the journal Frontiers in Soil Science

Received: *18 December 2021*
Accepted: *15 February 2022*
Published: *18 March 2022*

Citation:
Cheng L, DiTommaso A and Kao-Kniffin J (2022) Opportunities for Microbiome Suppression of Weeds Using Regenerative Agricultural Technologies. Front. Soil Sci. 2:838595. doi: 10.3389/fsoil.2022.838595

INTRODUCTION

Weed management in the United States (U.S.) has been largely reliant on synthetic herbicides since the 1970's. In some cropping systems, synthetic herbicides have reduced or eliminated the need for tillage or cultivation to manage weeds. Dependence on chemical weed control fuels a global herbicide industry that accounts for 40% of pesticide use worldwide (1). In the U.S. alone, the expenditure on herbicides exceeds $5 billion each year and accounts for 57% of the total pesticide use nationally (2). However, the continued sole reliance on chemical control has led to the evolved resistance of many weed species to an increasing number of widely used herbicides (3). Herbicide-resistant weeds, particularly weeds that are resistant to multiple herbicides, threaten agricultural productivity and sustainability.

The prevalence of single-tactic approaches to weed control stems largely from the early commercial success of glyphosate. Indeed, the development of glyphosate-resistant genetically modified (GM) crops expanded the use of glyphosate almost 15-fold by the twenty-first century (4) and encompassed multiple crops (**Figure 1**). Glyphosate applications in the U.S. exceed 1 billion kg/yr and now account for 67% of quantities used globally (4). As a result, severe outbreaks of

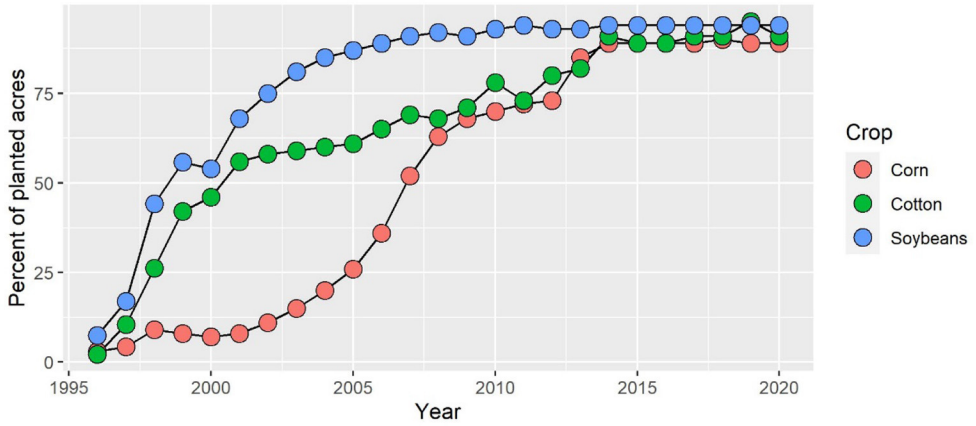


FIGURE 1 | Adoption of herbicide-tolerant crops in the United States since the 1990's. The crop data indicate herbicide-tolerant varieties. Source: USDA, Economic Research Service using data from the 2002 ERS report, Adoption of Bioengineered Crops (AER-810) for the years 1996–99 and National Agricultural Statistics Service (annual) June Agricultural Survey for the years 2000–20.

glyphosate-resistant or tolerant weed populations have been reported in 54 plant species (5), resulting in an expected annual cost of over $10 billion in increased chemical costs (6). Herbicide resistance in weeds has been developing rapidly, now compromising 21 of the 31 currently known herbicide sites of action (7). Many weed populations have evolved resistance to multiple sites of action (7). The second generation of GM crops that have stacked herbicide-resistant traits is likely to accelerate the evolution of weeds resistant to multiple sites of action and further compromise the efficacy of chemical control methods (3). Pivoting toward a regenerative agricultural system that limits or delays the development of herbicide-resistant weeds will require innovative approaches.

While there is an urgent need to develop new herbicide compounds with novel modes of action, the pace of discovery has been slow in the early twenty-first century. In 2020, inhibition of fatty acid thioesterase (Cinmethylin, branded as Luximo by BASF), became the first new mode of action approved in the past 35 years by HRAC (Herbicide Resistance Action Committee) (**Figure 2**). This compound was originally discovered synthetically in the early 1980's (8). Naturally occurring microorganisms that suppress weeds are a potential source of novel herbicides. Microorganisms associated with plants (collectively, the plant microbiome) likely co-evolved strategies to contend with neighboring plant competitors, so they may be a promising reservoir for compounds that inhibit plant growth. Several microorganisms have been formulated as bioherbicides to control weedy and invasive plants in agricultural and natural areas (9). An alternative to using living microorganisms for weed control is the application of compounds produced by these organisms, which are referred to as "natural products." Weed-suppressive and allelopathic compounds can be isolated as natural products derived from microorganisms and plants (10). Natural products isolated from microorganisms also play important roles outside of agriculture. For example, over 60% of FDA-approved anti-infective and anti-tumorigenic agents currently on the market were discovered from microorganisms found in natural environments, like soil and water (11, 12).

Natural product discovery has historically been limited by the fact that most soil microorganisms are not cultivable in a laboratory setting. The biosynthetic potential of soil microbiomes may be underestimated because research may be biased toward the few microbial phyla that have high representation of cultivable bacteria with full reference genomes (13). In addition, research efforts have sometimes overlooked environments that could serve as potential reservoirs of natural products. For example, an analysis of park soils from New York City showed a large diversity of natural product biosynthetic gene clusters (14). Soil microbiomes producing natural products relevant to agriculture are found worldwide and in a wide variety of environments.

Innovations in sequencing and molecular biology have enabled metagenomic approaches to be developed for isolation of microbial antibiotics and enzymes, which could lead to the discovery of new modes of action for weed control (15). Notably, shotgun metagenomics facilitates *de novo* sequencing of microbiomes (16). Metagenomic approaches to natural product discovery have been used to identify new antibiotics for the pharmaceutical industry (17). The same methods used for the isolation of antibiotics can accelerate the discovery of weed-suppressive compounds. For example, antibiotic compounds such as herbicidin, blasticidin and 5-hydro-xylmethyl-blasticidin S exhibit herbicidal activity (15). Compounds other than antibiotics, such as glufosinate, were also discovered from soil bacteria and subsequently synthesized in large quantities as commercial herbicides (18). Most recently, genome mining of soil fungal species led to the discovery of a potent weed-suppressive compound that could be developed as an herbicide having a novel mode of action (19). Once identified, natural products must be synthesized in large quantities to be used commercially. Microbial technologies focused on product biosynthesis are based on the concept of microorganisms serving as production factories for natural products.

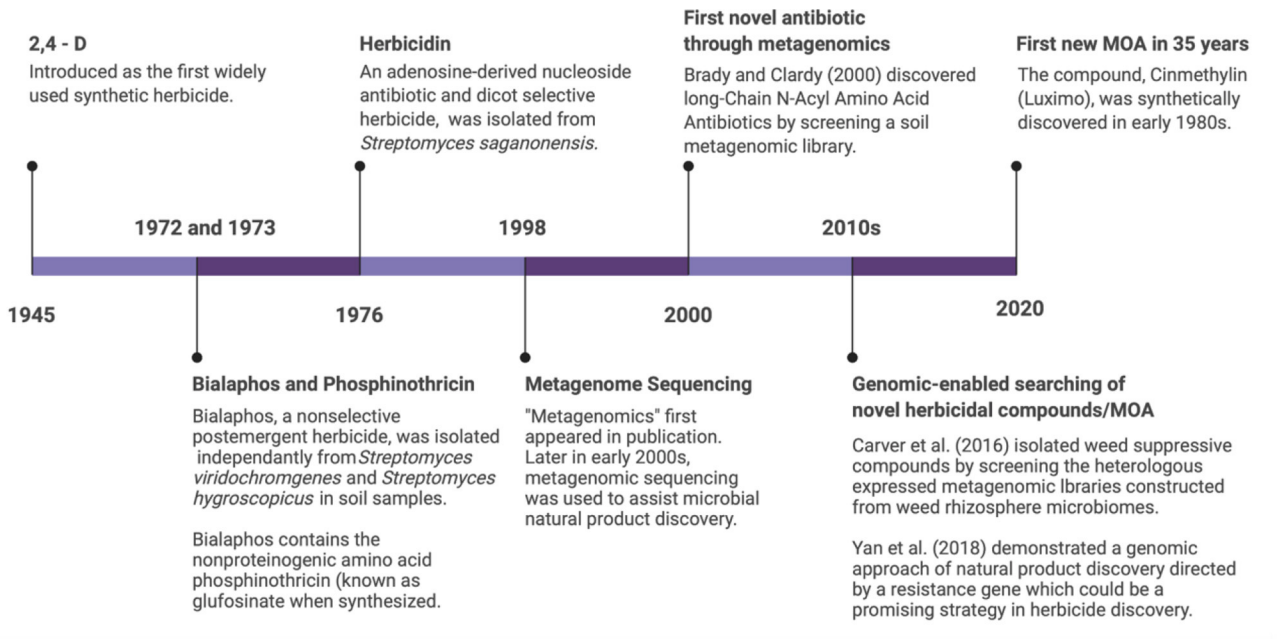


FIGURE 2 | Timeline of herbicide development indicating synthetic, natural, and genomics-enabled screening tools. Chemical synthesis approaches have stagnated since the start of the twenty-first century, while microbial natural product discovery and genomics show promising applications in weed management.

An alternative to the use of microbial natural products for weed control is the use of ecological management strategies that enhance microbiome function to suppress weeds. Integrated weed management (IWM), which combines various management practices based on ecological principles, can be an effective approach to manage herbicide-resistant weeds (3). An IWM approach focuses on managing herbicide-resistant weeds through mechanical and cultural practices, such as growing cover crops (20), cultivation, increased seeding rates, and reducing weed seed drop during harvest (21). However, IWM research has largely focused on aboveground processes, such as plant competition and herbivory, with more limited attention given to understanding the soil microbiome despite its significant influence on weed establishment and growth (1). The soil microbiome can be affected by land management practices. For example, agricultural practices such as tillage, crop rotation, cover crops, and fertilization can influence the diversity of arbuscular mycorrhizal fungi (22, 23), which in turn can impact a range of weed species (24, 25). This interaction suggests that field management practices could be used to create weed-suppressive soil microbiomes, supplementing current IWM strategies. Sequencing technologies could be harnessed to better understand the effects of agricultural practices on the soil microbiome and consequently on weed species and crop-weed interactions.

In this paper, we review: (1) the status and challenges of microbial biocontrol with bioherbicides; (2) current approaches of natural product discovery for novel herbicides; and (3) IWM strategies for managing the field microbiome and suppressing weeds with negative plant-soil feedbacks. Lastly, we address the need for soil microbiome research that uses emerging technologies and methodologies to discover novel weed-suppressive compounds. Our review differs from previous treatments of microbial biocontrol and natural products [e.g., (26, 27)] because we focus on the potential of emerging technologies to assist natural product discovery and the role of cropping system management in shaping the soil microbiome.

Microbial Biocontrol With Bioherbicide Agents

Decades of research have focused on bacteria and fungi for the control of undesirable plants. The microbial agents or their compounds are referred to as "bioherbicides" and suppress weeds through plant-pathogen interactions or allelopathy. For example, novel pathogens were able to accumulate and suppress a highly invasive species, Japanese stiltgrass (*Microstegium vimineum*), under field conditions (28). Most bioherbicides are target-specific pathogens that require large quantities of product (e.g., infective spores) to control mostly annual weeds in cropping or turfgrass systems (26). This approach is often referred to as "inundative" biological control. It is distinct from the "inoculative" or classical biological control approach, which typically uses imported insects to target non-native perennial weeds occupying extensive rangelands. Although some microbes might be candidates for the inoculative approach, this approach is challenging to implement and generally unsuitable for agricultural systems (27).

Among the most promising bioherbicides are microbial strains that can reduce the weed seedbank by promoting weed seed decay, inhibiting germination, or arresting germination. Such bioherbicides, which target the earliest stages of weed

establishment, have great potential in reduced-till or no-till cropping systems (26). For example, *Pseudomonas* strains isolated from weed rhizospheres have been developed into effective preemergent bioherbicides. A strain of *Pseudomonas fluorescens* formulated as a bioherbicide caused a 90% reduction in emergence of an annual weedy grass, green foxtail (*Setaria viridis*) (29). Many *Fusarium* strains with seed-decaying potential have been evaluated for their capacity to kill weed seeds (30). In contrast with these preemergent bioherbicides, postemergent bioherbicides control the weed seed bank by reducing seed production. For example, *Puccinia carduorum* suppressed musk thistle (*Carduus nutans*) seed production by as much as 57% (31). Other research showed that pseudomonas spp. developed for biocontrol reduced seed production of downy brome (*Bromus tectorum*) by 64% (32).

Several commercial biocontrol agents have been developed from weed-suppressive microorganisms isolated from soil (1). A limited number of bioherbicides (products of living or dead microorganisms) are currently registered in the United States (**Table 1**). The first registered bioherbicide in the United States was DeVine®, introduced in 1981. The product is a facultative fungal pathogen (*Phytophthora palmivora*) that causes root rot in strangler vine (*Morrenia odorata*). Since then, the number of biopesticides has increased around the world, but the market share of bioherbicides represents <10% of all biopesticides (26). The following paragraphs discuss reasons for the limited commercial success of bioherbicides: the difficulty of studying some microbial taxa, the need to screen numerous isolates, and the unpredictable behavior of some candidate agents under field conditions.

Bioherbicide development is impeded by challenges in accessing the pool of potential agents. Many surveys are restricted to a small subset of microbial taxa that are primarily fungi (1) and dependent on cultivation-based methods. The potential pool of biological agents for biocontrol is largely untapped, given the vast diversity of microbial taxa that have no reference genome (36). The identification and isolation of microorganisms that are elusive to standard laboratory cultivation may be facilitated by recent advances in cell capture technologies. One example is the "ichip" platform developed by Epstein et al., where cultivation in native soil habitats is achieved using diffusion chambers (37). Another example is using microfluidic techniques to simulate the chemical conditions and physical structures of native growth conditions (38). Greater focus on technologies to isolate microorganisms from their environments should expand the pool of candidate bioherbicides, increasing the likelihood that novel products for weed control will be discovered.

Another major challenge in bioherbicide discovery is the time- and resource- consuming process of isolate screening and testing. For instance, Kennedy and Stubbs (39) recovered more than 10,000 isolates using a conventional agar plating method. After several rounds of bioassay and growth chamber screening, only six strains showed promise and were field tested. Pathogenic isolates collected from diseased plants might reveal potential bioherbicides useful in downstream screening but require significant testing for off-target pathogenicity. A *Bipolaris bicolor* strain was isolated from severely diseased leaves of goosegrass (*Eleusine indica*) in a tea plantation system. Further tests on pathogenicity and host range demonstrated the potential of this strain as a biocontrol agent against Poaceae weeds in tea and broadleaf vegetable production (40). The initial field collection of 10 candidate isolates was obtained from 16 tea plantations through a time-consuming process. Soil microbiomes can serve as a pool of potential bioherbicides as well but could have similar limitations (time- and resource-intensive screening processes). For example, only one promising phytotoxic isolate was obtained after an herbicidal assay of 1,300 field-collected *Streptomyces* strains, even though *Streptomyces* are well-known for producing secondary metabolites relevant to natural product discovery (41). For bioherbicides to be successfully commercialized, additional testing and evaluation of host range, formulation, soil survival, production, and application need to be conducted to meet both consumer demand and regulatory requirements. Click or tap here to enter text.

Commercial use of biocontrol agents will be easiest if these bioherbicides, like most synthetic herbicides, are reliably effective when applied to the soil. However, the behavior of bioherbicide agents in soil can be unpredictable if the product is comprised of living organisms that are expected to perform a specific function. For example, a recent study on weed-suppressive *Pseudomonas fluorescens* strains showed that these strains reduced plant growth when grown on agar media but not in soil (42). Competition from other soil microorganisms was suggested as a possible reason for the loss of efficacy in the field. Indeed, microbial communities often work as a cohort and microbial functions that suppress weeds could be affected by both microbial interactions and environmental conditions. A single strain is less likely to be effective under field conditions. Recent studies have demonstrated that synthetic microbial communities (SynCom) designed with metagenomic data have tremendous potential in plant improvement (43). In fact, commercial biofertilizers or plant growth-promoting products on the market often include multiple microbial species or strains. An example is the Mammoth P consortium (Growcentia, Fort Collins, CO, USA), which is an assemblage of phosphorus-mobilizing bacteria from four taxa. The product showed higher rates of phosphate solubilization compared with single strains (44). Currently, all the registered bioherbicides in the United States are based on single species and strains. Outside of the United States, the only bioherbicide product that contains more than one strain is Organo-Sol®, registered in Canada (26). This product, which contains several species of lactic acid bacteria that produce lactic acid and citric acid, suppresses white clover (*Trifolium repens*) and red clover (*Trifolium pratense*) in lawns. Emerging technologies enabling researchers to study difficult-to-culture microorganisms may facilitate the development of novel SynCom-based bioherbicides. A more in-depth understanding of microbial interactions and their effects on microbiome function would help ensure that bioherbicides that are effective in lab and greenhouse settings can also suppress weeds in the field.

The rising cost of managing herbicide-resistant weeds and restrictions on the use of some herbicides should drive demand for innovations in weed biocontrol. The global bioherbicide market, valued at USD 1.28 billion in 2016, is expected to

TABLE 1 | Registered bioherbicides in the United States.

Product name and time	Active microbe species	Target	Mechanism	Status	References
DeVine™, 1981	*Phytophthora palmivora*	Strangler vine (*Morrenia odorata*) in citrus crops	Initiates a root infection in strangler vine that starts to kill the plant in 6–10 weeks	Not available	(26)
Collego™/LockDown™, 1982/2006	*Colletotrichum gloeosporioides* f.sp. *aeschynomene*	Northern jointvetch (*Aeschynomene virginica* L. B.S.P.) in rice and soybean	Primarily infects the stems of the weed, causes disease lesions that will completely encircle the stems of the northern jointvetch plants.	Not available	(33)
Dr. BioSedge®, 1987	*Puccinia canaliculala*	Yellow nutsedge (*Cyperus esculentus* L.) in soybean, potato, corn, and cotton	Inhibits yellow nutsedge flowering, reduces plant density and new tuber formation	Product failed due to mass production issue	(34)
Woad Warrior®, 2002	*Puccinia thlaspeos* woad	Dyer's woad (*Isatis tinctoria* L.)	Fungal rust that reproduces and spreads using only dyer's woad as a host.	Not commercially available	(35)
Myco-Tech® Paste /Chontrol® Paste, 2005/2020	*Chondrostereum purpureum* HQ1/ PFC2139*	Susceptible deciduous tree species in forests	Colonizes the stump and inhibits sprouting and regrowth, causes subsequent wood decomposition. The reduced stem density helps mechanical cut.	Commercially available	(34)
Smoulder® G/WP, 2008	*Alternaria destruens* 059	Dodders (*Cuscuta* spp.) in fields and ornamental nurseries	Infects live or dead dodder plant tissue, suppressing dodder at early and late stages of growth	Not commercially available	(26)
SolviNix™, 2009	Tobacco mild green mosaic tobamovirus (TMGMV U2)	Tropical soda apple (*Solanum viarum*) in rangelands	The virus enters the plant cells through minute injuries and kills tropical soda apple by triggering a systemic lethal hypersensitive plant response.	Commercially available	(34)
Phoma P/H/TECH, 2012	*Phoma macrostoma* 94-44B	Dicots in golf courses, agriculture and agroforestry	Produces macrocidins that cause photobleaching in dicots.	Commercially available	(26)
Opportune™/MBI-005, 2012*	*Streptomyces acidiscabies*	Broadleaves and sedges in turf, wheat, rice, and corn	The fermentation produces Thaxtomin A that inhibits cellulose biosynthesis in the meristem of sensitive plant species.	Undergoing further formulation refinement	EPA, Marrone Bio Innovations Inc
Battalion Pro, 2020	*Pseudomonas fluorescens* ACK55	Downy brome (*Bromus tectorum*), medusahead (*Taeniatherum caput-medusae*), jointed goatgrass (*Aegilops cylindrica*)	The bacteria affect the roots, seeds, or young seedlings and inhibit root-cell elongation. The suppressive compound inhibits lipopolysaccharide production in the cell wall and membrane and reduces root-cell wall elongation.	Absence of an industrial partner	EPA
Venerate /MBI-012, 2021*†	*Burkholderia rinojensis* A396	Pigweed family (*Amaranthaceae*)	Produces herbicidal compounds including Templamide A/B and Templazole A/B	Registered as bioinsecticide. Spectrum of herbicidal activity and crop safety to be determined. WDG formulation in development.	EPA, Marrone Bio Innovations Inc

Adapted from Cordeau et al. (26), Aneja et al. (9), and Abbas et al. (34).

**Opportune™/MBI-005 and Venerate/MBI-012 are microorganism-based products that contains non-viable microbe cells. According to Marrone Bio Innovations Inc, WDG (water dispersible granules) formulation is being developed for commercially viability.*

†*Venerate/MBI-012 is registered as a bioinsecticide but the same strain possesses herbicidal activity. The product is sold as Venerate or Venerate XC. The herbicidal activity is from multiple metabolites (undisclosed) produced during fermentation (Marrone Bio Innovations Inc at IR-4 western regional workshop).*

reach USD 4.14 billion by 2024 (45). However, challenges in bioherbicide commercialization still exist [reviewed by (27)]. Although bioherbicides have been the focus of research for decades, their modes of action are not well-understood [reviewed by (26, 46)]. Environmental factors, such as temperature and humidity, and their interactions can significantly affect bioherbicide efficacy under field conditions (47). Microbial interactions could also impact the organism's virulence through quorum sensing (47). Other factors impeding commercialization may include inconsistent product quality with scaled-up

production (47) and concerns about non-target dispersal through adaptation to the new environment and host exposure over time. The potential to release a bioherbicide agent that becomes a non-target pathogen is a major limitation to wider adoption of practice. One solution to concerns about non-target dispersal is to apply active compounds produced by microorganisms (i.e., natural products) rather than releasing live microorganisms as biocontrol agents.

Discovery of Weed-Suppressive Natural Products

Microbial natural products have been a prolific source of compounds for medical and agricultural uses (12). Natural products are secondary metabolites produced by organisms that are not involved in primary growth, reproduction, and development. Some secondary metabolites increase fitness by altering interactions with other organisms. For example, secondary metabolites can be used as biological weaponry to outcompete other organisms for resources. Microorganisms in the plant rhizosphere, which is a narrow band (1–2 mm) of soil surrounding plant roots, could co-evolve with their plant hosts by generating novel allelopathic compounds that suppress competing plant species. Because these compounds suppress plants, they could be a rich reservoir for the discovery of natural products with novel herbicidal properties. Some natural products isolated from microbes can be used as herbicides without modification, while others can be modified or used to identify new herbicide targets (27).

Research on natural products developed rapidly after the discovery of penicillin, one of the world's first antibiotics (10). This discovery ushered in the "Golden Age" of natural product discovery of the 1950's and 1960's, which focused primarily on microorganisms and plants. During this early period, researchers developed systematic screening processes for soils, typically including acquisition of environmental samples, culturing and isolation of microorganisms, followed by testing of the fermentation broth or purified products against test organisms. More than 1,000 natural products with antibacterial or antifungal activities were discovered during this time (10). Most of these natural products were produced by organisms in the bacterial phylum Actinomycetes, containing the highly cultivable members of the *Streptomyces* genus. The many species of *Streptomyces* are widely distributed across nearly all ecosystems, including the microbiomes inhabited by higher eukaryotes. At present, actinobacteria produce two thirds of all known antibiotics (48). Actinobacteria also produce a vast array of anti-cancer compounds, immunosuppressants, anthelmintics, antiviral compounds, and extracellular enzymes (48). Compounds produced by several actinobacteria species have also led to novel herbicide discovery (**Figure 2**). These compounds include bialaphos produced by *Streptomyces hygroscopicus* SF1293, herbicidins produced by *Streptomyces saganonensis*, phosphinothricin produced by *Streptomyces viridochromogenes* (known as glufosinate when synthesized), and Thaxtomin A from *Streptomyces acidiscabies* (49) (**Figure 2**).

Natural product discovery continued after the Golden Age. An estimated 10–20 million microbial isolates were screened from 1950 to 2000, with efforts mostly focused on discovering antibacterial and antifungal compounds (10). There was also interest in finding natural products for the treatment of various human diseases and development of agrichemicals. Sample collection was expanded to sources beyond soils; for example, some anti-cancer compounds were successfully derived from marine samples (50). However, the pace of natural product discovery eventually slowed, partially because screening processes that require isolating organisms as pure cultures in a laboratory setting are laborious. Such cultivation-dependent screening processes are still widely used. For example, 14 phytotoxic secondary metabolites were obtained from *in vitro* cultures of two fungal pathogens of buffelgrass (*Cenchrus ciliaris*) (51). Among these compounds, radicinin was identified as a promising bioherbicide that showed target-specific toxic activities on buffelgrass. Another study conducted herbicidal assays on 1,300 field-collected *Streptomyces* strains and found only two herbicidal compounds from one phytotoxic isolate (41). Research in the decades after the Golden Age of natural product discovery has revealed more bioactive actinomycete species; however, most bacteria in soil remain uncultivable using standard lab culturing techniques (10, 15). Therefore, the likelihood of finding new natural products *via* traditional methods is decreasing. The rate of novel antibiotic discovery in the phylum Actinomycetes using traditional screening has been estimated at <1 per million (48).

Advances in genome sequencing during the twenty-first century have brought new opportunities for natural product discovery. Genome sequencing can be used to identify novel biosynthetic gene clusters (BGC) coding the production of secondary metabolites (**Figure 2**). Genomic sequencing data revealed that BGCs in microbial genomes are much more abundant than predictions based on expressed secondary metabolites (48). A recent study of BGC diversity and potential bioactivity in urban park soils of New York City demonstrated a higher-than-expected level of chemical novelty, suggesting that urban soils could be a valuable source of natural products (14). Genomic datasets are typically large and complex, particularly with microbiome samples or when many microbial strains are collected. To find potentially useful natural products within these datasets, bioinformatic tools such as antiSMASH6 (52) or PRISM4 (53) can be used to predict and identify novel BGCs. For example, antiSMASH was used to predict which gene clusters in actinomycetes might yield new antibiotics (54). This approach led to the discovery of corbomycin, which is an antibiotic with a novel mode of action (54). Several other bioinformatic tools have been developed to assist BCG mining. For example, with the putative gene clusters identified by antiSMASH, BiG-SCAPE can build gene cluster families (55). Additionally, MIBiG is a database of known BGCs and their products that could be used for sequence-based dereplication of the gene clusters (56). Deep learning approaches are being developed for screening of chemical libraries; these approaches can improve predictions of natural product functions based on their structures (57).

The rapid-growing datasets of BGCs discovered through high-throughput genome mining could be a challenge in natural product discovery. Because these datasets contain numerous BGCs of unknown function, many of which are irrelevant to the desired purpose or functionally redundant with known BGCs,

it is difficult to determine which BGCs merit experimental study. One strategy is to cluster putative BGCs into gene cluster families (GCFs) for the purpose of dereplication and to avoid rediscovery of known compounds in the downstream experimental characterization. However, the increasingly large number of GCFs with no known functions still makes it difficult to focus downstream screening on groups that are likely to produce natural products of interest. For example, a large-scale genomic study on 3,080 bacterial genomes from the Actinomycetes phylum found nearly 18,000 GCFs, most of which have no known products (55). To increase the likelihood of obtaining chemical novelty, some researchers have tried prioritizing microbial taxa that are less well-characterized, or sampling extreme or unusual environments. For example, rare Actinomycetes bacteria of marine and wetland ecosystems were suggested to be promising sources of novel natural products (58, 59). A recent global soil survey of polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs), which are enzymes involved in the biosynthesis of numerous peptide and peptide-like natural products, found that geographic distance and biome type were associated with diversity in BGCs (11). However, knowledge about the environmental and taxonomic distributions of BGCs is still lacking (48).

Although metagenomic approaches provide great opportunities for identifying novel BGCs, it is still a challenge to link orphan (i.e., unknown corresponding metabolites) BGCs to their associated natural products (60). To address this challenge, BGCs are often cloned or transferred to heterologous expression hosts such as *Streptomyces* species that are known for their natural product production abilities. The heterologous expression method typically involves three steps: isolation and cloning of the DNA fragments that contain BGCs in the original host, expression in a heterologous host organism, and genetic manipulation of the cloned pathway for interrogation or activation. Zhang et al. (60) provide a comprehensive review of heterologous expression methods for microbial natural product discovery. One advantage of heterologous expression of BGCs is that it enables the expression of BGCs from uncultivable microorganisms derived from environmental samples. Another advantage is that a good heterologous expression host can provide a clean secondary metabolite background, which will make it easier to isolate and identify the compound encoded by the BGC of interest. *Streptomyces coelicolor* M1152/M1154, *Streptomyces avermitilis* SUKA17/22, and *Streptomyces lividans* SBT5 are good expression hosts that lack competing BGCs (61). A third advantage is that genetic tools can be used to activate cryptic BGCs in the heterologous expression host; these tools are typically not available in the original host.

Cloning is a challenging step in the heterologous expression of microbial natural products due to the large size, repetitiveness, and high GC-content of many microbial BGCs (60). Library-based cloning and heterologous expression methods provide an alternative top-down approach. The strategy is widely used to clone microbial BGCs from metagenome or environmental DNA samples, where complete genome information is lacking. Genomic DNA are randomly sheared into small fragments of ∼40 kb that could contain BGCs and ligated into cosmid or fosmid vectors. The vectors are then transformed into heterologous hosts such as *E. coli* or *Streptomyces lividans* strains to be expressed. The BGCs in the random 40 kb fragments are often incomplete and need to be combined and trimmed before use (60). However, a complete BGC can be obtained using this expression method and the biosynthetic pathway can be expressed. This approach was first used by Brady and Clardy to discover novel antibiotics from uncultivated microorganisms in soil samples (62). Following the same methodology, Carver et al. (63) isolated weed-suppressive compounds by using heterologous expression to screen fosmid libraries constructed from weed rhizosphere microbiomes (**Figure 2**). Library vectors that hold longer DNA fragments (>55 kb) than cosmids and fosmids, such as bacterial artificial chromosomes (BACs) and P1 artificial chromosomes (PACs), could be transferred to *E. coli* directly by electroporation (60). Because these vectors hold longer DNA, they make it easier to screen complete biosynthetic pathways or larger BGCs. The best example is the largest heterologously expressed BGC, the quinolidomicin A1, which is over 200 kb and cloned in a BAC library (64).

Assembly and direct cloning methods provide powerful alternatives to library cloning (60). These methods, based on synthetic biology, either assemble BGCs *in vitro* or reconstruct larger BGCs *in vivo*. Examples include Gibson assembly, Golden Gate assembly, and yeast recombination (65). A recent study successfully expressed two synthesized BGCs from human metagenomic sequences in various heterologous hosts and obtained five novel antibacterial compounds (66). In addition to these cloning methods, the increased feasibility of *de novo* DNA synthesis is providing new opportunities for research on BGCs. *De novo* DNA synthesis was used to access and refactor BGCs (i.e., reorganize the cluster structure to achieve stable function) <10 kb; this approach could greatly facilitate capturing and characterizing BGCs in the future (60). Although these advances are promising, it is still challenging to express natural products in heterologous host systems, where precursors or cofactors may be lacking (67).

It is often difficult to induce BCG expression because the factors controlling activation and expression are typically not well-understood. It was estimated that <10% of BGCs are expressed under laboratory culture conditions where microorganisms are grown in artificial, simplistic environments consisting of agar petri plates and liquid broth (10). Expression of BGCs may require environmental signals and cues, such as cues from microbial interactions with hosts or other organisms (68, 69). For example, unique chemical production patterns were observed in *Streptomyces coelicolor* interacting with other Actinomycetes bacteria by nanospray desorption electrospray ionization (NanoDESI) and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) imaging mass spectrometry (68). A recent study showed that fungal infection of plant roots induced the production of non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) by unknown BGCs in the root endophytic microbiome (70). Computational tools such as PREDetector that predict regulatory elements of BGC expression (71), along with other meta-omics

data, could be used to systematically study the triggers of BGC expression (60).

A recent paper demonstrated a genomic approach to natural product discovery directed by a resistance gene, which could be a promising strategy for herbicide discovery (19). Specifically, the authors discovered and verified the mode of action of aspterric acid (a fungal sesquiterpenoid with herbicidal activity) based on the co-clustering of its corresponding self-resistance gene in the BGC responsible for aspterric acid biosynthesis. The rationale behind this approach is that, if the fungal aspterric acid targets a plant enzyme that is also essential to fungi, the fungus might have a resistance gene in the same BGC as the genes that produce aspterric acid. Although aspterric acid was previously known as a phytotoxic compound and not suitable as a commercial herbicide due to inadequate herbicidal strength and chemical complexity, this study provides proof of concept for a resistance gene-directed approach to genome mining for novel herbicide discovery (72).

Agronomic Microbiome Management for Weed Suppression

Soil microorganisms play an important role in sustaining healthy soils that promote crop production and suppress pests and weeds. Management practices that enhance soil biodiversity and cause desirable changes in soil community composition are likely to increase agricultural sustainability (73). In the context of agricultural weed control, managers may be able to create weed-suppressive soils through enrichment of weed-inhibiting microorganisms (74, 75). This section focuses on identifying practices that may be useful in creating weed-suppressive soils, which include reduced tillage, reduced agrichemical inputs, and maintenance of high soil organic matter (76). In addition, we discuss the role of plant-soil feedbacks (PSF) in weed control. It is worth noting that these topics can now be studied with next-generation sequencing technology [e.g., (77, 78)], which provides a much clearer picture of the soil microbiome than the laboratory cultivation-based methods that historically limited most studies to individual bacterial species or strains.

Standard agricultural practices such as tillage and heavy use of agrichemical inputs (fertilizers and pesticides) significantly alter soil microbiomes, sometimes with undesirable results (77, 78). A recent study suggested that cropping practices of organic and conventional farming with different tillage intensities accounted for 10% of total variation in wheat rhizosphere microbial communities (79). Cropping system affected not only individual microbial groups but also microbial co-occurrences, indicating that soil microbial interactions can also be affected by common agricultural practices. A better understanding of which microbiome members influence crop and weed performance and how to optimize the microbiome could markedly improve weed management in cropping systems.

Another agronomic strategy that can be developed within a microbial weed management approach is the addition of carbon amendments or incorporation of plant residues into soil (80–82). Soil microorganisms respond rapidly to carbon additions with enhanced growth, increasing their demand for nitrogen, phosphorus, and other limiting nutrients required for primary metabolism (83). This response to increased carbon availability is referred to as "nutrient immobilization" because limiting nutrients other than carbon become immobilized in microbial cells and therefore are unavailable for biological uptake by other organisms.

Nutrient immobilization can be harnessed for weed control because plants can be poor competitors for nutrients, relative to soil microorganisms. These differences could be exploited to control weed populations that preferentially grow on highly fertile soils (84, 85). Several field studies investigating the effects of carbon addition on invasive weeds showed reduced nitrogen availability in carbon-amended soils, likely resulting from microbial nitrogen immobilization in response to carbon stimulation (86–90). These findings demonstrate the potential for soil carbon addition to promote microbial competition for nutrients during the critical period of weed control. In agronomic settings, it is important to examine which weed species are most responsive to soil carbon addition and the best timing for carbon applications and nutrient immobilization in relation to crop establishment. Future research should also describe the indirect, microorganism-mediated impacts of cover crop management on weed communities. Cover crops have been used to sequester carbon and nitrogen in soil and provide weed suppression. Several recent studies reported that soil microbial communities, including functional groups such as arbuscular mycorrhizal fungi, were positively affected by cover crop management (43). However, the role of these changes to microbial communities in influencing weed suppression by cover crops has not been examined.

Plant-soil feedback (PSF) occurs when a plant species alters biotic or abiotic conditions in soil, which in turn impact the growth of the same plant species or a different plant species (91). Negative PSF (PSF that inhibits plant growth) typically results from either allelopathic effects or accumulated host-specific pathogens in soil (92–94). Negative feedback is believed to be more common than positive feedback and plays an important role in species coexistence and the maintenance of plant biodiversity in ecosystems (91, 95).

While PSF has been primarily studied in natural environments, PSF is also important in agricultural environments and responsive to agricultural management practices. As discussed, practices such as tillage and fertilization can affect the composition and structure of the soil microbiome. These changes to the microbiome may affect PSF. van der Putten et al. (96) proposed a triangular framework consisting of symbionts, decomposers, and enemies (e.g., pathogens) to analyze the shifts in PSF due to environmental changes. The authors used this framework to predict the effects of agricultural practices (agrichemical inputs and land management in conventional and organic systems) on the soil microbiome and PSF. This study highlighted the role that PSF plays in agricultural systems but their potential for weed suppression was not discussed.

A research focus on PSF in agricultural systems could have important implications for weed management (**Figure 3**). There is increasing empirical evidence for a role of soil microbiomes and PSF in governing weed population dynamics. Understanding

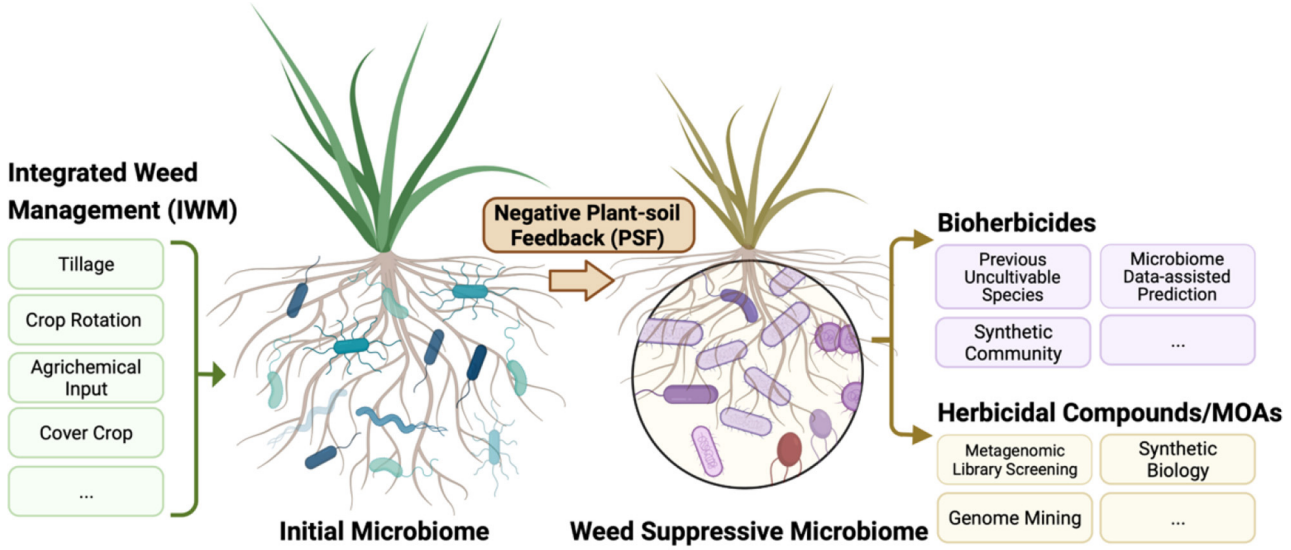


FIGURE 3 | A schematic overview of approaches harnessing the soil microbiome and negative plant-soil feedback to promote weed management in regenerative agriculture. Agricultural practices alter the soil microbiome, so practices that promote weed suppression by microorganisms could be included in integrated weed management programs. Negative plant-soil feedbacks occurs when plants cause biotic and abiotic changes in the soil, which inhibit further plant growth. Research on these feedback processes may help identify potential microbial agents or compounds involved in plant growth suppression.

how management practices affect PSF could therefore improve ecological weed management strategies. This research is likely to be complex because PSF processes are likely to vary among weed species and cropping systems. For example, the rate at which plants accumulate species-specific pathogens and the effects of these pathogens vary among plant species (93). Plants that quickly accumulate pathogens might be more vulnerable to PSF processes limiting plant density (93). However, it is still not clear if the strength of negative PSF on a plant species corresponds with field abundance of the species, as research has provided contradictory results (97, 98). A more in-depth understanding of how weed density influences negative feedback pressures is likely to improve the efficacy of weed management strategies.

More generally, the direction and strength of PSF may depend on plant species or functional group (95, 99, 100). In a study of 48 grassland species, grasses and small herbs showed negative PSF, tall herbs exhibited positive PSF, and legumes showed neutral PSF, suggesting a strong correlation between PSF direction and plant functional group (99). While most weed species are small to mid-sized herbs or grasses, it is not clear if their PSF patterns are species-dependent or tend to be negative. For example, Himalayan balsam (*Impatiens glandulifera*), one of the most widespread weeds in the United Kingdom, showed positive PSF (101). In a study of 12 grass and forb species, early-successional species showed negative PSF while late-successional species exhibited positive PSF (102). Because PSF patterns appear to be species-specific, it is unlikely that any management program could promote negative PSF for all weed species in an agricultural field. A more realistic goal might be to promote negative PSF for most weeds or for particularly troublesome weeds, such as herbicide-resistant populations. Future research should investigate patterns in PSF strength and direction for these troublesome weeds. As sequencing technologies continue to improve in resolution and cost, the ability to characterize soil microbiomes as contributing to positive or negative PSF could improve integrated weed management strategies. For example, there may be opportunities to select cover crop species or crops that prime the soil for enrichment of weed-suppressive microbiomes and allelopathic exudates.

Although PSF processes can affect any plant species, research on PSF may be particularly important to understanding invasive plants. Many weeds are invasive species that were introduced into geographically novel ranges (with novel soil biota) and successfully competed against plants native to these novel ranges. It has long been recognized that microorganisms and PSF often play an important role in plant invasions (93, 103). Multiple hypotheses have been proposed to explain the success of plant invasions *via* belowground microbial effects. The "enemy release" hypothesis proposes that invasive plant species in a novel region are released from the factors limiting their population sizes in their native ranges. For example, the absence of soil-borne pathogens or growth-inhibiting soil biota in invaded regions can result in the proliferation of a plant species in these regions (104). Enemy release has been largely a theoretical concept, but there are convincing empirical studies that support this hypothesis. The establishment of Chinese tallow (*Triadica sebifera*), native to Asia, in the United States was influenced by soil biota: this invader was more negatively affected by soil pathogens in the native range and mycorrhizal colonization was higher in the invaded range (105). Another field study revealed that, when the invasive Lodgepole pine (*Pinus contorta*) is grown in soils without its native soil biota, this species can rapidly

grow and spread into new regions (106). Common ragweed (*Ambrosia artemisiifolia*), a highly invasive annual weed native to North America, was able to escape from both aboveground and belowground enemies at a more local scale [new sites within North America; (107)]. The concept of escaping from "enemies" could become a management tool for protecting crops in situations where soil pathogen composition can be identified rapidly.

Alternatives to the enemy release hypothesis focus on soil microorganisms that are present in invaded regions. For example, invasive plant species could gain an advantage in their new ranges by stimulating the growth and abundance of pathogens that negatively affect resident competing plant species [i.e., the "accumulation of local pathogens" hypothesis; (108)]. In cropping systems, it is valuable to identify soil biota that are deleterious to weeds but not harmful to crop species. This area of study warrants further research attention. Invasive species research has also revealed that invasive plants can accumulate beneficial mutualists of their own ("enhanced mutualist" hypothesis) or suppress beneficial mutualists of resident species to gain a competitive advantage ("mutualism disruption" hypothesis). Garlic mustard (*Alliaria petiolata*), a noxious invasive weed, was shown to inhibit fungal mutualists of North American native plants; the authors observed stronger inhibition in its invaded range than in its native range (109). Continued research on interactions between invasive plants and soil microorganisms will improve our understanding of invasive agricultural weeds and plant-microbe interactions more broadly. Translating ecological theory into agricultural practice will require innovative approaches to identify factors influencing PSF processes, including plant, microbial, and environmental characteristics.

CONCLUSION

Managing weeds effectively and sustainably is essential to agricultural productivity. However, weed management in the twenty-first century is challenged by the increasing number of herbicide-resistant weeds, many of which are resistant to multiple herbicide modes of action. A key concept in regenerative agriculture is to use the most appropriate technologies to effectively manage agroecosystems. In the context of weed management, it is clear that these technologies must not be limited to synthetic herbicides. Several promising approaches are based on the soil microbiome, including bioherbicides, natural products derived from microbes, and manipulation of the existing microbiome through agricultural practices. Early research based on cultivation-dependent methods of microbial biocontrol paved the way for recent advances in genomics-enabled natural product discovery. Natural products that inhibit seed germination or arrest seedling growth can enable creative strategies for weed seedbank management. Research on microbes that contribute to weed suppression in the field can reveal additional natural products and suggest improvements to management programs (**Figure 3**). Continued advances in metagenomic sequencing will accelerate research on the microbial management of agricultural weeds. In addition, advances in digital agriculture will help incorporate microbiome data into predictions about crop performance and pest pressure. These technological advances are crucial to understanding how soil microbiomes affect agricultural productivity and how they might be harnessed to promote regenerative agriculture.

AUTHOR CONTRIBUTIONS

LC wrote the manuscript. JK-K and AD contributed to revisions. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Controlling Weedy and Invasive Plants Program (grant no. 2016-67014-24859) from the USDA National Institute of Food and Agriculture and a Cornell Institute for Digital Agriculture Student Research Grant award to LC.

ACKNOWLEDGMENTS

We thank Sofia Kashtelyan and Kristopher Smith for assisting with research that led to discussions on microbial natural product discovery in weed management. Thanks also to A. Sophie Westbrook for her helpful suggestions on an earlier version of this manuscript.

REFERENCES

1. Trognitz F, Hackl E, Widhalm S, Sessitsch A. The role of plant-microbiome interactions in weed establishment and control. *FEMS Microbiol Ecol.* (2016) 92:fiw138. doi: 10.1093/femsec/fiw138
2. Atwood D, Paisley-Jones C. *Pesticides Industry Sales and Usage 2008–2012*. Washington, DC: United States Environmental Protection Agency (2017).
3. Mortensen DA, Egan JF, Maxwell BD, Ryan MR, Smith RG. Navigating a critical juncture for sustainable weed management. *Bioscience.* (2012) 62:75–84. doi: 10.1525/bio.2012.62.1.12
4. Benbrook CM. Trends in glyphosate herbicide use in the United States and globally. *Environ Sci Europe.* (2016) 28:70. doi: 10.1186/s12302-016-0070-0
5. Heap I. *The International Herbicide-Resistant Weed Database*. Available online at: www.weedscience.org (accessed March 2, 2022).
6. Varah A, Ahodo K, Coutts SR, Hicks HL, Comont D, Crook L, et al. The costs of human-induced evolution in an agricultural system. *Nat Sustainabil.* (2020) 3:8. doi: 10.1038/s41893-019-0450-8
7. Heap I. *The International Survey of Herbicide Resistant Weeds*. (2022). Available online at: www.weedscience.org (accessed January 19, 2022).
8. Duke SO, Baerson SR, Rimando AM. Herbicides: glyphosate. In: JR Plimmer, DW Gammon, NN Ragsdale, editors, *Encyclopedia of Agrochemicals*. New York, NY: John Wiley & Sons, Inc. (2003). p. 850–63. doi: 10.1002/047126363X.agr119
9. Aneja KR, Khan SA, Aneja A. Bioherbicides: strategies, challenges and prospects. In: T Satyanarayana, S Deshmukh, B Johri, editors, *Developments in Fungal Biology and Applied Mycology*. Singapore: Springer (2017). p. 449–70. doi: 10.1007/978-981-10-4768-8_23

10. Katz L, Baltz RH. Natural product discovery: past, present, and future. *J Indus Microbiol Biotechnol.* (2016) 43:5. doi: 10.1007/s10295-015-1723-5
11. Charlop-Powers Z, Owen JG, Reddy BVB, Ternei M, Guimaraes DO, de Frias UA, et al. Global biogeographic sampling of bacterial secondary metabolism. *eLife.* (2015) 2015:e05048. doi: 10.7554/eLife.05048
12. Pham Jv, Yilma MA, Feliz A, Majid MT, Maffetone N, et al. A review of the microbial production of bioactive natural products and biologics. *Front Microbiol.* (2019) 10:1404. doi: 10.3389/fmicb.2019.01404
13. Crits-Christoph A, Diamond S, Butterfield CN, Thomas BC, Banfield JF. Novel soil bacteria possess diverse genes for secondary metabolite biosynthesis. *Nature.* (2018) 558:440–4. doi: 10.1038/s41586-018-0207-y
14. Charlop-Powers Z, Pregitzer CC, Lemetre C, Ternei MA, Maniko J, Hover BM, et al. Urban park soil microbiomes are a rich reservoir of natural product biosynthetic diversity. *Proc Natl Acad Sci USA.* (2016) 113:14811–6. doi: 10.1073/pnas.1615581113
15. Kao-Kniffin J, Carver SM, DiTommaso A. Advancing weed management strategies using metagenomic techniques. *Weed Sci.* (2013) 61:171–84. doi: 10.1614/WS-D-12-00114.1
16. Libis V, Antonovsky N, Zhang M, Shang Z, Montiel D, Maniko J, et al. Uncovering the biosynthetic potential of rare metagenomic DNA using co-occurrence network analysis of targeted sequences. *Nat Commun.* (2019) 10:1–9. doi: 10.1038/s41467-019-11658-z
17. Hutchings M, Truman A, Wilkinson B. Antibiotics: past, present and future. *Curr Opin Microbiol.* (2019) 51:72–80. doi: 10.1016/j.mib.2019.10.008
18. Dayan FE, Duke SO. Natural compounds as next-generation herbicides. *Plant Physiol.* (2014) 166:1090–105. doi: 10.1104/pp.114.239061
19. Yan Y, Liu Q, Zang X, Yuan S, Bat-Erdene U, Nguyen C, et al. Resistance-gene-directed discovery of a natural-product herbicide with a new mode of action. *Nature.* (2018) 559:415–8. doi: 10.1038/s41586-018-0319-4
20. Davis VM, Gibson KD, Bauman TT, Weller SC, Johnson WG. Influence of weed management practices and crop rotation on glyphosate-resistant horseweed (*Conyza canadensis*) population dynamics and crop yield-years III and IV. *Weed Sci.* (2009) 57:417–26. doi: 10.1614/WS-09-006.1
21. Walsh MJ, Owen MJ, Powles SB. Frequency and distribution of herbicide resistance in *Raphanus raphanistrum* populations randomly collected across the Western Australian wheatbelt. *Weed Res.* (2007) 47:542–50. doi: 10.1111/j.1365-3180.2007.00593.x
22. Alguacil MM. The Impact of tillage practices on arbuscular mycorrhizal fungal diversity in subtropical crops. *Ecological Applications.* (2008) 18:527–36. doi: 10.1890/07-0521.1
23. van der Heyde M, Ohsowski B, Abbott LK, Hart M. Arbuscular mycorrhizal fungus responses to disturbance are context-dependent. *Mycorrhiza.* (2017) 27:431–40. doi: 10.1007/s00572-016-0759-3
24. Rinaudo V, Bàrberi P, Giovannetti M, van der Heijden MGA. Mycorrhizal fungi suppress aggressive agricultural weeds. *Plant Soil.* (2010) 333:202. doi: 10.1007/s11104-009-0202-z
25. el Omari B, el Ghachtouli N. Arbuscular mycorrhizal fungi-weeds interaction in cropping and unmanaged ecosystems: a review. *Symbiosis.* (2021) 83:9. doi: 10.1007/s13199-021-00753-9
26. Cordeau S, Triolet M, Wayman S, Steinberg C, Guillemin JP. Bioherbicides: dead in the water? A review of the existing products for integrated weed management. *Crop Protection.* (2016) 87:44–9. doi: 10.1016/j.cropro.2016.04.016
27. Duke SO, Pan Z, Bajsa-Hirschel J, Douglas Boyette C. The potential future roles of natural compounds and microbial bioherbicides in weed management in crops. *Adv Weed Sci*. (2022) 40:e020210054. doi: 10.51694/AdvWeedSci/2022
28. Stricker KB, Harmon PF, Goss EM, Clay K, Flory SL. Emergence and accumulation of novel pathogens suppress an invasive species. *Ecol Lett*. (2016) 19:469–77. doi: 10.1111/ele.12583
29. Daigle DJ, Connick WJ, Boyetchko SM. Formulating a weed-suppressive bacterium in "Pesta" 1. *Weed Technol.* (2002) 16:407–13. doi: 10.1614/0890-037X(2002)0160407:FAWSBI2.0.CO;2
30. Müller-Stöver D, Nybroe O, Baraibar B, Loddo D, Eizenberg H, French K, et al. Contribution of the seed microbiome to weed management. *Weed Res.* (2016) 56:335–9. doi: 10.1111/wre.12218
31. Baudoin ABAM, Abad RG, Kok LT, Bruckart WL. Field evaluation of *Puccinia carduorum* for biological control of musk thistle. *Biol Control.* (1993) 3:136–52. doi: 10.1006/bcon.1993.1009
32. Kennedy AC, Young FL, Elliott LF, Douglas CL. Rhizobacteria suppressive to the weed downy brome. *Soil Sci Soc Am J.* (1991) 55:722–7. doi: 10.2136/sssaj1991.03615995005500030014x
33. Bowers RC. Commercialization of Collego ^{TM} –an industrialist's view. *Weed Sci.* (1986) 34:24–5. doi: 10.1017/S0043174500068326
34. Abbas T, Zahir ZA, Naveed M, Kremer RJ. Limitations of existing weed control practices necessitate development of alternative techniques based on biological approaches. In: DL Sparks, editor, *Advances in Agronomy*. Cambridge, MA: Academic Press (2018). p. 239–80. doi: 10.1016/bs.agron.2017.10.005
35. Kropp BR, Hansen DR, Thomson Sv. Establishment and dispersal of *Puccinia thlaspeos* in field populations of dyer's woad. *Plant Dis.* (2002) 86:241–6. doi: 10.1094/PDIS.2002.86.3.241
36. Hug LA, Thomas BC, Sharon I, Brown CT, Sharma R, Hettich RL, et al. Critical biogeochemical functions in the subsurface are associated with bacteria from new phyla and little studied lineages. *Environ Microbiol.* (2016) 18:159–73. doi: 10.1111/1462-2920.12930
37. Berdy B, Spoering AL, Ling LL, Epstein SS. In situ cultivation of previously uncultivable microorganisms using the ichip. *Nat Protoc*. (2017) 12:2232–42. doi: 10.1038/nprot.2017.074
38. Aleklett K, Kiers ET, Ohlsson P, Shimizu TS, Caldas VE, Hammer EC. Build your own soil: Exploring microfluidics to create microbial habitat structures. *ISME J.* (2018) 12:318–9. doi: 10.1038/ismej.2017.184
39. Kennedy AC, Stubbs TL. Management effects on the incidence of jointed goatgrass inhibitory rhizobacteria. *Biol Control*. (2007) 40:213–21. doi: 10.1016/j.biocontrol.2006.10.006
40. Xiao W, Li J, Zhang Y, Guo Y, Fang W, Valverde BE, et al. A fungal Bipolaris bicolor strain as a potential bioherbicide for goosegrass (*Eleusine indica*) control. *Pest Manag Sci.* (2021) 78:1251–64. doi: 10.1002/ps.6742
41. Bo AB, Kim JD, Kim YS, Sin HT, Kim HJ, Khaitov B, et al. Isolation, identification and characterization of Streptomyces metabolites as a potential bioherbicide. *PLoS ONE.* (2019) 14:e222933. doi: 10.1371/journal.pone.0222933
42. Lazarus BE, Feris K, Germino MJ. Weed-suppressive bacteria effects differ in culture compared to in soils and with or without microbial competition and separation of active ingredient. *Biol Control.* (2021) 152:104422. doi: 10.1016/j.biocontrol.2020.104422
43. Ray P, Lakshmanan V, Labbé JL, Craven KD. Microbe to microbiome: a paradigm shift in the application of microorganisms for sustainable agriculture. *Front Microbiol.* (2020) 11:622926. doi: 10.3389/fmicb.2020.622926
44. Baas P, Bell C, Mancini LM, Lee MN, Conant RT, Wallenstein MD. Phosphorus mobilizing consortium Mammoth P^{TM} enhances plant growth. *PeerJ.* (2016) 3:e1621v1. doi: 10.7287/peerj.preprints.1621v1
45. Grand View Research, Inc. *Bioherbicides Market Size Worth $4.14 Billion By 2024*. (2018). Available online at: https://www.grandviewresearch.com/press-release/global-bioherbicides-market (accessed December 19, 2021).
46. Masteling R, Lombard L, de Boer W, Raaijmakers JM, Dini-Andreote F. Harnessing the microbiome to control plant parasitic weeds. *Curr Opin Microbiol.* (2019) 49:26–33. doi: 10.1016/j.mib.2019.09.006
47. Harding DP, Raizada MN. Controlling weeds with fungi, bacteria and viruses: a review. *Front Plant Sci.* (2015) 6:659. doi: 10.3389/fpls.2015.00659
48. van Bergeijk DA, Terlouw BR, Medema MH, van Wezel GP. Ecology and genomics of Actinobacteria: new concepts for natural product discovery. *Nat Rev Microbiol.* (2020) 18:546–88. doi: 10.1038/s41579-020-0379-y
49. Duke SO, Abbas HK. Natural products with potential use as herbicides. In: K Inderjit, MM Dakshini, FA Einhellig, editors, *Allelopathy*. Washington, DC: ACS Publications (1994). p. 348–62. doi: 10.1021/bk-1995-0582.ch025
50. Khalifa SAM, Elias N, Farag MA, Chen L, Saeed A, Hegazy MEF, et al. Marine natural products: a source of novel anticancer drugs. *Mar Drugs.* (2019) 17:491. doi: 10.3390/md17090491
51. Masi M, Freda F, Sangermano F, Calabrò V, Cimmino A, Cristofaro M, et al. Radicinin, a fungal phytotoxin as a target-specific bioherbicide for invasive buffelgrass (*Cenchrus ciliaris*) control. *Molecules.* (2019) 24:1086. doi: 10.3390/molecules24061086

52. Blin K, Shaw S, Kloosterman AM, Charlop-Powers Z, van Wezel GP, Medema MH, et al. AntiSMASH 6.0: improving cluster detection and comparison capabilities. *Nucl Acids Res.* (2021) 49:gkab335. doi: 10.1093/nar/gkab335
53. Skinnider MA, Merwin NJ, Johnston CW, Magarvey NA. PRISM 3: expanded prediction of natural product chemical structures from microbial genomes. *Nucl Acids Res.* (2017) 45:gkx320. doi: 10.1093/nar/gkx320
54. Culp EJ, Waglechner N, Wang W, Fiebig-Comyn AA, Hsu YP, Koteva K, et al. Evolution-guided discovery of antibiotics that inhibit peptidoglycan remodelling. *Nature.* (2020) 578:582–7. doi: 10.1038/s41586-020-1990-9
55. Navarro-Muñoz JC, Selem-Mojica N, Mullowney MW, Kautsar SA, Tryon JH, Parkinson EI, et al. A computational framework to explore large-scale biosynthetic diversity. *Nat Chem Biol.* (2020) 16:60–8. doi: 10.1038/s41589-019-0400-9
56. Kautsar SA, Blin K, Shaw S, Navarro-Muñoz JC, Terlouw BR, van der Hooft JJJ, et al. MIBiG 2.0: a repository for biosynthetic gene clusters of known function. *Nucl Acids Res.* (2020) 48:gkz882. doi: 10.1093/nar/gkz882
57. Stokes JM, Yang K, Swanson K, Jin W, Cubillos-Ruiz A, Donghia NM, et al. A deep learning approach to antibiotic discovery. *Cell.* (2020) 180:688–702. doi: 10.1016/j.cell.2020.01.021
58. Benhadj M, Gacemi-Kirane D, Menasria T, Guebla K, Ahmane Z. Screening of rare actinomycetes isolated from natural wetland ecosystem (Fetzara Lake, northeastern Algeria) for hydrolytic enzymes and antimicrobial activities. *J King Saud Univ Sci.* (2019) 31:706–12. doi: 10.1016/j.jksus.2018.03.008
59. Subramani R, Sipkema D. Marine rare actinomycetes: a promising source of structurally diverse and unique novel natural products. *Mar Drugs.* (2019) 17:249. doi: 10.3390/md17050249
60. Zhang JJ, Tang X, Moore BS. Genetic platforms for heterologous expression of microbial natural products. *Nat Prod Rep.* (2019) 36:1313–32. doi: 10.1039/C9NP00025A
61. Xu M, Wang Y, Zhao Z, Gao G, Huang SX, Kang Q, et al. Functional genome mining for metabolites encoded by large gene clusters through heterologous expression of a whole-genome bacterial artificial chromosome library in *Streptomyces spp*. *Appl Environ Microbiol.* (2016) 82:5795–805. doi: 10.1128/AEM.01383-16
62. Brady SF, Clardy J. Long-chain N-acyl amino acid antibiotics isolated from heterologously expressed environmental DNA. *J Am Chem Soc*. (2007) 122:12903–4. doi: 10.1128/AEM.66.6.2541-2547.2000
63. Carver SM, Nikulin N, Kao-Kniffin J. Uncovering plant growth-mediating allelochemicals produced by soil microorganisms. *Weed Sci.* (2016) 64:119–28. doi: 10.1614/WS-D-15-00095.1
64. Hashimoto T, Hashimoto J, Kozone I, Amagai K, Kawahara T, Takahashi S, et al. Biosynthesis of Quinolidomicin, the largest known macrolide of terrestrial origin: Identification and heterologous expression of a biosynthetic gene cluster over 200 kb. *Org Lett.* (2018) 20:7996–9. doi: 10.1021/acs.orglett.8b03570
65. Heng E, Tan LL, Zhang MM, Wong FT. CRISPR-Cas strategies for natural product discovery and engineering in actinomycetes. *Process Biochem.* (2021) 102:261–8. doi: 10.1016/j.procbio.2021.01.007
66. Sugimoto Y, Camacho FR, Wang S, Chankhamjon P, Odabas A, Biswas A, et al. A metagenomic strategy for harnessing the chemical repertoire of the human microbiome. *Science.* (2019) 366:eaax9176. doi: 10.1126/science.aax9176
67. Smanski MJ, Bhatia S, Zhao D, Park YJ, Woodruff LBA, Giannoukos G, et al. Functional optimization of gene clusters by combinatorial design and assembly. *Nat Biotechnol.* (2014) 32:1241–9. doi: 10.1038/nbt.3063
68. Traxler MF, Watrous JD, Alexandrov T, Dorrestein PC, Kolter R. Interspecies interactions stimulate diversification of the *Streptomyces coelicolor* secreted metabolome. *MBio.* (2013) 4:e00459–13. doi: 10.1128/mBio.00459-13
69. Traxler MF, Kolter R. Natural products in soil microbe interactions and evolution. *Nat Prod Rep.* (2015) 32:956. doi: 10.1039/C5NP00013K
70. Carrión VJ, Perez-Jaramillo J, Cordovez V, Tracanna V, de Hollander M, Ruiz-Buck D, et al. Pathogen-induced activation of disease-suppressive functions in the endophytic root microbiome. *Science.* (2019) 366:606–12. doi: 10.1126/science.aaw9285
71. Hiard S, Marée R, Colson S, Hoskisson PA, Titgemeyer F, van Wezel GP, et al. PREDetector: a new tool to identify regulatory elements in bacterial genomes. *Biochem Biophys Res Commun.* (2007) 357:861–4. doi: 10.1016/j.bbrc.2007.03.180
72. Duke SO, Stidham MA, Dayan FE. A novel genomic approach to herbicide and herbicide mode of action discovery. *Pest Manag Sci.* (2019) 75:314–7. doi: 10.1002/ps.5228
73. Bender SF, Wagg C, van der Heijden MGA. An underground revolution: biodiversity and soil ecological engineering for agricultural sustainability. *Trends Ecol Evol.* (2016) 31:440–52. doi: 10.1016/j.tree.2016.02.016
74. Kennedy AC. Soil microorganisms for weed management. *J Crop Prod.* (1999) 2:123–38. doi: 10.1300/J144v02n01_07
75. Kremer RJ, Li J. Developing weed-suppressive soils through improved soil quality management. *Soil Tillage Res.* (2003) 72:193–202. doi: 10.1016/S0167-1987(03)00088-6
76. Li J, Kremer RJ. Rhizobacteria associated with weed seedlings in different cropping systems. *Weed Sci.* (2000) 48:731–41. doi: 10.1614/0043-1745(2000)0480734:RAWWSI2.0.CO;2
77. Carbonetto B, Rascovan N, Álvarez R, Mentaberry A, Vázquez MP. Structure, composition and metagenomic profile of soil microbiomes associated to agricultural land use and tillage systems in Argentine Pampas. *PLoS ONE.* (2014) 9:e99949. doi: 10.1371/journal.pone.0099949
78. Hartmann M, Frey B, Mayer J, Mäder P, Widmer F. Distinct soil microbial diversity under long-term organic and conventional farming. *ISME J.* (2015) 9:1177–94. doi: 10.1038/ismej.2014.210
79. Hartman K, van der Heijden MGA, Wittwer RA, Banerjee S, Walser JC, Schlaeppi K. Cropping practices manipulate abundance patterns of root and soil microbiome members paving the way to smart farming. *Microbiome.* (2018) 6:14. doi: 10.1186/s40168-017-0389-9
80. Perry LG, Galatowitsch SM, Rosen CJ. Competitive control of invasive vegetation: a native wetland sedge suppresses *Phalaris arundinacea* in carbon-enriched soil. *J Appl Ecol.* (2004) 41:151–62. doi: 10.1111/j.1365-2664.2004.00871.x
81. Eschen R, Mortimer SR, Lawson CS, Edwards AR, Brook AJ, Igual JM, et al. Carbon addition alters vegetation composition on ex-arable fields. *J Appl Ecol.* (2007) 44:95–104. doi: 10.1111/j.1365-2664.2006.01240.x
82. Cole RJ, Soper FM, Litton CM, Knauf AE, Sparks K, Gerow KG, et al. Restoration benefits of soil nutrient manipulation and weeding in invaded dry and wet tropical ecosystems in Hawai?i. *Restorat Ecol.* (2021) 29:1464. doi: 10.1111/rec.13390
83. Sinsabaugh RL, Manzoni S, Moorhead DL, Richter A. Carbon use efficiency of microbial communities: stoichiometry, methodology and modelling. *Ecol Lett.* (2013) 16:930–9. doi: 10.1111/ele.12113
84. Paschke MW, McLendon T, Redente EF. Nitrogen availability and old-field succession in a shortgrass steppe. *Ecosystems.* (2000) 3:144–58. doi: 10.1007/s100210000016
85. Blumenthal DM, Jordan NR, Russelle MP. Soil carbon addition controls weeds and facilitates prairie restoration. *Ecol Appl.* (2003) 13:605–15. doi: 10.1890/1051-0761(2003)0130605:SCACWA2.0.CO;2
86. McLendon T, Redente EF. Effects of nitrogen limitation on species replacement dynamics during early secondary succession on a semiarid sagebrush site. *Oecologia.* (1992) 91:312–7. doi: 10.1007/BF00317618
87. Reever Morghan KJ, Seastedt TR. Effects of soil nitrogen reduction on nonnative plants in restored grasslands. *Restorat Ecol.* (1999) 7:51–5. doi: 10.1046/j.1526-100X.1999.07106.x
88. Baer SG, Blair JM, Collins SL, Knapp AK. Plant community responses to resource availability and heterogeneity during restoration. *Oecologia.* (2004) 139:617–29. doi: 10.1007/s00442-004-1541-3
89. Uddin MN, Robinson RW, Asaeda T. Nitrogen immobilization may reduce invasibility of nutrient enriched plant community invaded by *Phragmites australis*. *Sci Rep.* (2020) 10:1601. doi: 10.1038/s41598-020-58523-4
90. Knauf AE, Litton CM, Cole RJ, Sparks JP, Giardina CP, Gerow KG, et al. Nutrient-use strategy and not competition determines native and invasive species response to changes in soil nutrient availability. *Restorat Ecol.* (2021) 29:e13374. doi: 10.1111/rec.13374
91. Bever JD, Dickie IA, Facelli E, Facelli JM, Klironomos J, Moora M, et al. Rooting theories of plant community ecology in microbial interactions. *Trends Ecol Evol.* (2010) 25:468–78. doi: 10.1016/j.tree.2010.05.004

92. Mills KE, Bever JD. Maintenance of diversity within plant communities: soil pathogens as agents of negative feedback. *Ecology.* (1998) 79:1595–601. doi: 10.1890/0012-9658(1998)0791595:MODWPC2.0.CO;2
93. Klironomos JN. Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature.* (2002) 417:67–70. doi: 10.1038/417067a
94. Mangan SA, Schnitzer SA, Herre EA, Mack KML, Valencia MC, Sanchez EI, et al. Negative plant-soil feedback predicts tree-species relative abundance in a tropical forest. *Nature.* (2010) 466:752–5. doi: 10.1038/nature09273
95. Bennett J, Maherali H, Reinhart K, Lekberg Y, Hart M, Klironomos J. Plant-soil feedbacks and mycorrhizal type influence temperate forest population dynamics. *Science.* (2017) 355:181–4. doi: 10.1126/science.aai8212
96. van der Putten WH, Bradford MA, Pernilla Brinkman E, van de Voorde TFJ, Veen GF, Bailey JK. Where, when and how plant-soil feedback matters in a changing world. *Funct Ecol.* (2016) 30:1062–71. doi: 10.1111/1365-2435.12657
97. Reinhart KO. The organization of plant communities: negative plant-soil feedbacks and semiarid grasslands. *Ecology.* (2012) 93:2377–85. doi: 10.1890/12-0486.1
98. Maron JL, Smith AL, Ortega YK, Pearson DE, Callaway RM. Negative plant-soil feedbacks increase with plant abundance, and are unchanged by competition. *Ecology.* (2016) 97:2055–63. doi: 10.1002/ecy.1431
99. Cortois R, Schröder-Georgi T, Weigelt A, van der Putten WH, de Deyn GB, van der Heijden M. Plant-soil feedbacks: role of plant functional group and plant traits. *J Ecol.* (2016) 104:1608–17. doi: 10.1111/1365-2745.12643
100. Teste FP, Kardol P, Turner BL, Wardle DA, Zemunik G, Renton M, et al. Plant-soil feedback and the maintenance of diversity in Mediterranean-climate shrublands. *Science.* (2017) 176:173–6. doi: 10.1126/science.aai8291
101. Pattison Z, Rumble H, Tanner RA, Jin L, Gange AC. Positive plant-soil feedbacks of the invasive *Impatiens glandulifera* and their effects on above-ground microbial communities. *Weed Res.* (2016) 56:198–207. doi: 10.1111/wre.12200
102. Kardol P, Martijn Bezemer T, Van Der Putten WH. Temporal variation in plant-soil feedback controls succession. *Ecol Lett.* (2006) 9:1080–8. doi: 10.1111/j.1461-0248.2006.00953.x
103. Callaway RM, Thelen GC, Rodriguez A, Holben WE. Soil biota and exotic plant invasion. *Nature.* (2004) 427:731–3. doi: 10.1038/nature02322
104. Van der Putten WH, Klironomos JN, Wardle DA. Microbial ecology of biological invasions. *ISME J.* (2007) 1:28–37. doi: 10.1038/ismej.2007.9
105. Yang Q, Carrillo J, Jin H, Shang L, Hovick SM, Nijjer S, et al. Plant-soil biota interactions of an invasive species in its native and introduced ranges: Implications for invasion success. *Soil Biol Biochem.* (2013) 65:78–85. doi: 10.1016/j.soilbio.2013.05.004
106. Gundale MJ, Kardol P, Nilsson MC, Nilsson U, Lucas RW, Wardle DA. Interactions with soil biota shift from negative to positive when a tree species is moved outside its native range. *New Phytol.* (2014) 202:415–21. doi: 10.1111/nph.12699
107. MacKay J, Kotanen PM. Local escape of an invasive plant common ragweed (*Ambrosia artemisiifolia L*), from above-ground and below-ground enemies in its native area. *J Ecol.* (2008) 96:1152–61. doi: 10.1111/j.1365-2745.2008.01426.x
108. Eppinga MB, Rietkerk M, Dekker SC, de Ruiter PC, van der Putten WH. Accumulation of local pathogens: a new hypothesis to explain exotic plant invasions. *Oikos.* (2006) 114:168–76. doi: 10.1111/j.2006.0030-1299.14625.x
109. Rati DAP, Tinson KRS, Lironomos JOHNK. Novel weapons: invasive plant suppresses fungal mutualists in America but not in its native Europe. *Ecology.* (2008) 89:1043–55. doi: 10.1890/07-0370.1

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Soil Science

ORIGINAL RESEARCH
published: 31 March 2022
doi: 10.3389/fsoil.2022.837508

Effect of Long-Term Agricultural Management on the Soil Microbiota Influenced by the Time of Soil Sampling

*Gabriela Fernandez-Gnecco[1,2], Fernanda Covacevich[1], Veronica F. Consolo[1], Jan H. Behr[3], Loreen Sommermann[4], Narges Moradtalab[5], Lorrie Maccario[6], Søren J. Sørensen[6], Annette Deubel[4], Ingo Schellenberg[4], Joerg Geistlinger[4], Günter Neumann[5], Rita Grosch[3], Kornelia Smalla[2] and Doreen Babin[2]**

[1] Instituto de Investigaciones en Biodiversidad y Biotecnología (INBIOTEC), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Mar del Plata, Buenos Aires, Argentina, [2] Julius Kühn Institute (JKI) – Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Braunschweig, Germany, [3] Leibniz Institute of Vegetable and Ornamental Crops (IGZ), Plant-Microbe Systems, Großbeeren, Germany, [4] Department of Agriculture, Ecotrophology and Landscape Development, Institute of Bioanalytical Sciences (IBAS), Anhalt University of Applied Sciences, Bernburg, Germany, [5] Department of Nutritional Crop Physiology, Institute of Crop Science, University of Hohenheim, Stuttgart, Germany, [6] Section of Microbiology, Department of Biology, University of Copenhagen, Copenhagen, Denmark

OPEN ACCESS

Edited by:
Patricia Dorr De Quadros, University of Waterloo, Canada

Reviewed by:
Simone Raposo Cotta, University of São Paulo, Brazil
Daniel Kumazawa Morais, Academy of Sciences of the Czech Republic (ASCR), Czechia

***Correspondence:**
Doreen Babin
doreen.babin@julius-kuehn.de

Specialty section:
This article was submitted to Soil Biology, Ecosystems and Biodiversity, a section of the journal Frontiers in Soil Science

Received: *16 December 2021*
Accepted: *07 February 2022*
Published: *31 March 2022*

Citation:
Fernandez-Gnecco G, Covacevich F, Consolo VF, Behr JH, Sommermann L, Moradtalab N, Maccario L, Sørensen SJ, Deubel A, Schellenberg I, Geistlinger J, Neumann G, Grosch R, Smalla K and Babin D (2022) Effect of Long-Term Agricultural Management on the Soil Microbiota Influenced by the Time of Soil Sampling. Front. Soil Sci. 2:837508. doi: 10.3389/fsoil.2022.837508

Application of agrochemicals and mechanization enabled increasing agricultural productivity yet caused various environmental and soil health-related problems. Agricultural practices affect soil microorganisms, which are the key players of many ecosystem processes. However, less is known about whether this effect differs between time points. Therefore, soil was sampled in winter (without crop) and in summer (in the presence of maize) from a long-term field experiment (LTE) in Bernburg (Germany) managed either under cultivator tillage (CT) or moldboard plow (MP) in combination with either intensive nitrogen (N)-fertilization and pesticides (Int) or extensive reduced N-fertilization without fungicides (Ext), respectively. High-throughput sequencing of 16S rRNA gene and fungal ITS2 amplicons showed that changes in the microbial community composition were correlated to differences in soil chemical properties caused by tillage practice. Microbial communities of soils sampled in winter differed only depending on the tillage practice while, in summer, also a strong effect of the fertilization intensity was observed. A small proportion of microbial taxa was shared between soils from the two sampling times, suggesting the existence of a stable core microbiota at the LTE. In general, taxa associated with organic matter decomposition (such as *Actinobacteria*, *Bacteroidetes*, *Rhizopus*, and *Exophiala*) had a higher relative abundance under CT. Among the taxa with significant changes in relative abundances due to different long-term agricultural practices were putative pathogenic (e.g., *Gibellulopsis* and *Gibberella*) and beneficial microbial genera (e.g., *Chitinophagaceae*, *Ferruginibacter*, and *Minimedusa*). In summary, this study suggests that the effects of long-term agricultural management practices on the soil microbiota are influenced by the soil sampling time, and this needs to be kept in mind in future studies for the interpretation of field data.

Keywords: tillage practice, fertilization intensity, high-throughput amplicon sequencing, 16S rRNA gene, fungal ITS2 region

INTRODUCTION

Agricultural production has been intensified globally through the use of irrigation, fertilizers, biocides, and mechanization to meet the growing demands for food, feed, and fiber (1). However, intensive agricultural management contributes to soil erosion, salinization, nutrient depletion, and imbalance and a decline in water-holding capacity and soil structure (2). Furthermore, loss of soil biodiversity as well as increases of soil-borne plant pathogens are reported as principal consequences of conventional intensive agriculture (3, 4). Applying a sustainable crop management, e.g., highly diverse crop rotations and reduced tillage coupled with lower agrochemical input, is suggested to conserve soil quality and health as well as biodiversity (5, 6).

Soil properties in agricultural systems are affected by multiple environmental and anthropogenic factors, resulting in temporal variability, i.e., variability between years and within growing seasons (7, 8). In particular, biological soil properties are highly spatiotemporally variable. For instance, temporal dynamics of soil microbial communities and fluctuations in microbial (relative) abundances during the growing season of a crop have been reported (9–12). In contrast, other features, such as soil pH, texture, and porosity, are considered to be rather static (8).

Soil microorganisms are involved in nutrient cycling, organic matter (OM) decomposition, pathogen suppression, and maintenance of the soil structure (13). Therefore, the soil microbiota is essential for soil ecosystem functioning and for plant growth and health (14). There is increasing evidence that agricultural management practices affect the soil microbial community structure and composition (12, 15–18). Previous studies show that the type of tillage is one of the main drivers of the microbial community composition (19–21), leading to changes in the relative abundance of certain taxa. For instance, actinobacterial taxa (e.g., *Nocardioides, Rubrobacter*), known to contribute to OM decomposition (22), exhibited a higher relative abundance in soils under cultivator tillage (CT), whereas acidobacterial taxa (such as order Gp4) were higher under conventional moldboard plow (MP) tillage (21). Regarding putative plant pathogens, tillage practices exert different effects on soil microbial communities. For instance, *Fusarium* or *Phoma* were shown to be enhanced under CT or MP practices, respectively (20). Fertilizer quantity and quality as well as pesticide input also shape the soil microbiota (20, 21, 23, 24). Nitrogen (N) fertilization intensity was shown to increase the relative abundance and community structure of bacteria and fungi (25) as well as of plant beneficial microorganisms, such as arbuscular mycorrhizal fungi (AMF) (20). Many studies usually focus on the soil microbiota of one growing season disregarding temporal variability between years. This is of particular concern as an improved understanding of the temporal dependency of the farming practice effect on the soil microbiota under field conditions could also help to understand the mechanism behind plant–soil feedback and agricultural legacies (26, 27). This could make a critical contribution to the development of microbial-based solutions for sustainable farming practices.

In the present study, we used a long-term field experiment (LTE) established in Bernburg (Saxony-Anhalt, Germany) in 1992. This LTE facilitates comparing two different tillage practices, i.e., CT vs. MP. Additionally, two different intensities of N-fertilization and pesticide use, i.e., standard N-fertilization with pesticide application (Int) vs. reduced N-fertilization without growth regulators/fungicides (Ext), are applied per tillage practice. The effects of these agricultural managements on the LTE soil microbiota have already been well described (18, 20, 21), but previous studies lack information on the temporal variability of the soil microbiota. Therefore, the objectives of the present study are a) to determine the effect of tillage and N-fertilization intensity on the soil microbiota (here: bacteria, archaea, and fungi) depending on the soil sampling time and b) to evaluate whether soils under different management and sampling time share common microorganisms (core microbiota). We hypothesized that (i) tillage practice is the main driver of the soil microbiota in winter while, in summer, also the fertilization intensity exerts a strong effect. Moreover, we hypothesized that (ii) long-term conservation practices (CT, Ext) exhibit a higher microbial diversity and more potentially beneficial microbes compared with the conventional practices (MP, Int) independent of the sampling time.

MATERIALS AND METHODS

Site Description and Soil Sampling

The LTE is located at the Anhalt University of Applied Sciences, Bernburg, Saxony-Anhalt, Germany, and it was established in 1992 to evaluate an annual rotation system consisting of winter wheat (*Triticum aestivum* L.) / maize (*Zea mays* L.) / winter wheat / winter barley (*Hordeum vulgare* L.) / winter rapeseed (*Brassica napus* L.) under two different tillage practices and fertilization intensities. The LTE consists of five plots (1.2 ha each, divided into four subplots [replicates]). The experimental station (51.82°N and 11.70°E, 511 mm mean annual rainfall, 9.7°C mean temperature [1981–2010], 80 m above sea level) was previously described (28). Briefly, the soil is a loess chernozem over limestone (22% clay, 70% silt, and 8% sand) in the plowed upper horizon (20).

Two different tillage practices [CT (12–15 cm depth) vs. MP (20–30 cm depth)] are applied in combination with two intensities of N-fertilization (Int vs. Ext). This results in four treatments (CT.Ext, CT.Int, MP.Ext, and MP.Int), each with four replicates (**Table 1**).

Soil sampling was carried out in the season 2018/2019 in the field used for maize cultivation. The preceding crop was winter wheat (after rapeseed), which was harvested in July 2018. Soil management with CT or MP was applied on 5 November and soil samples were collected on 28 November 2018, from the fallow field (in the following referred to as winter sampling). Maize (cv. Benedictio) was sown on 23 April 2019, with a single-grain seed drill. After sowing, 100 or 40 N kg ha^{-1} in a water solution of urea and ammonium nitrate (UAN 28; 14% N as carbamide, 7% N as NH_4^+, 7% N as NO_3^-) were applied as Int or Ext treatment, respectively. As postemergence herbicides, S-metolachlor, atrazine with mesotrione, and prosulfuron were

TABLE 1 | Overview of long-term agricultural practices (treatments; $n = 4$ replicates) studied at LTE Bernburg.

Acronym	Sampling time	Tillage practice	N-fertilization intensity
W.CT.Ext	Winter (W)	Cultivator tillage (CT)	50% reduced N-fertilization without use of fungicides/growth regulators (Ext)
W.CT.Int	Winter (W)	Cultivator tillage (CT)	Standard N-fertilization with use of pesticides/growth regulators (Int)
W.MP.Ext	Winter (W)	Moldboard plow (MP)	50% reduced N-fertilization without use of fungicides/growth regulators (Ext)
W.MP.Int	Winter (W)	Moldboard plow (MP)	Standard N-fertilization with use of pesticides/growth regulators (Int)
S.CT.Ext	Summer (S)	Cultivator tillage (CT)	50% reduced N-fertilization without use of fungicides/growth regulators (Ext)
S.CT.Int	Summer (S)	Cultivator tillage (CT)	Standard N-fertilization with use of pesticides/growth regulators (Int)
S.MP.Ext	Summer (S)	Moldboard plow (MP)	50% reduced N-fertilization without use of fungicides/growth regulators (Ext)
S.MP.Int	Summer (S)	Moldboard plow (MP)	Standard N-fertilization with use of pesticides/growth regulators (Int)

applied two months before soil sampling in both Int and Ext treatments. The second soil sampling was carried out on 2 July 2019, at the vegetative stage of maize [stem elongation 31–34 BBCH scale (29)], in the following referred to as summer sampling.

For each sampling and treatment, 20 random subsamples per replicate ($n = 4$) were taken with a soil corer (5 cm diameter) from 0 to 20 cm depth in winter. In order to reduce the effect of the standing crop in summer, the soil loosely adhering to maize roots was sampled which was obtained by digging out and shaking nine plants per replicate. Soil subsamples were combined per replicate and homogenized by sieving (mesh size 2 mm). Thus, a total number of 16 samples (four treatments, each four replicates) for each sampling time were collected and stored at −20°C until total community-DNA (TC-DNA) extraction.

Soil Chemical Properties

Total N (TN), total C (TC), OM (for soils sampled in summer), total organic C (TOC), K_2O, P_2O_5, and pH were analyzed according to standard protocols of VDLUFA and DIN (Association of German Agricultural Analytic and Research Institutes e. V. and German Institute for Standardization, respectively). Soil OM was converted to TOC using a conversion factor of 1.724 (30). K_2O and P_2O_5 were converted into K and P, respectively, according to their molecular mass.

Total Community-DNA Extraction

The TC-DNA was extracted from 0.5 g of soil (fresh weight) from each replicate by harsh lysis using a FastPrep-24 bead-beating system and the FastDNA Spin Kit for Soil and then purified using GeneClean Spin Kit according to the manufacturer's instructions (both MP Biomedicals, Santa Ana, California, USA). The TC-DNA quality and yields were checked by 1% agarose gel electrophoresis using 0.5X TBE buffer and stained with 0.005% ethidium bromide. The extracted and purified TC-DNA was stored at −20°C.

Quantification of Bacterial 16S rRNA Gene and Fungal ITS Fragment Copies by Quantitative Real-Time PCR (qPCR)

Quantification of the bacterial 16S rRNA gene was carried out using the primer pair Bact1369F (5'-CGGTGAATACGTTCYCGG-3') and Prok1492R (5'-GGWTACCTTGTTACGACTT-3') (31). The detection of bacterial genes was based on the release of a fluorescence signal from the TaqMan-probe TM1389F [5'-CTTGTACACACCGCCCGTC-3'; (31)] containing the FAM fluorophore attached to the 5'-end and a TAMRA quencher at the 3'-end. Amplifications were performed in 50 μL reaction volume as described in Vogel et al. (32) with the modification of using 1.25 U Hot Start Taq Polymerase (New England BioLabs, Inc., Ipswich, Massachusetts, USA). Serial dilutions of the gel-purified 16S rRNA gene from *Escherichia coli* (1,467 bp) cloned into pGEM-T vector (Promega, Fitchburg, Wisconsin, USA; optical density $OD_{260} = 0.513$) were used for the generation of standard curves (average efficiency = 112.3%; $R^2 = 0.982$).

Fungal ITS fragments were quantified according to the protocol established by Gschwendtner et al. (33) with the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCGCTTATTGATATGC-3') (34). The detection of fungal fragments was carried out with the fluorescent dye EvaGreen (Biotium, San Francisco, California, USA). Amplifications were performed in 50 μL reaction volume as described in Vogel et al. (32) with the modification of 4% DMSO. The serially diluted gel-purified ITS fragment from *Phomopsis* sp. cloned into pGEM-T vector was used for generating standard curves (technical triplicates; average efficiency = 81.4%; $R^2 = 0.998$). The specificity of EvaGreen detection was checked by melting curve analysis. Amplifications and detections were performed in the Thermocycler CFX96 Real Time PCR System (Bio-Rad Laboratories, Inc., Hercules, California, USA). Logarithmic transformed data was related to the soil dry weight.

16S rRNA Gene and ITS2 Fragment Amplicon Sequencing

As described in Fernandez-Gnecco et al. (12), amplicon sequencing libraries were prepared using a two-step PCR targeting the V3-V4 region of the bacterial and archaeal 16S rRNA gene or the ITS2 region for fungal community profiling. Briefly, the V3-V4 region of the 16S rRNA gene was amplified using primers Uni341F (5'-CCTAYGGGRBGCASCAG-3') and Uni806R (5'- GGACTACHVGGGTWTCTAAT-3') (35–37) with PCR conditions as described in Babin et al. (21). The first ITS2 PCR was performed with the primers gITS7 (5'-GTGARTCATCGARTCTTTG-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (38) with PCR conditions as described in Fernandez-Gnecco et al. (12). PCR products were checked by

1% agarose gel electrophoresis using 0.5X TBE buffer and stained with 0.005% ethidium bromide.

Illumina sequencing adapters and sample-specific dual indexes (IDT Integrated DNA Technologies, Coralville, Iowa, USA) were added in a second PCR using PCRBIO HiFi (PCR Biosystems Ltd., London, UK) for 15 amplification cycles. As for the first PCR, amplification products were purified using HighPrep PCR clean-up (MagBio Genomics, Gaithersburg, USA, ratio 0.65:1). SequalPrep Normalization Plate (96) Kits were used to normalize sample concentrations (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The libraries were then pooled and concentrated using DNA Clean and Concentrator-5 Kit (Zymo Research, Irvine, California, USA). After determining 16S rRNA gene and ITS2 pool concentrations using the Quant-iT High-Sensitivity DNA Assay Kit (Life Technologies, Carlsbad, California, USA), the libraries were denatured and diluted to 8 pM. Sequencing was performed on an Illumina MiSeq platform using Reagent Kit v2 (2 × 250 cycles; Illumina, San Diego, California, USA) following the manufacturer's instructions.

Sequence Analysis

Cutadapt version 2.3 (39) was used to remove primer sequences from first PCR, and only read pairs containing both primer sequences were kept. Reads were further processed for error correction, merging, and generation of amplicon sequence variants (ASVs) using DADA2 version 1.10.0 (40) plugin for QIIME2 (41) with the following parameters: truncL = 0, truncR = 0; trimL = 8, trimR = 8, a minimum overlap of five nucleotides, and otherwise default parameters. Each ASV was taxonomically annotated using q2-feature-classifier classify-sklearn module trained with SILVA SSU rel. 132 database (42), trimmed for the V3-V4 region for bacterial and archaeal community analysis or with the untrimmed UNITE database v7.2 dynamic (43) for fungal community analysis.

Singletons, potential contaminants based on the negative control, and non-target reads (chloroplasts and mitochondria for 16S rRNA gene data) were removed. Additionally, to account for PCR and sequencing artifacts, microbial ASVs with fewer than five reads across the full data set were excluded from further analyses. For 16S sequencing data, curation consisted of ambiguous taxonomy renaming. Decontam R package (44) was used to filter ASVs identified as PCR contaminants using the "prevalence" method. Only for ITS data, a potential contaminant ASV, identified as *Penicillium* sp., was found and removed in winter samples, which represented up to 51% of reads. The resulting final number of ASVs and quality-filtered reads per sample and treatment can be found in **Supplementary Tables 1–3**. For 16S rRNA gene data, the cleaning resulted in a final number of 3,814 ASVs and 421,991 high-quality reads for the winter sampling and 4,970 ASVs and 519,482 reads for the summer sampling. For ITS data, a total of 175 ASVs and 85,081 reads for the winter sampling and 885 ASVs and 1,516,993 reads for the summer sampling were obtained.

Statistical Data Analysis

According to the experimental design, tillage practice and N-fertilization intensity were treated as fixed factors while sampling time was considered random. Therefore, for each sampling time separately, a two-factorial model was used for the statistical analysis of soil chemical characteristics, microbial gene copy numbers, and alpha- and beta-diversity metrics. Soil chemical characteristics, alpha-diversity indices and qPCR results were subjected to analysis of variance (ANOVA) after checking whether data meets ANOVA assumptions (Shapiro-Wilk's and Levene's tests). When ANOVA assumptions failed, data was log10 transformed to achieve a Gaussian distribution. To conduct comparison between two individual treatments (CT.Ext vs. CT.Int, MP.Ext vs. MP.Int, CT.Ext vs. MP.Ext, and CT.Int vs. MP.Int), Student's *t*-test was performed. Alpha-diversity indices (Species richness, Shannon diversity, and Pielou's evenness) were calculated per sample based on 100 times randomly to the least number of sequences per data set (bacterial and archaeal community: winter $n = 13{,}905$ or summer $n = 3{,}962$ reads; fungal community: winter $n = 3{,}460$ or summer $n = 65{,}376$ reads) subsampled read count data. In order to test the effect of tillage and N-fertilization intensity on the microbial community composition (beta-diversity), a permutational multivariate analysis of variance [PERMANOVA; (45)] was used. The PERMANOVA analysis was based on Bray–Curtis dissimilarity matrices using 10,000 permutations calculated from logarithmic transformed data. Differences among community compositions were visualized using non-metric multidimensional scaling (NMDS) and constrained analysis of principal coordinates (CAP). Both methods were based on Bray–Curtis dissimilarities and were performed with logarithmic transformed data. To correlate microbial community composition with soil chemical parameters, the function envfit (package vegan) was used.

To test for microbial genera with significantly different relative abundance between CT vs. MP or Int vs. Ext, respectively, a likelihood ratio test under negative binomial distribution and generalized linear models (FDR-corrected $p < 0.05$) was carried out separately per sampling time and for each N-fertilization intensity or tillage type, respectively. The normalization of count data was performed using correction factors for the library size as recommended by the developers (edgeR), and only genera present in more than three samples across the data set were considered (relative abundance > 0.5%). To graphically display the relative abundance distribution of the most abundant genera affected by agricultural practices, a heat map based on relative abundances for each taxon was drawn (horizontal clustering based on Euclidean distance). For each sampling time, a Venn diagram was generated to compare the presence of ASVs in the different agricultural practices and to identify the core microbiota, i.e., ASVs present in all treatments. Fungal trophic modes at genus level were predicted using the software tool FUNGuild following developers' recommendations (46).

All analyses were carried out with RStudio version 3.6.1 (https://www.r-project.org/) using the following R packages: vegan (47), agricolae (48), rcompanion (49), car (50), edgeR (51), ggplot2 (52), rioja (53), phyloseq (54), labdsv (55), mvabund (56), pheatmap (57), BiocManager (58), and VennDiagram (59).

TABLE 2 | Chemical properties in soils under different long-term tillage practices (cultivator tillage vs. moldboard plow) and N-fertilization intensities (intensive vs. extensive) in LTE Bernburg.

Soil chemical properties	Season	Cultivator tillage		Moldboard plow	
		Extensive	Intensive	Extensive	Intensive
TN (%)	Winter	0.2 ± 0 aA	0.2 ± 0 aA	0.1 ± 0 aB	0.1 ± 0 aB
	Summer	0.2 ± 0.0 aA	0.2 ± 0 aA	0.1 ± 0 aB	0.1 ± 0 aB
TC (%)	Winter	2.2 ± 0 aA	2.1 ± 0 aA	1.8 ± 0.1 aB	1.9 ± 0.1 aB
	Summer	2.2 ± 0.1 aA	2.3 ± 0.0 aA	1.7 ± 0.0 bB	1.9 ± 0.0 aB
TOC (%)	Winter	2.0 ± 0 aA	1.9 ± 0 aA	1.4 ± 0 aB	1.4 ± 0.1 aB
	Summer	2.0 ± 0.1 aA	2.2 ± 0 aA	1.4 ± 0 aB	1.5 ± 0 aB
Available K (mg kg^{-1})	Winter	483.4 ± 21.49 aA	388.7 ± 12.68 bA	164.6 ± 6.48 aB	148.3 ± 6.21 aB
	Summer	411.7 ± 6.19 aA	397.9 ± 8.01 aA	153.9 ± 1.91 aB	147.4 ± 2.05 aB
Available P (mg kg^{-1})	Winter	115.9 ± 12.4 aA	126.4 ± 16 aA	84.3 ± 3.8 aB	76.7 ± 4.1 aB
	Summer	168.3 ± 8.4 aA	196.6 ± 9.7 aA	103.6 ± 7 aB	78.2 ± 4.5 bB
pH	Winter	7.3 ± 0.1 aB	7.3 ± 0.1 aB	7.5 ± 0.0 aA	7.5 ± 0.0 aA
	Summer	7.3 ± 0.1 aB	7.3 ± 0.1 aB	7.5 ± 0.0 aA	7.6 ± 0.0 aA

TN, total nitrogen; TC, total carbon; TOC, total organic carbon; available potassium (K) and phosphorus (P) content. Means (n = 4) are displayed ± standard error. Different lowercase letters indicate significant differences among N-fertilization intensities tested separately per tillage practice. Different uppercase letters indicate significant differences among tillage practices tested separately per N-fertilization intensity (paired t-test, $p < 0.05$).

RESULTS

Soil Chemical Properties

Observed effects of tillage practice on soil chemical properties were independent of the sampling time. Two-way ANOVA showed that TN, TC, TOC, and available K and P as well as pH (all $p < 0.001$) were significantly affected by the tillage practice. Pairwise comparisons showed that the amount of TN, TC, TOC, and available K and P were significantly higher in CT compared with MP irrespective of the N-fertilization intensity while pH showed the opposite trend (7.5 or 7.3 for MP or CT, respectively; **Table 2**). Regarding N-fertilization intensity, effects on soil chemical properties depended on the sampling time. Two-way ANOVA showed that the N-fertilization intensity significantly affected the amount of available K ($p = 0.001$) in soils sampled in winter while TC content and available P levels (both $p < 0.001$) were affected by N-fertilization intensity in soils sampled in summer. Pairwise comparisons indicated higher levels of available K in CT.Ext vs. CT.Int in soils sampled in winter and higher TC content in MP.Int vs. MP.Ext and higher available P levels in MP.Ext vs. MP.Int in soils sampled in summer. Other parameters (pH, TOC, TN) were not affected by N-fertilization intensity.

Quantification of Bacterial and Fungal Markers

Two-way ANOVA showed that the 16S rRNA gene copy numbers were in both sampling times affected by neither tillage practice nor N-fertilization intensity. Bacterial 16S rRNA gene copy numbers ranged in winter from 9×10^8 to 1×10^9 and in summer from 7×10^8 to 1×10^9 per gram of dry soil (**Supplementary Figure 1A**).

In contrast, two-way ANOVA showed that ITS fragment copy numbers were significantly affected by the tillage practice in winter ($p = 0.001$) and summer ($p = 0.004$) but not by N-fertilization intensity. Fungal ITS copy numbers ranged in winter from 1×10^7 to 1×10^8 and in summer from 6×10^6 to 1×10^7 per gram of dry soil (**Supplementary Figure 1B**). Pairwise comparisons showed that soils from CT exhibited higher ITS copy numbers compared with MP under both N-fertilization intensities (Ext and Int) in winter, whereas in summer, this observation was only made in Int.

Soil Microbial Alpha-Diversity Patterns

Rarefaction curves (**Supplementary Figure 2**) showed that the sequencing depth was sufficient to cover the microbial diversity present in each sample.

Two-way ANOVA revealed that both tillage practice and N-fertilization intensity significantly affected the alpha-diversity of bacterial and archaeal communities in soils sampled in winter (Species richness and Shannon diversity, all $p < 0.001$; **Figures 1A,B**). Diversity estimators showed higher diversity in MP vs. CT and in Int vs. Ext. Pielou's evenness did not differ between Int vs. Ext in winter but was significantly higher in CT vs. MP ($p < 0.001$). Bacterial and archaeal Species richness and Shannon diversity in soils sampled in winter were significantly higher in MP.Int compared with CT.Int (**Figures 1A,B**) but the opposite was observed for Pielou's diversity (**Figure 1C**). In soils sampled in summer, two-way ANOVA revealed that both, tillage practice and N-fertilization intensity, affected the alpha-diversity of bacterial and archaeal communities (Species richness and Shannon diversity, all $p < 0.01$), resulting in higher bacterial/archaeal diversity in CT vs. MP. Pielou's evenness was only affected by tillage practice ($p = 0.02$), resulting in higher bacterial/archaeal diversity in Ext vs. Int. Contrary to winter, diversity estimators (Species richness, Shannon diversity) for bacterial and archaeal communities in soils sampled in summer from CT.Int had a significantly higher alpha-diversity compared

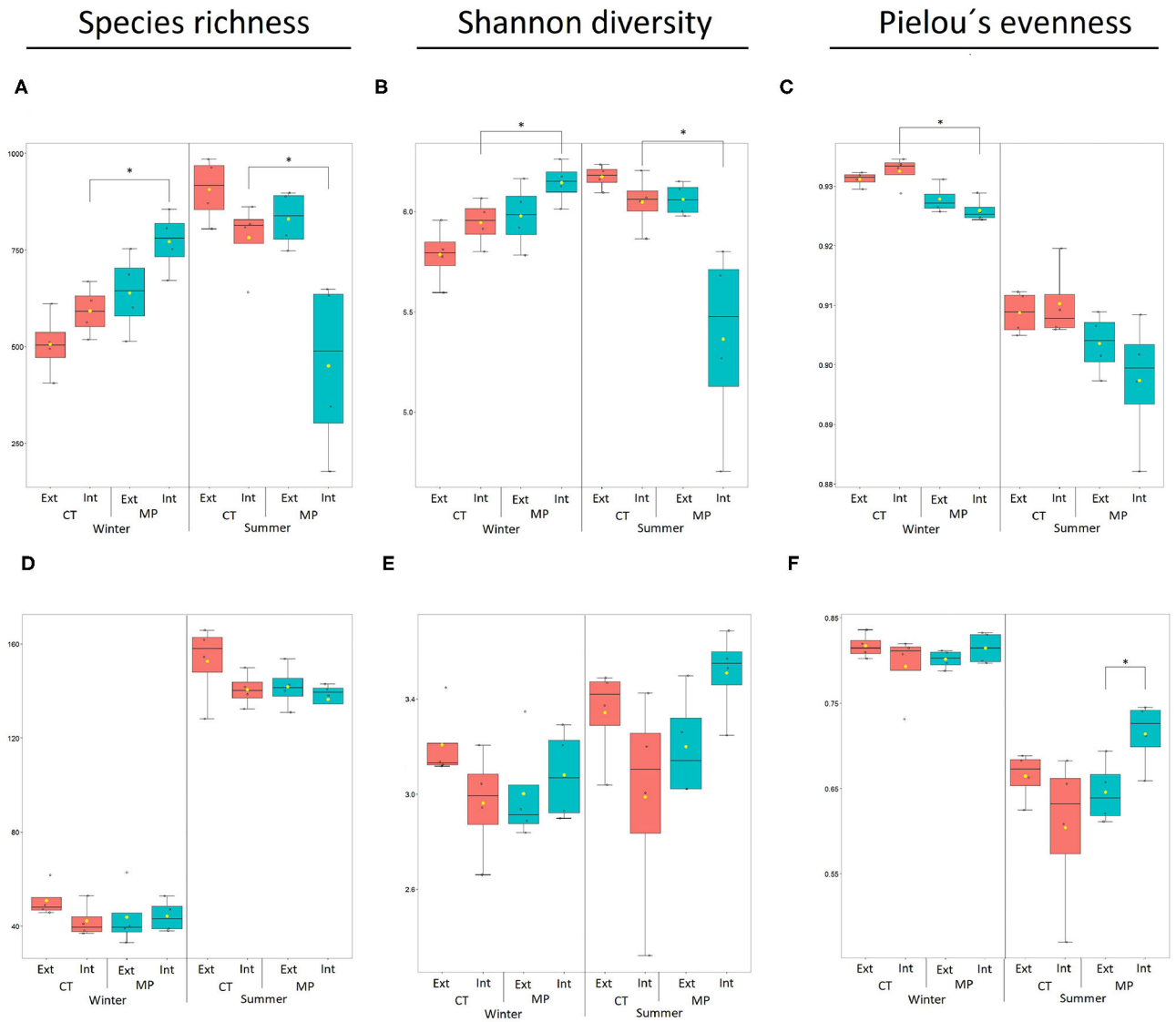


FIGURE 1 | Diversity indices of bacterial/archaeal **(A–C)** and fungal **(D–F)** communities in soils sampled in LTE Bernburg in winter or summer under different long-term tillage practices (CT, Cultivator Tillage vs. MP, Moldboard Plow) and N-fertilization intensities (Int, intensive vs. Ext, extensive). Boxplots show the mean (yellow dot), min./max. (whiskers), and replicates per sample ($n = 4$; black dots). Asterisk indicates significant differences between treatments (paired t-test, $p < 0.05$).

with MP.Int, while no significant differences among individual treatments were observed for Pielou's evenness (**Figure 1C**).

Regarding the alpha-diversity of fungal communities in soils sampled in winter, two-way ANOVA revealed that diversity estimators (Species richness, Shannon diversity, and Pielou's evenness) were affected by neither tillage practice nor N-fertilization intensity. In soils sampled in summer, two-way ANOVA revealed a significant interaction between tillage practice and N-fertilization intensity on fungal Shannon diversity and Pielou's evenness (both $p = 0.04$), resulting in higher indices in CT.Ext vs. MP.Ext and MP.Int vs. CT.Int treatments. Pairwise comparisons showed that the Pielou estimator was higher in MP.Int than in MP.Ext in soils sampled in summer (**Figures 1D–F**).

Soil Microbial Beta-Diversity Patterns

Soil microbial communities under different tillage practice and N-fertilization intensity were analyzed by PERMANOVA, which showed that both the bacterial/archaeal and fungal community composition in soils sampled in winter were significantly affected by tillage practice but not by N-fertilization intensity (**Table 3**). In summer, both factors significantly affected the microbial community composition with tillage being a stronger driver than N-fertilization intensity.

NMDS ordination showed a clear tillage-dependent clustering at both sampling times for bacterial/archaeal communities (**Supplementary Figures 3A,B**). Fungal communities were grouped by tillage practice with subclusters corresponding to the N-fertilization intensity in summer, but

TABLE 3 | Effect of long-term tillage practice and N-fertilization intensity on soil microbial communities in LTE Bernburg.

	Bacterial/archaeal communities				Fungal communities			
	Winter		Summer		Winter		Summer	
Factors	Explained variance (%)	*p*-value	Explained variance (%)	*p*-value	Explained variance (%)	*p*-value	Explained variance (%)	*p*-value
Tillage	16.81	9.99e-05*	16.60	9.99e-05*	11.03	0.01*	28.81	9.99e-05*
N-Fertilization intensity	7.47	0.08	13.79	0.001*	5.75	0.63	10.52	0.01*
Tillage: N-Fertilization intensity	6.18	0.24	6.85	0.13	4.94	0.80	6.15	0.14
Residuals	69.52		62.68		78.27		54.5	

Asterisk indicates significant influence of the factor analyzed ($p < 0.05$).

no treatment-dependent clustering was observed in winter (**Supplementary Figures 3C,D**).

CAP analysis confirmed PERMANOVA results for both 16S rRNA gene (**Figures 2A,B**) and ITS data (**Figures 2C,D**). Tillage practice shaped the microbial community composition, resulting in a distinct clustering of MP and CT samples at both sampling times. Microbial communities in CT soils were significantly positively correlated with P (except for ITS data in winter), K, TOC, TN, and TC content, whereas microbial communities in MP soils were significantly positively correlated with the soil pH (all $p < 0.01$), independently of the sampling time.

Effects of Tillage and N-Fertilization Intensity on Soil Bacterial/Archaeal Taxa

The taxonomic composition at phylum level (**Supplementary Figure 4A**) was dominated by Bacteroidetes (9 or 37%, winter or summer, respectively), Proteobacteria (25 or 26%), Acidobacteria (17 or 8%), Actinobacteria (23 or 0.5%), Thaumarchaeota (12 or 9%), and Firmicutes (3 or 7%). In winter, most of the differences in phylum relative abundance among the treatments were due to the tillage practice, resulting in a significantly higher relative abundance of Actinobacteria ($p = 0.002$) in CT compared with MP. In summer, in contrast, the phylum Firmicutes exhibited a significantly higher relative abundance ($p = 0.01$) in MP soils compared with CT. Furthermore, also the N-fertilization intensity affected the bacterial/archaeal phylum composition in summer. For instance, Proteobacteria ($p = 0.007$), Acidobacteria ($p = 0.04$), Actinobacteria ($p = 0.01$), and Thaumarchaeota ($p = 0.005$) exhibited higher relative abundances in Int vs. Ext treatments, whereas Bacteroidetes showed the opposite trend ($p = 0.01$).

At the genus level, in total, 592 bacterial/archaeal genera were detected, and sequences with closest affiliation to *Nitrososphaeraceae* (Thaumarchaeota; 11 or 9%, winter or summer, respectively), *Microscillaceae* (Bacteroidetes; 1 or 8%), *Chitinophagaceae* (Bacteroidetes; 1 or 7%), *Bacillus* (Firmicutes; 2 or 5%), and *Sphingomonas* (Proteobacteria; 3 or 4%) were predominant.

In winter, several bacterial (but not archaeal) genera with significantly different relative abundances in MP vs. CT were identified (**Supplementary Table 4**; **Figure 3**), whereas no N-fertilization intensity-dependent genera were detected (**Supplementary Table 5**; **Figure 3**). For instance, acidobacterial taxa (*RB41* and Subgroup 7) had significantly higher relative abundances in MP treatments and actinobacterial genera (*Rubrobacter* and *Nocardioides*) in CT treatments, irrespective of the N-fertilization intensity. Additionally, *Microvirga* (Proteobacteria) had a higher relative abundance in CT.Int compared with MP.Int, and sequences with the closest affiliation to *Gemmatimonadaceae* (Gemmatimonadetes) were higher in MP.Ext compared with CT.Ext indicating interaction effects between tillage and N-fertilization intensity.

In contrast, in summer, many genera with significantly different relative abundances between N-fertilization intensities were detected, and only a few genera (under 1% of relative abundance) were found to differ in relative abundance depending on tillage practice (**Figure 3**; **Supplementary Tables 4, 5**). For instance, *Ferruginibacter* and sequences with the closest affiliation to *Chitinophagaceae* (both belonging to the phylum Bacteroidetes) were significantly enriched under Ext compared with Int independent of the tillage practice. In MP soils, a significantly higher relative abundance of sequences with the closest affiliation to *Saccharimonadales* (Patescibacteria) was observed in Ext compared with Int.

Effects of Tillage and N-Fertilization Intensity on Soil Fungal Taxa

The taxonomic composition at the phylum level (**Supplementary Figure 4B**) was dominated by the phylum Ascomycota (51 or 57%, winter or summer, respectively), Basidiomycota (33 or 12%), Mucoromycota (1 or 22%), Mortierellomycota (13 or 6%), Chytridiomycota (0.8 or 1%), and Glomeromycota (0.05 or 0.3%). At both sampling times, the phylum Mucoromycota exhibited a significantly higher relative abundance in CT compared with MP while Glomeromycota showed the opposite trend (both $p < 0.02$). In summer, Basidiomycota had a higher relative abundance in MP compared with CT ($p = 0.01$).

At genus level, 241 fungal genera were detected, among which *Exophiala* (Ascomycota, 11 or 16%, winter or summer, respectively), *Rhizopus* (Mucoromycota, 2 or 22%), *Bolbitius* (Basidiomycota, 20 or 0.05%), and *Mortierella* (Mortierellomycota, 13 or 6%) were the most abundant.

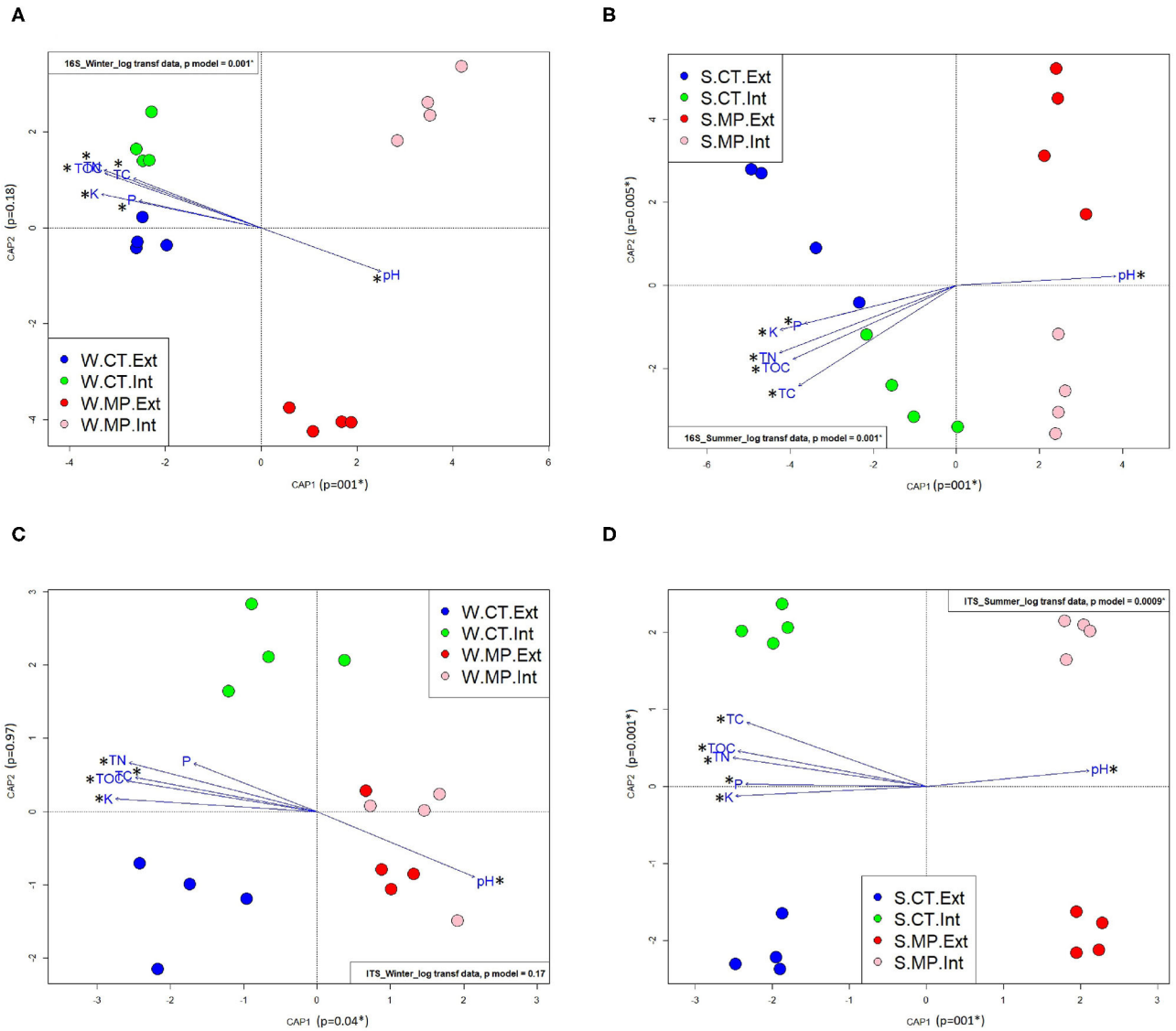


FIGURE 2 | Constrained analysis of principal coordinates (CAP) using Bray–Curtis distance of bacterial/archaeal **(A,B)** and fungal **(C,D)** communities in soils sampled in LTE Bernburg in winter (W) or summer (S) under different long-term tillage practices (CT, Cultivator Tillage vs. MP, Moldboard Plow) and N-fertilization intensities (Int, intensive vs. Ext, extensive). Asterisks indicate a significant correlation of the chemical parameter with the community composition ($p < 0.05$). Soil chemical parameters: total nitrogen (TN), total carbon (TC), total organic carbon (TOC), available potassium (K), and phosphorus (P) content and pH.

In order to obtain further insights into the ecological assignment of detected fungal genera, a tentative classification into pathotrophic, saprotrophic or symbiotrophic, or multiple trophic modes was made using FUNGuild (**Supplementary Figure 5**). Circa 77% of fungal reads could be classified at the genus level. In soils sampled in winter, fungal trophic modes were affected by tillage practice and the interaction with N-fertilization intensity, resulting in the highest relative abundance of saprotrophic fungi in CT.Ext ($p = 0.03$). In soils sampled in summer, the fungal trophic assignment was influenced by N-fertilization intensity and the interaction with tillage practice, resulting in the lowest relative abundance of genera classified as pathotroph-saprotroph in MP.Int ($p = 0.03$).

The analysis of differentially abundant fungal genera among treatments showed that, in soils sampled in winter, the genus *Rhizopus* (classified as pathotroph-saprotroph-symbiotroph, Mucoromycota) was most strongly affected by tillage practice, displaying higher relative abundances in CT than MP (**Figure 4**; **Supplementary Table 6**). No fungal genera significantly responding to N-fertilization intensity were detected in winter (**Supplementary Table 7**). In soils sampled in summer, differentially abundant fungal taxa were found to be affected by tillage practice and N-fertilization

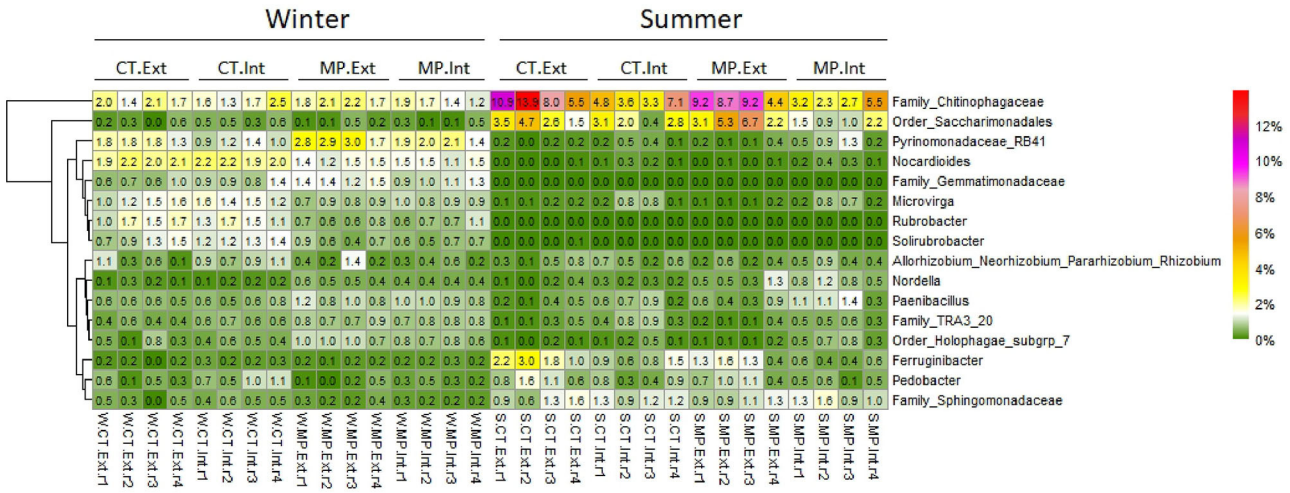


FIGURE 3 | Heatmap displaying the relative abundance distribution of bacterial genera (responder) significantly affected (FDR < 0.05) by different long-term management practices (CT, Cultivator Tillage vs. MP, Moldboard Plow; Int, intensive vs. Ext, extensive) in soils sampled in LTE Bernburg in winter (W) or summer (S). Most abundant responders with average ($n = 4$) relative abundance per treatment >0.5% are shown. Pairwise comparisons between treatments are presented in **Supplementary Tables 4, 5**.

intensity. For instance, the genus *Gibberella* (pathotroph; Ascomycota) showed a significantly higher relative abundance in CT compared with MP. *Metarhizium* (pathotroph-symbiotroph; Ascomycota) was enriched under CT.Ext compared with MP.Ext (**Supplementary Table 6**). *Minimedusa* (no trophic mode assigned; Basidiomycota) and *Gibellulopsis* (pathotroph; Ascomycota) were significantly enriched under Int compared with Ext. Soils under MP.Int exhibited a higher relative abundance of the genus *Exophiala* (pathotroph-symbiotroph; Ascomycota) compared with MP.Ext, whereas *Penicillium* (no trophic mode assigned; Ascomycota) showed the opposite trend. Genus *Rhizopus* showed different responses to N-fertilization intensity depending on the tillage type (higher relative abundance in CT.Int vs. CT.Ext but higher in MP.Ext vs. MP.Int; **Figure 4**; **Supplementary Table 7**).

Soil Bacterial/Archaeal Core Microbiota in the LTE Across Different Agricultural Practices

Regardless of tillage practice or N-fertilization intensity, a soil core microbiota, defined here as ASVs present in soils of all four investigated treatments (CT.Ext, CT.Int, MP.Ext, and MP.Int), was detected at each sampling time. The winter bacterial/archaeal core microbiota consisted of 484 ASVs (**Figure 5A**), representing 13% of the total ASVs detected in all soils. Most of these bacterial/archaeal core ASVs were affiliated to the phyla Actinobacteria, Proteobacteria, and Acidobacteria (all ca. 25%; **Figure 5C**). Classification at lower taxonomic levels revealed *RB41* (Acidobacteria; 2%) and unclassified genera of *Micrococcaceae* (3%) and Deltaproteobacteria (3%) as major core bacteria. The summer core microbiota consisted of 555 ASVs (**Figure 5B**), representing 12% of the total ASVs. Among all summer core ASVs, 35% were affiliated to the phylum Bacteroidetes, 29% to Proteobacteria and 10% to Acidobacteria (**Figure 5D**). Most of these ASVs belonged to unclassified genera of the families *Microscillaceae* (8%), *Methylophilaceae* (1%), and *Blastocatellaceae* (3%).

The core microbiota shared between winter and summer comprised 177 ASVs (data not shown). These sampling time-independent ASVs were mainly affiliated to *Chitinophagaceae* (13%), Acidobacteria_subgrp_6 (7%), and *Sphingomonadaceae* (5%).

Soil Fungal Core Microbiota in the LTE Across Different Agricultural Practices

Regardless of tillage practice or N-fertilization intensity, a winter fungal core microbiota consisting of 45 ASVs was identified (**Figure 6A**), representing 26% of the total ASVs. Most of the core ASVs belonged to the phyla Ascomycota (58%) and Basidiomycota (29%) (**Figure 6C**). At lower taxonomic levels, fungal core ASVs at the winter sampling were classified mainly as genera *Bolbitius* (20%) and *Exophiala* (10%). The summer core microbiota consisted of 125 ASVs (**Figure 6B**), representing 15% of the total ASVs. The phyla Ascomycota (63%) and Basidiomycota (21%) were again most represented among the core ASVs (**Figure 6D**). At lower taxonomic levels, most of the fungal core ASVs at the summer sampling were affiliated to the genera *Exophiala* (16%) and *Solicoccozyma* (3%).

The comparison between fungal core microbiota in winter and summer showed 21 common ASVs (data not shown), which were mainly affiliated to the genera *Mortierella* (15%), *Bolbitius* (5%), and *Exophiala* (5%).

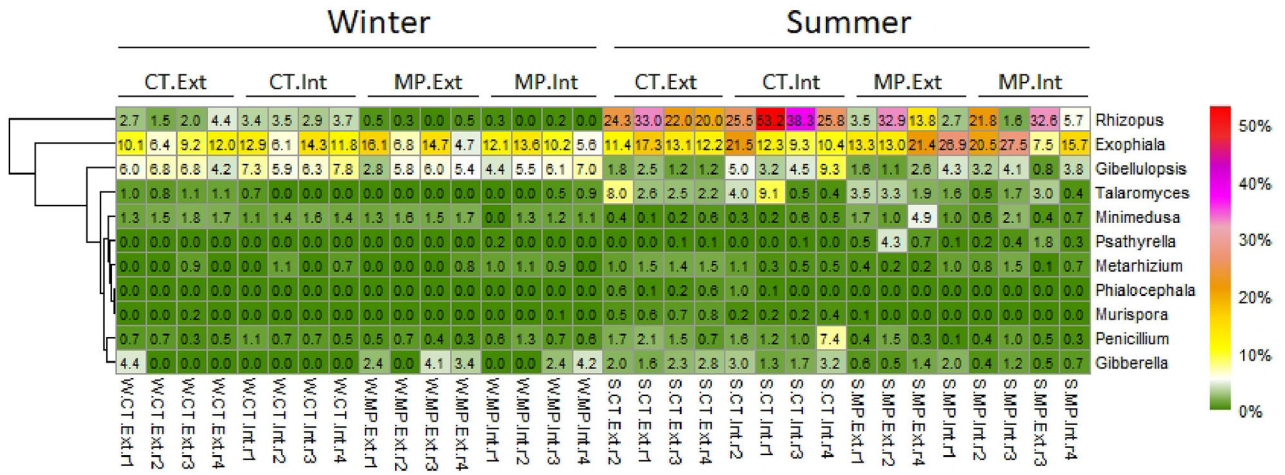


FIGURE 4 | Heatmap displaying the relative abundance distribution of fungal genera (responder) significantly affected (FDR < 0.05) by different long-term management practices (CT, Cultivator Tillage vs. MP, Moldboard Plow; Int, intensive vs. Ext, extensive) in soils sampled in LTE Bernburg in winter (W) or summer (S). Most abundant responders with average ($n = 4$) relative abundance >0.5% are shown. Pairwise comparisons between treatments with assignment into functional guilds are presented in **Supplementary Tables 6, 7**.

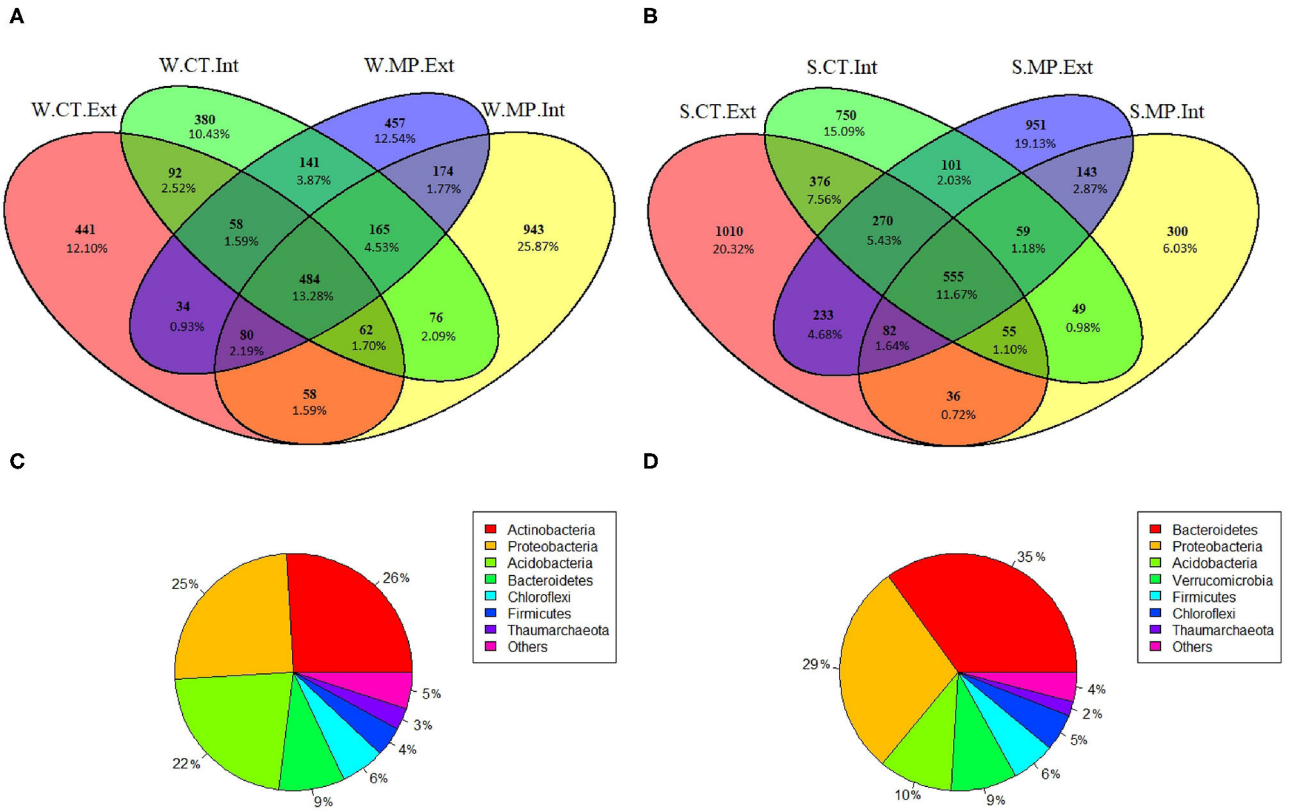


FIGURE 5 | Venn Diagrams **(A,B)** showing unique and shared bacterial/archaeal ASVs among soils sampled in LTE Bernburg in winter (W) or summer (S) under different long-term tillage practices (CT, Cultivator Tillage vs. MP, Moldboard Plow) and N-fertilization intensities (Int, intensive vs. Ext, extensive). Taxonomic classification **(C,D)** at the phylum level of ASVs shared among all four treatments in winter or summer, respectively.

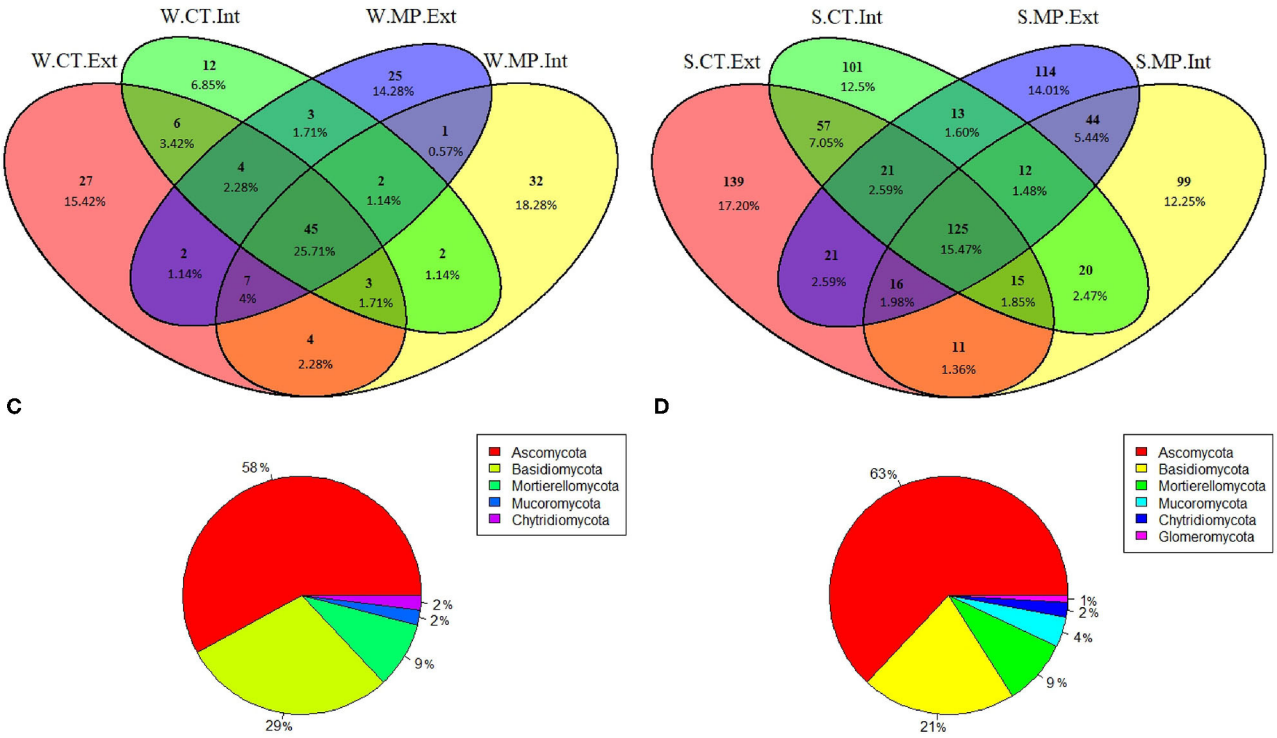


FIGURE 6 | Venn Diagrams **(A,B)** showing unique and shared fungal ASVs among treatments in soils sampled in LTE Bernburg in winter (W) or summer (S) under different long-term tillage practices (CT, Cultivator Tillage vs. MP, Moldboard Plow) and N-fertilization intensities (Int, intensive vs. Ext, extensive). Taxonomic classification **(C,D)** at phylum level of shared ASVs in winter or summer, respectively.

DISCUSSION

In this study, we aimed to determine to what extent long-term intensive and conserved agricultural management practices (tillage practice and N-fertilization intensity) affect soil chemical and microbial parameters depending on the sampling time. Soil samples were taken in absence of plants in November (winter; two weeks after tillage) and in the following growing season in July in presence of maize (summer; one month after N-fertilization).

We observed that agricultural management shaped the soil microbiota, but the effects detected were sampling time-dependent. Tillage practice was the main driver of the soil microbiota in winter while, in summer, also the N-fertilization intensity exerted a strong effect (alpha- and beta-diversity, responder analysis). This confirms our first hypothesis and is likely a direct short-term response to the applied agricultural management. The studied model crop was maize, which does not require vernalization. Therefore, the winter sampling was carried out in fallow soils shortly after soil tillage while the summer sampling took place in presence of maize under the influence of respective fertilization/plant protection measures. Agricultural practices such as crop rotation, periodic fertilization, and pesticide use result in temporal and spatial changes in soil chemical properties and, therefore, in differences in nutrient availability for microorganisms (60). It has been shown previously that different long-term tillage practices shape the soil microbial community structure (20, 21, 61). Tillage is known to make protected OM available for microbial degradation (62). Furthermore, tillage results in a destruction and transformation of microhabitats by mechanical breakup of soil aggregates (niche condition homogenization) and dislocation of the soil microbiota along the soil profile (63).

Apart from these short-term responses, our results support a legacy management effect in summer (several months after MP or CT application) suggesting that the soil disturbance had a long-lasting influence contrary to N-fertilization intensity. Schlüter et al. (64) showed for the same LTE that tillage changed the soil structure and hydraulic properties in the long term. This legacy likely promotes different conditions for microbial survival and growth and, therefore, resulted in the observed long-lasting effect on the soil microbiota. Therefore, the legacy of agricultural practices needs to be considered as an additional variable in plant-soil feedback loops (26, 27).

Contrary to our results obtained from the summer sampling, previous studies in the LTE show that N-fertilization intensity had little or no effect on the soil microbial community (18, 20, 21). In the LTE, mineral N-fertilization is performed when the

crop starts growing (for maize: in spring). In comparison to Sommermann et al. (20) and Babin et al. (21), who sampled soils from winter wheat fields in the late generative phase at harvest (July), where roots are largely inactive, we analyzed an earlier time point of the maize growing cycle during vegetative growth, characterized by high root activity and nutrient uptake (65). Particularly, during vegetative growth with intense nutrient uptake, different fertilization intensity can influence the type and extension of root-induced changes in the nearby soil chemistry and the composition and quantity of rhizodeposits with potential feedback loops on rhizosphere microbial communities. However, it should be kept in mind that the observed N-fertilization intensity effect on the soil microbiota at the summer sampling could be caused by the different levels of N-fertilization or long-term fungicide use. Mineral N fertilizer (urea and ammonia) are known to affect soil pH (66). Although no differences in soil pH in Int vs. Ext were detected here, temporal effects shortly after fertilizer application cannot be excluded. Furthermore, the high N inputs in Int could have inhibited some soil microorganisms (66). The long-term fungicide use in Int might have had direct negative effects on the fungal communities in these soils.

Only a small proportion of the detected ASVs was permanently detected in the soils, i.e., independent of the sampling time and management. This core microbiota was constituted of taxa typically associated with agricultural soils (e.g., *Sphingomonadaceae*, *Chitinophagaceae, Mortierella*, *Bolbitius*, and *Exophiala*). *Sphingomonadaceae*, *Chitinophagaceae*, and *Mortierella* were previously detected in the LTE soils (20, 21). This indicates that these taxa are specific for the soil site and are not responding to physicochemical changes caused by the agricultural management. At the different sampling times, a different core microbiota was detected. This shows that, besides dynamics in the soil microbial community compositions due to agricultural practices, there is also a season-dependent succession in the composition of the soil microbial communities.

For both sampling times, distinct soil microbial communities were observed in CT and MP. This was in concordance with previous studies in the LTE (18, 20, 21). Differences in the microbiota are likely linked to the observed tillage-dependent differences in soil chemical parameters. In this study, we could show that the soil microbial community composition under CT was strongly positively correlated with soil nutrients (e.g., K, P, TOC, and TC) but negatively correlated with pH. In fact, it was shown previously that CT changes the labile carbon pool in the soil (67). Soils under long-term reduced tillage with residues remaining on the soil surface promote soil stratification as observed for CT in the LTE, e.g., in terms of available P and K (28). Our data suggest that the higher TOC content in CT compared with MP in the topsoil layer (0–20 cm) promoted fungal growth and the enrichment of saprotrophs. Consistently, we observed higher fungal ITS fragment copy numbers in CT vs. MP. Soils under CT.Ext exhibited also a higher relative abundance of predicted saprotrophs in winter in comparison with MP.Ext. The saprotrophic fungus *Rhizopus* (Mucoromycota) had a higher relative abundance in soils under CT practice independent of the sampling time. Members of this fungal genus produce a variety of enzymes that enable them to utilize a wide range of nutrients and, therefore, to play a key role in the decomposition of organic materials (68, 69). Since plant residues are accumulated in the topsoil under CT, we, therefore, suggest that *Rhizopus* had a competitive advantage over other saprotrophs and established in the long term. This is in agreement with Srour et al. (61) who show that the accumulation of crop residues on the surface resulted in an increase in soil OM in the top layer promoting the proliferation of obligate saprotrophic fungi. In agreement with Babin et al. (21), acidobacterial taxa (such as *RB41*, Subgroup 7) had higher relative abundances in the MP treatment in comparison with CT. It is reported that a high relative abundance of Acidobacteria is indicative for oligotrophic soils with lower C and nutrient levels (70). The enrichment of actinobacterial genera, which are able to degrade complex organic compounds (22) in CT treatments in comparison with conventional MP tillage was likely related to the decomposition of crop residues remaining on the soil surface. As the decomposition of OM plays a critical role in the supply of crops with nutrients (13), we propose that CT fosters soil fertility.

Furthermore, CT and MP soils differed in the relative abundance of potential plant beneficial and pathogenic microorganisms. For instance, the relative abundance of the putative beneficial pathotroph-symbiotroph fungi *Metarhizium* (phylum Ascomycota) was significantly increased under CT tillage practice. Members of the genus *Metarhizium* are reported to have plant growth-promoting traits as well as biological control activity against insect pathogens (71, 72). The fact that the plant beneficial symbiotic AMF exhibited highest relative abundance in soils under MP tillage could be linked with the lower available P level found in these treatments in comparison with CT. This is in accordance with previous reports that stated that AMF are negatively affected (soil diversity and root colonization) by high available P levels in soils (73, 74). In addition, plow tillage can influence AMF activity by disrupting hyphal networks causing dispersion of propagules (75). Soils under CT practice also showed a high relative abundance of *Gibberella* in summer irrespective of the N-fertilization intensity. Sommermann et al. (20) reported that the relative abundance of *Gibberella*/*Fusarium* was enriched in CT.Int soils cultivated with wheat after maize in the LTE. Members of *Gibberella* are known plant pathogens, such as *Gibberella zeae* (anamorph = *Fusarium graminearum* Schwabe), which causes a mycotoxin contamination in maize, the so-called "ear rot" disease. The pathogen has the ability to survive in crop residues on the soil surface (76, 77), which presents a risk of managing soils by CT.

Irrespective of tillage practice, soils under Ext fertilization exhibited high relative abundances of sequences with the closest affiliation to *Chitinophagaceae* and the genus *Ferruginibacter* (both phylum Bacteroidetes). Members of the Bacteroidetes phylum were reported to have plant-beneficial characteristics (78). Additionally, Bacteroidetes species have the ability to degrade complex organic compounds, such as fungal cell walls (79), which means that they can act as antagonists toward fungal pathogens. Soils under Int fertilization showed a high relative abundance of *Gibellulopsis* in summer irrespective of the tillage practice. This genus contains saprophytes and

opportunistic plant pathogens, such as *Gibellulopsis nigrescens* [basionym: *Verticillium nigrescens* Pethybridge (80)], which causes vascular wilt diseases in numerous hosts and can survive in soil or on dead plant material (81). However, also the potential beneficial genus *Minimedusa,* which was reported as antagonistic toward *Fusarium* sp. (82), was higher under Int fertilization practice. Based on metagenomic analysis, Srour et al. (61) revealed that long-term plow tillage and intensive N-fertilization management shifted soil microbial communities toward fast-growing competitors (such as pathogenic species). In the present study, the cosmopolitan fungi *Exophiala* (predicted as pathotroph-symbiotroph; phylum Ascomycota) showed higher relative abundance in MP.Int vs. MP.Ext. *Exophiala* has been recently reported in similarly high relative abundance (10–15%) in agricultural soils under intensive management in the Argentinean Humid Pampas (12).

Intensive mineral N-fertilization is reported to be one of the main factors that decreases microbial diversity and number of genera (83). We could confirm this in the present study only for the bacterial/archaeal alpha-diversity in MP.Int soils sampled in summer compared to MP.Ext and contrarily even showed that fungal evenness increased in MP.Int compared to MP.Ext. This suggests that other factors, such as studied organism group, combination with agricultural practices (e.g., long-term fungicide use, tillage practice), and the sampling time point play a role when evaluating the effect of fertilization on the microbial diversity. Therefore, further studies are needed to validate these observations and elucidate the underlying mechanisms.

At the summer sampling time point, significant differences in maize shoot dry mass were recorded (see **Supplementary Table 8**) with higher dry masses in Ext than Int. This could be due to the abovementioned higher relative abundance of taxa with plant-beneficial characteristics in soils under Ext fertilization. However, this did not influence average grain yields at harvest, which were similarly low in all treatments [4.5 t ha^{-1} (MT.Ext); 4.6 t ha^{-1} (CT.Ext); 4.9 t ha^{-1} (CT.Int); 5.1 t ha^{-1} (MP.Int)]. The very low maize yields recorded in the growing season under study (2019) compared with previous years was likely due to the low temperature and water deficit that plants faced during early growth (data not shown) resulting in a deficiency of P in plants of all treatments (**Supplementary Table 9**).

Finally, whether the conserved agricultural practices of CT and Ext have positive effects on soil microbial communities and consequently on soil quality and plant performance depends on various factors. Therefore, further studies under field conditions are needed that consider additional aspects such as local weather, soil type, and the combination with other agricultural practices (e.g., crop rotation).

CONCLUDING REMARKS

For the LTE Bernburg, we showed here that the observed responses of the soil microbiota to tillage practices and N-fertilization intensity (with or without growth regulator/fungicide use) differed according to the sampling time. Cultivator tillage (CT) promoted taxa associated with organic matter decomposition resulting in higher nutrient contents, which could foster soil fertility. Moldboard plow (MP) promoted taxa typically associated with oligotrophic environments. Putative beneficial (such as *Chitinophagaceae*, *Ferruginibacter*, *Minimedusa*, and *Exophiala*) or pathogenic (such as *Gibellulopsis* and *Gibberella*) microbial genera were detected responding differently to the agricultural practices. However, further studies considering, e.g., different soil types, crops, and climatic conditions are needed to obtain more insights into how conserved agricultural practices affect plant beneficials and pathogens in soil. Understanding how agricultural management influences soil microbial communities will help to steer the soil microbiota to a desired beneficial state, which can be harnessed for the development of more sustainable agricultural practices and an improved plant performance.

DATA AVAILABILITY STATEMENT

Data sets presented in this study can be found in the NCBI sequence read archive (https://www.ncbi.nlm.nih.gov/). The accession number is PRJNA742917 or PRJNA742938 for 16S rRNA gene or ITS amplicon sequencing datasets, respectively.

AUTHOR CONTRIBUTIONS

GF-G, DB, FC, VC, and KS contributed to the conception and design of the present research. AD is in charge of the LTE in Bernburg. GF-G performed the experiments and analyzed the data. LM and SS performed the sequencing and contributed to the sequence data analysis. LS, IS, and JG helped with the interpretation of fungal data. JB, NM, GN, and RG analyzed the chemical properties. GF-G wrote the manuscript with DB. All authors contributed to refine the manuscript and approved the final version.

FUNDING

GF-G was financially supported by the National Scientific and Technical Research Council (CONICET, Argentina) and German Academic Exchange Service (DAAD, Germany). This study was financially supported by DAAD and the German Federal Ministry of Education and Research (BMBF, Germany) within the framework of the project DiControl (http://dicontrol.igzev.de/de/; grant number 031B0514C) as part of the BonaRes funding initiative Soil as a sustainable resource for the bioeconomy (https://www.bonares.de/).

ACKNOWLEDGMENTS

The present research work is part of the thesis by GF-G in partial fulfillment of the requirements for a Doctor's degree (Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata, Argentina). We would like to thank Julian Dege and

Gabriel Moyano for their helpful assistance with programming language for data analysis. We would like to thank the Biocomputing Core Facility from the department of Biology at the University of Copenhagen for providing computing power for amplicon analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsoil.2022.837508/full#supplementary-material

REFERENCES

1. Foley J, Ramankutty N, Brauman K, Cassidy E, Gerber J, Johnston M, et al. Solutions for a cultivated planet. *Nature.* (2011) 478:337–42. doi: 10.1038/nature10452
2. Zabel F, Delzeit R, Schneider J, Seppelt R, Mauser W, Václavík T. Global impacts of future cropland expansion and intensification on agricultural markets and biodiversity. *Nat Commun.* (2019) 10:2844. doi: 10.1038/s41467-019-10775-z
3. Tsiafouli M, Drakou E, Orgiazzi A, Hedlund K, Ritz K. Editorial: optimizing the delivery of multiple ecosystem goods and services in agricultural systems. *Front Ecol Evol.* (2017) 5:97. doi: 10.3389/fevo.2017.00097
4. Wolfgang A, Taffner J, Guimarães R, Coyne D, Berg G. Novel strategies for soil-borne diseases: exploiting the microbiome and volatile-based mechanisms toward controlling *Meloidogyne*-based disease complexes. *Front Microbiol.* (2019) 10:190. doi: 10.3389/fmicb.2019.01296
5. Tamburini G, Bommarco R, Cherico T, Kremen C, van der Heijden M, Liebman M, et al. Agricultural diversification promotes multiple ecosystem services without compromising yield. *Sci Adv.* (2020) 6:eaba1715. doi: 10.1126/sciadv.aba1715
6. Zhang K, Maltais-Landry G, Liao H. How soil biota regulate C cycling and soil C pools in diversified crop rotations. *Soil Biol Biochem.* (2021) 156:108219. doi: 10.1016/j.soilbio.2021.108219
7. Atreya K, Sharma R, Bajracharya N. Developing a sustainable agro-system for central Nepal using reduced tillage and straw mulching. *J Environ Manag.* (2008) 88:547–55. doi: 10.1016/j.jenvman.2007.03.017
8. Piotrowska-Długosz A, Lemanowicz J, Długosz J, Spychaj-Fabisiak E, Gozdowski D, Rybacki M. Spatio-temporal variations of soil properties in a plot scale: a case study of soil phosphorus forms and related enzymes. *J Soils Sediments.* (2016) 16:62–76. doi: 10.1007/s11368-015-1180-9
9. Barboza A, Pylro V, Jacques R, Gubiani P, de Quadros F, Trindade J, et al. Seasonal dynamics alter taxonomical and functional microbial profiles in Pampa biome soils under natural grasslands. *PeerJ.* (2018) 6:e4991. doi: 10.7717/peerj.4991
10. Shigyo N, Umeki K, Hirao T. Seasonal dynamics of soil fungal and bacterial communities in cool-temperate montane forests. *Front Microbiol.* (2019) 10:1944. doi: 10.3389/fmicb.2019.01944
11. Taye Z, Helgason B, Bell J, Norris C, Vail S, Robinson S, et al. Core and differentially abundant bacterial taxa in the rhizosphere of field grown brassica napus genotypes: implications for canola breeding. *Front Microbiol.* (2020) 10:3007. doi: 10.3389/fmicb.2019.03007
12. Fernandez-Gnecco G, Smalla K, Maccario L, Sørensen S, Barbieri P, Consolo V, et al. Microbial community analysis of soils under different soybean cropping regimes in the Argentinean south-eastern Humid Pampas. *FEMS Microbiol Ecol.* (2021) 97:fiab007. doi: 10.1093/femsec/fiab007
13. Fierer N. Embracing the unknown: Disentangling the complexities of the soil microbiome. *Nat Rev Microbiol.* (2017) 15:579–90. doi: 10.1038/nrmicro.2017.87
14. Compant S, Samad A, Faist H, Sessitsch A. A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. *J Adv Res.* (2019) 19:29–37. doi: 10.1016/j.jare.2019.03.004
15. Hartmann M, Frey B, Mayer J, Mäder P, Widmer F. Distinct soil microbial diversity under long-term organic and conventional farming. *ISME J.* (2015) 9:1177–94. doi: 10.1038/ismej.2014.210
16. Benitez M, Osborne S, Lehman R. Previous crop and rotation history effects on maize seedling health and associated rhizosphere microbiome. *Sci Rep.* (2017) 7:15709. doi: 10.1038/s41598-017-15955-9
17. Babin D, Sommermann L, Chowdhury S, Behr J, Sandmann M, Neumann G, et al. Distinct rhizomicrobiota assemblages and plant performance in lettuce grown in soils with different agricultural management histories. *FEMS Microbiol Ecol.* (2021) 97:fiab027. doi: 10.1093/femsec/fiab027
18. Bziuk N, Maccario L, Douchkov D, Lueck S, Babin D, Sørensen S, et al. Tillage shapes the soil and rhizosphere microbiome of barley-but not its susceptibility towards *Blumeria graminis* f. sp. *hordei*. *FEMS Microbiol Ecol.* (2021) 97: fiab018. doi: 10.1093/femsec/fiab018
19. Degrune F, Theodorakopoulos N, Colinet G, Hiel M, Bodson B, Taminiau B, et al. Temporal dynamics of soil microbial communities below the seedbed under two contrasting tillage regimes. *Front Microbiol.* (2017) 8:1127. doi: 10.3389/fmicb.2017.01127
20. Sommermann L, Geistlinger J, Wibberg D, Deubel A, Zwanzig J, Babin D, et al. Fungal community profiles in agricultural soils of a long-term field trial under different tillage., fertilization and crop rotation conditions analyzed by high-throughput ITS-amplicon sequencing. *PLoS ONE.* (2018) 13:e0195345. doi: 10.1371/journal.pone.0195345
21. Babin D, Deubel A, Jacquiod S, Sørensen J, Geistlinger J, Grosch R, et al. Impact of long-term agricultural management practices on soil prokaryotic communities. *Soil Biol Biochem.* (2019) 129:17–28. doi: 10.1016/j.soilbio.2018.11.002
22. Jacquiod S, Franqueville L, Cécillon S, Vogel T, Simonet P. Soil bacterial community shifts after chitin enrichment: an integrative metagenomic approach. *PLoS ONE.* (2013) 8:e79699. doi: 10.1371/journal.pone.0079699
23. Verma J, Jaiswal D, Sagar R. Pesticide relevance and their microbial degradation: a-state-of-art. *Rev Environ Sci Biotechnol.* (2014) 13:429–66. doi: 10.1007/s11157-014-9341-7
24. Zhao Z, He J, Quan Z, Wu C, Sheng R, Zhang L, et al. Fertilization changes soil microbiome functioning, especially phagotrophic protists. *Soil Biol Biochem.* (2020) 148:107863. doi: 10.1016/j.soilbio.2020.107863
25. Villamil MB, Kim N, Riggins CW, Zabaloy MC, Allegrini M, Rodríguez-Zas SL. Microbial signatures in fertile soils under long-term n management. *Front Soil Sci.* (2021) 1:765901. doi: 10.3389/fsoil.2021.765901
26. van der Putten W, Bardgett R, Bever J, Bezemer T, Casper B, Fukami T, et al. Plant-soil feedbacks: the past, the present and future challenges. *J Ecol.* (2013) 101:265–76. doi: 10.1111/1365-2745.12054
27. Lapsansky E, Milroy A, Andales M, Vivanco J. Soil memory as a potential mechanism for encouraging sustainable plant health and productivity. *Curr Opin Biotech.* (2016) 38:137–42. doi: 10.1016/j.copbio.2016.01.014
28. Deubel A, Hofmann B, Orzessek D. Long-term effects of tillage on stratification and plant availability of phosphate and potassium in a loess chernozem. *Soil Tillage Res.* (2011) 117:85–92. doi: 10.1016/j.still.2011.09.001
29. Meier U. *Growth Stages of Mono- and Dicotyledonous Plants: BBCH Monograph: Open Agrar Repositorium*. (2001). Available online at: https://www.politicheagricole.it/flex/AppData/WebLive/Agrometeo/MIEPFY800/BBCHengl2001.pdf (accessed October 10, 2021).
30. Wolff E. Entwurf zur Bodenanalyse. *Die Landwirtschaftlichen Versuchsstationen.* (1864) 6:141–71.
31. Suzuki M, Taylor L, De Long E. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. *Appl Environ Microbiol.* (2000) 4605–14. doi: 10.1128/AEM.66.11.4605-4614.2000
32. Vogel C, Babin D, Pronk G, Heister K, Smalla K, Kögel-Knabner I. Establishment of macro-aggregates and organic matter turnover by microbial communities in long-term incubated artificial soils. *Soil Biol Biochem.* (2014) 79:57–67. doi: 10.1016/j.soilbio.2014.07.012
33. Gschwendtner S, Reichmann M, Müller M, Radl V, Munch JC, Schloter M. Effects of genetically modified amylopectin-accumulating potato plants on the abundance of beneficial and pathogenic microorganisms in the rhizosphere. *Plant Soil.* (2010) 335:413–22. doi: 10.1007/s11104-010-0430-2
34. White T, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: M Innis, D

Gelfand, J Sninsky, T White, editors, *PCR Protocols: A Guide to Methods and Applications*. New York, NY: Academic Press (1990). p. 315–22. doi: 10.1016/B978-0-12-372180-8.50042-1
35. Yu Y, Lee C, Kim J, Hwang S. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng.* (2005) 89:670–9. doi: 10.1002/bit.20347
36. Sundberg C, Al-Soud W, Larsson M, Alm E, Yekta S, Svensson B, et al. 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. *FEMS Microbiol Ecol.* (2013) 85:612–26. doi: 10.1111/1574-6941.12148
37. Caporaso J, Lauber C, Walters W, Berg-Lyons D, Lozupone C, Turnbaugh P, et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA*. (2011) 108(Suppl.1):4516-22. doi: 10.1073/pnas.1000080107
38. Ihrmark K, Bödeker I, Cruz-Martinez K, Friberg H, Kubartova A, Schenck J, et al. New primers to amplify the fungal ITS2 region – evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiol Ecol.* (2012) 82:666–77. doi: 10.1111/j.1574-6941.2012.01437.x
39. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* (2011) 17:10–2. doi: 10.14806/ej.17.1.200
40. Callahan B, McMurdie P, Rosen M, Han A, Johnson A, Holmes S. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods*. (2016) 13:581–3. doi: 10.1038/nmeth.3869
41. Bolyen E, Rideout J, Dillon M, Bokulich N, Abnet C, Al-Ghalith G, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol.* (2019) 37:852–7. doi: 10.1038/s41587-019-0209-9
42. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* (2013) 41:D590–6. doi: 10.1093/nar/gks1219
43. Nilsson R, Larsson K, Taylor A, Bengtsson-Palme J, Jeppesen T, Schigel D, et al. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Res.* (2019) 47:D259–64. doi: 10.1093/nar/gky1022
44. Davis N, Proctor D, Holmes S, Relman D, Callahan B. Simple statistical identification and removal of contaminant sequences in markergene and metagenomics data. *Microbiome.* (2018) 6:226. doi: 10.1186/s40168-018-0605-2
45. Anderson M. A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* (2001) 26:32–46. doi: 10.1046/j.1442-9993.2001.01070.x
46. Nguyen NH, Song Z, Bates ST, Branco S, Tedersoo L, Menke J, et al. FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecol.* (2016) 20:241–8. doi: 10.1016/j.funeco.2015.06.006
47. Oksanen J, Blanchet F, Friendly M. *Vegan: Community Ecology Package. R package version 2.5-6*. (2019). Available online at: https://cran.r-project.org/web/packages/vegan/index.html (accessed January 18, 2021).
48. De Mendiburu F. *Agricolae: Statistical Procedures for Agricultural Research. R package version 1.3-2*. (2020). Available online at: https://CRAN.R-project.org/package=agricolae (accessed March 26, 2021).
49. Mangiafico S. *Rcompanion: Functions to Support Extension Education Program Evaluation. R package version 2.3.26*. (2020). Available online at: https://CRAN.R-project.org/package=rcompanion (accessed February 26, 2021).
50. Fox F, Weisberg S. *An R Companion to Applied Regression*. 3rd ed. Thousand Oaks CA: Sage (2019).
51. Robinson M, McCarthy D, Smyth G. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* (2010) 26:139–40. doi: 10.1093/bioinformatics/btp616
52. Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag New York. (2016). Available online at: https://ggplot2.tidyverse.org (accessed February 24, 2021).
53. Juggins S. *Rioja: Analysis of Quaternary Science Data, R package version (0.9-21)*. (2017). Available online at: http://cran.r-project.org/package=rioja (accessed February 18, 2021).
54. McMurdie P, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE.* (2013) 8:e61217. doi: 10.1371/journal.pone.0061217
55. Roberts D. *labdsv: Ordination and Multivariate Analysis for Ecology. R package version 2.0-1*. (2019). Available online at: https://CRAN.R-project.org/package=labdsv (accessed March 10, 2021).
56. Wang Y, Naumann U, Eddelbuettel D, Wilshire J, Warton D, Byrnes J, et al. *mvabund: Statistical Methods for Analysing Multivariate Abundance Data. R package version 4.1.3*. (2020). Available online at: https://CRAN.R-project.org/package=mvabund (accessed March 12, 2021).
57. Kolde R. *pheatmap: Pretty Heatmaps. R package version 1.0.12.2019*. (2019). Available online at: https://CRAN.R-project.org/package=pheatmap (accessed March 26, 2021).
58. Morgan M. *BiocManager: Access the Bioconductor Project Package Repository. R package version 1.30.10*. (2019). Available online at: https://CRAN.R-project.org/package=BiocManager (accessed January 18, 2021).
59. Chen H, Boutros P. VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinformatics*. (2011) 12:35. doi: 10.1186/1471-2105-12-35
60. Carbonetto B, Rascovan N, Álvarez R, Mentaberry A, Vázquez M. Structure, composition and metagenomic profile of soil microbiomes associated to agricultural land use and tillage systems in Argentine Pampas. *PLoS ONE.* (2014) 9:e99949. doi: 10.1371/journal.pone.0099949
61. Srour A, Ammar H, Subedi A, Pimentel M, Cook R, Bond J, et al. Microbial communities associated with long-term tillage and fertility treatments in a corn-soybean cropping system. *Front Microbiol.* (2020) 11:1363. doi: 10.3389/fmicb.2020.01363
62. Six J, Elliott E, Paustian K. Soil macroaggregate turnover and microaggregate formation: a mechanism for C sequestration under no-tillage agriculture. *Soil Biol Biochem.* (2000) 32:2099–103. doi: 10.1016/S0038-0717(00)00179-6
63. Or D, Keller T, Schlesinger W. Natural and managed soil structure: on the fragile scaffolding for soil functioning. *Soil Tillage Res.* (2021) 208:104912. doi: 10.1016/j.still.2020.104912
64. Schlüter S, Großmann C, Diel J, Wu G, Tischer S, Deubel A, et al. Long-term effects of conventional and reduced tillage on soil structure, soil ecological and soil hydraulic properties. *Geoderma.* (2018) 332:10–9. doi: 10.1016/j.geoderma.2018.07.001
65. Marschner H. *Marschner's Mineral Nutrition of Higher Plants*. London: Elsevier (2012).
66. Geisseler D, Scow K. Long-term effects of mineral fertilizers on soil microorganisms – a review. *Soil Biol Biochem.* (2014) 75:54–63. doi: 10.1016/j.soilbio.2014.03.023
67. Shanmugam G, Buehring N, Prevost J, Kingery W. Soil bacterial community diversity and composition as affected by tillage intensity treatments in corn-soybean production systems. *Microbiol Res.* (2021) 12:157–72. doi: 10.3390/microbiolres12010012
68. Lennartsson P, Taherzadeh M, Edebo L. Rhizopus. In: C Batt, M Tortorello, editors, *Encyclopedia of Food Microbiology*. 2nd ed. New York, NY: Academic Press (2014). p. 284–90. doi: 10.1016/B978-0-12-384730-0.00391-8
69. Yadav D, Mir N, Wadhwa R, Tushir S, Sethi S, Anurag R, et al. Hydrolysis of peanut (*Arachis hypogea* L.) protein concentrate by fungal crude protease extract: effect on structural., functional and *in-vitro* protein digestibility. *J Food Sci Technol.* (2021) 21:5225. doi: 10.1007/s13197-021-05225-y
70. Fierer N, Bradford M, Jackson R. Toward an ecological classification of soil bacteria. *Ecology.* (2007) 88:1354–64. doi: 10.1890/05-1839
71. Barelli L, Waller A, Behie S, Bidochka M. Plant microbiome analysis after *Metarhizium* amendment reveals increases in abundance of plant growth-promoting organisms and maintenance of disease-suppressive soil. *PLoS ONE.* (2020) 15:e0231150. doi: 10.1371/journal.pone.0231150
72. Patel T. *Metarhizium*. In: N Amaresan, M Senthil Kumar, K Annapurna, K Kumar, A Sankaranarayanan, editors, *Beneficial Microbes in Agro-Ecology*. New York, NY: Academic Press (2020). p. 593–610. doi: 10.1016/B978-0-12-823414-3.00029-0
73. Gosling P, Hodge A, Goodlass G, Bending G. Arbuscular mycorrhizal fungi and organic farming. *Agric Ecosyst Env.* (2006) 113:17–35. doi: 10.1016/j.agee.2005.09.009

74. Covacevich F, Echeverría H, Aguirrezábal L. Soil available phosphorus status determines indigenous mycorrhizal colonization of field and glasshouse-grown spring wheat from Argentina. *Appl Soil Ecol.* (2007) 35:1–9. doi: 10.1016/j.apsoil.2006.06.001
75. Schalamuk S, Cabello M. Arbuscular mycorrhizal fungal propagules from tillage and no-tillage systems: possible effects on Glomeromycota diversity. *Mycologia.* (2010) 102:261–8. doi: 10.3852/08-118
76. Pereyra S, Dill-Macky R, Sims A. Survival and inoculum production of *Gibberella zeae* in wheat residue. *Plant Dis.* (2004) 88:724–30. doi: 10.1094/PDIS.2004.88.7.724
77. Wahdan S, Hossen S, Tanunchai B, Schädler M, Buscot F, Purahong W. Future climate significantly alters fungal plant pathogen dynamics during the early phase of wheat litter decomposition. *Microorganisms.* (2020) 8:908. doi: 10.3390/microorganisms8060908
78. Lidbury I, Borsetto C, Murphy A, Bottrill A, Jones A, Bending G, et al. Niche-adaptation in plant-associated Bacteroidetes favours specialisation in organic phosphorus mineralisation. *ISME J.* (2021) 15:1040–55. doi: 10.1038/s41396-020-00829-2
79. Wieczorek AS, Schmidt O, Chatzinotas A, von Bergen M, Gorissen A, Kolb S. Ecological functions of agricultural soil bacteria and microeukaryotes in chitin degradation: a case study. *Front Microbiol.* (2019) 10:1293. doi: 10.3389/fmicb.2019.01293
80. Zare R, Gams W, Starink-Willemse M, Summerbell R. *Gibellulopsis*, a suitable genus for *Verticillium nigrescens*, and *Musicillium*, a new genus for *V. theobromae*. *Nova Hedwigia.* (2007) 85:463–89. doi: 10.1127/0029-5035/2007/0085-0463
81. Klosterman S, Subbarao K, Kang S, Veronese P, Gold S, Thomma B, et al. Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens. *PLoS Patholog.* (2011) 7:e1002137. doi: 10.1371/journal.ppat.1002137
82. Beale R, Pitt D. The antifungal properties of *Minimedusa polyspora*. *Mycol Res.* (1995) 99:337–42. doi: 10.1016/S0953-7562(09)80910-6
83. Semenov M, Krasnov G, Semenov V, van Bruggen A. Long-term fertilization rather than plant species shapes rhizosphere and bulk soil prokaryotic communities in agroecosystems. *Appl Soil Ecol.* (2020) 154:103641. doi: 10.1016/j.apsoil.2020.103641

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Soil Science

ORIGINAL RESEARCH
published: 05 April 2022
doi: 10.3389/fsoil.2022.749212

A Single Application of Compost Can Leave Lasting Impacts on Soil Microbial Community Structure and Alter Cross-Domain Interaction Networks

Steven Heisey[1], *Rebecca Ryals*[2,3], *Tai McClellan Maaz*[1] *and Nhu H. Nguyen*[1]*

[1] Department of Tropical Plant and Soil Sciences, University of Hawai'i at Mānoa, Honolulu, HI, United States, [2] Department of Natural Resources and Environmental Management, University of Hawai'i at Mānoa, Honolulu, HI, United States, [3] School of Natural Sciences, University of California, Merced, Merced, CA, United States

Our current understanding suggests that nutrient management strategies applied to agricultural soils over multiple years are required to cause major and stable shifts in soil microbial communities. However, some studies suggest that agricultural soils can benefit even from sporadic, single additions of organic matter. Here we investigate how single additions of high-quality organic matter can cause significant shifts in microbial soil communities over multiple cropping cycles. We grew radishes in a tropical Oxisol soil for six crop cycles after a single application of a high-nitrogen compost or urea. At planting and before biomass harvest, we sampled soils influenced by the radish rhizosphere and sequenced bacterial and archaeal 16S and fungal ITS rDNA marker genes. We measured microbial richness and diversity, community composition and structure, and constructed correlation networks to predict cross-domain microbial interactions. We found that a single application of compost, compared to urea or control, resulted in a persistent improved plant biomass response and led to sustained changes in the soil microbial community throughout the duration of the 227-day study. Compost altered the structure of both the fungal and prokaryotic microbial communities, introduced new microorganisms that persisted in the resident soil system, and altered soil microbial correlation network structure and hub taxa. In contrast, fertilization with urea did not significantly alter the structure of soil microbial communities compared to the control but reduced network complexity and altered hub taxa. This study highlights the significant impacts that high-quality organic matter fertilization can exert on agricultural soil microbiomes and adds to the growing body of knowledge on using organic fertilizers as a way to steer the soil microbiome toward a healthier soil.

Keywords: agriculture, tropical, soil, microbiome, network, Oxisol, Hawaii

OPEN ACCESS

Edited by:
Nigel Victor Gale,
University of Toronto, Canada

Reviewed by:
German Andres Estrada-Bonilla,
Colombian Corporation for Agricultural Research (AGROSAVIA), Colombia
José A. Siles,
Spanish National Research Council (CSIC), Spain

***Correspondence:**
Nhu H. Nguyen
nhu.nguyen@hawaii.edu

Specialty section:
This article was submitted to Soil Biology, Ecosystems and Biodiversity, a section of the journal Frontiers in Soil Science

Received: *29 July 2021*
Accepted: *07 March 2022*
Published: *05 April 2022*

Citation:
Heisey S, Ryals R, Maaz TM and Nguyen NH (2022) A Single Application of Compost Can Leave Lasting Impacts on Soil Microbial Community Structure and Alter Cross-Domain Interaction Networks. Front. Soil Sci. 2:749212. doi: 10.3389/fsoil.2022.749212

INTRODUCTION

Soil microbes are fundamental drivers of soil nutrient dynamics, and our ability to understand and predict microbial composition and function in agricultural soils is essential for the maintenance of healthy and sustainable soil ecosystems (1). Soil health, defined as the ability of agricultural soils to continue to provide ecosystem services while optimizing agricultural yields, consists of

soil physical, chemical, and biological components (2). Some common indicators for soil health include soil physical and chemical characteristics like pH, aggregate stability, water infiltration, bulk density, soil organic matter content, and biological indicators like microbial biomass and activity, microbial diversity, and carbon and nitrogen cycling potentials (2, 3). Indicators like microbial diversity and community structure are linked in part to properties like soil pH (4) and soil organic matter (5–7) highlighting the importance in managing these soil qualities when considering the health of the soil microbiome.

Nitrogen management in agricultural systems has a strong impact on soil quality and the soil microbiome (8–11). Urea fertilizer, otherwise known as carbamide $CO(NH_2)_2$, is the most common synthetic nitrogen source applied to agricultural soils to meet crop N requirements (12). Urea is a simple compound, composed of 46% N, that hydrolyzes rapidly by soil urease enzyme into carbonic acid and ammonia (13, 14). As a result of these fast reactions, the promotion of plant growth and soil microbial community shift is most pronounced immediately after application (15). The impact of urea on the soil microbiome, both direct and indirect, however, is less predictable. Some studies showed that repeated application of urea may decrease soil health, in part due to an increase in ureolytic prokaryotes whose presence decreases plant nitrogen use efficiency by promoting N losses through gaseous forms (i.e., ammonia volatilization following urea hydrolysis, denitrification following ammonia oxidation), as well as a general decrease in microbial diversity that provide other ecosystem services (16, 17). However, other studies suggest that the application of urea fertilizers can increase plant growth and the resulting abundance of root exudates can in-turn stimulate microbial activity and increase nitrogen cycling (9, 16).

In contrast, complex nutrient sources such as composts are also known to boost agricultural productivity directly by increasing soil plant-available nitrogen (18–20). However, compost also has indirect benefits to agricultural productivity primarily associated with its more complex organic compounds, including enhanced nitrogen use efficiency via the re-coupling of carbon and nitrogen cycles (21), increased microbial abundance and activity (18, 22), improved nutrient cycling and disease suppression (23, 24), better nutrient retention (25), and greater overall soil quality (26). Therefore, maintaining soil organic matter via fertilization is a means to both increase soil fertility and diversify the soil microbiome that can help to maintain that fertility. However, trends in how the soil microbiome shifts in response to compost fertilization can vary depending on soil properties and type of compost (27, 28). However, it is unclear how the interconnected communities of dominant soil microorganisms (such as fungi and bacteria) respond to different quality of fertilizers.

Among the statistical tools available that allow us to connect the complex interactions in the soil environment, network analyses offer a promising and unique opportunity to predict interactions among microbial individuals. Using co-occurrence patterns, researchers have studied how microbial networks differ between ecosystems or habitat niches (29, 30), evaluated network dynamics in response to fertilization (31) or drought (32), and gained insight into organisms associated with specific ecosystem functions (33). "Hub" species, that is, taxa that are highly connected in an ecosystem network by centrality measurements like degree and closeness centrality (34) may provide insights into complex microbiomes such as those in soils. Most previous soil microbial network studies have used single gene amplicon analyses to gain important insight on prokaryotic and eukaryotic network interactions. Current inference techniques exist that allow for analyses among multiple marker genes, giving insight into associations across biological domains and elucidate, for instance, the importance of fungi in stabilizing bacterial network connections in the human lung microbiome (35), or across soil profiles (36) and rhizospheres after wetups (37). Cross-domain network analyses applied to the soil microbiome offer unique opportunities to hypothesize interactions among communities of fungi, bacteria, and archaea, and how these interactions may ultimately lead to biological insights relevant to soil health such as the movement and cycling of nutrients in soil environment.

Despite the active research in this area, making the connection between nitrogen fertilizer sources to changes in the microbiome is still not trivial. This study aims to fill knowledge gaps associated with how soil microbial communities respond to simple and complex organic nitrogen fertilizers. We employed high-throughput sequencing techniques as well as recently developed cross-domain co-occurrence networks to measure community responses to a single fertilization event of urea and a high N compost across six planting cycles in a nutrient-poor tropical Oxisol soil. We hypothesize that complex nitrogen fertilizers such as compost, when applied to soils, have a detectable impact on plant growth, soil microbial diversity, community structure, network topography, and change network hub taxa as compared to urea, a simple, synthetic nitrogen fertilizer.

MATERIALS AND METHODS

Experimental Design

Plants were grown in a repeated measures design in a climate-controlled greenhouse with mean daytime temperature of 28°C and night time of 22°C at the University of Hawai'i at Mānoa. The Lahaina series (Very-fine, kaolinitic, isohyperthermic Rhodic Eutrustox, pH 7.9) was used as the growth medium, chosen for its naturally low fertility but agricultural importance in Hawai'i. This Oxisol is comparable to other Oxisols in tropical regions of the world (38), although the alkalinity, prior use for sugarcane production, and anecdotal evidence suggests it had been previously limed. The soil was collected from the 0 to 30 cm depth from an uncultivated field directly adjacent to an organically managed agricultural system in Waialua, HI (21.555 °N, −158.117 °W). This location had a homogenous vegetation cover of Guinea grass (*Megathyrsus maximus*) that helped to limit the amount of variation in the microbial community due to plant diversity (39, 40). Collected soil was air-dried, homogenized, and sieved to 2 mm. Plastic pots (7.6 L) with drainage holes, covered with a fiberglass screen mesh, were filled with 1 kg of acid-washed sand, followed by 3.5 kg of the homogenized soil mixed with each fertilizer treatment (urea, compost, and a no fertilizer control) at an application rate of 100 kg/ha plant-available nitrogen, which

was determined as the sum of inorganic N content and estimated mineralizable N. We used a 3% total N EcoSan compost (3–3.7–1.8, N-P-K), a product of aerobic, thermophilic composting process from human feces and sugarcane bagasse (41, 42). An estimated N mineralization rate of 7% was used for compost based on a recent review of N mineralization in composted biosolids (43). As such, we added 215.4 g of compost (dry weight) to each pot (equivalent to 47.6 metric tons/ha). We used urea (46-0-0, N-P-K) as a contrasting synthetic fertilizer. All N in urea was considered plant-available. Fertilizers were applied only once, immediately prior to the first planting. Each treatment was replicated three times ($n = 3$) for a total of nine pots ($N = 9$). Pots were arranged in a randomized control block design to account for possible environmental heterogeneity in the greenhouse. Because the soil had been dried, pots were watered to field capacity and allowed to equilibrate for 2 days prior to planting. Nine organic radish seeds (*Raphanus raphanistrum* subsp. *sativus* 'Cherry Belle', Burpee) were planted in each pot and grown for seven days; they were then thinned to three plants per pot. We chose radishes because of their fast growth from sowing to harvesting that allows for multiple growing cycles within a short period of time. Plants were hand-irrigated with deionized water (100–300 ml/day depending on the weather and stage of growth) to maintain approximate field capacity. Each cycle of radishes was grown for 36 days from seed to maturity, at which point they were harvested. The plants were uprooted, washed with deionized water, and divided at the crown for below and aboveground biomass. All three plants per pot were summed for a total biomass per replicate. Aboveground (shoots) and belowground (taproots) samples were put into paper bags and dried in an oven at 65°C for 2 and 7 days, respectively, for dry weight measurements. Large radish roots were sliced in half to facilitate the drying process. At the end of each harvest, pots were watered and allowed to equilibrate for 2 days prior to the sowing of the next cycle. These plantings and harvesting cycles were repeated for a total of 6 crop cycles, or 227 days, beginning on August 22, 2017.

Soil Sampling

Soil cores were collected immediately after treatment application (C0) and immediately prior to harvest on the 36th day for each of the six crop cycles (C1–C6). From each pot, three cores (1 cm diameter x 10 cm deep, total 63 samples) were taken about 2 cm from each radish taproot. After collection, these cores were composited and homogenized in a clean plastic bag. A subsample was put into a 10 mL transfer tube and stored in a −20°C freezer for microbial analysis. The remaining samples were air-dried and analyzed for pH using a slurry method with a 2 to 1 ratio of deionized water:soil. Soil samples collected after the sixth crop cycle (C6) were analyzed for total carbon, total nitrogen, and exchangeable cations (**Table 1**). Soil carbon and nitrogen concentrations were analyzed by combustion on an elemental analyzer (Costech 4100 Elemental Analyzer) at the University of Hawai'i at Hilo Analytical Laboratory. Exchangeable calcium, magnesium, potassium, and sodium were extracted from soils using the ammonium acetate method buffered at pH 7.0 (44) and analyzed on a Thermo iCAP DUO 7400 ICP-OES. Cation exchange capacity was calculated as the sum of base cations.

Amplicon Library Preparation

DNA was extracted from 0.25 g of frozen soil using the DNeasy PowerSoil kit (QIAGEN, Germany) following the manufacturer's standard protocol. Fungal and bacterial mock communities served as positive controls (45). Primer design and sample barcoding followed a two-step amplification, dual barcoding system using a combination of a P5/P7 Illumina Adapter, 8 bp barcodes attached to each adapter, and partial P5/P7 overhangs (**Supplementary Figure 1**). For bacteria and archaea, the 16S rRNA gene (V4 region) was targeted using updated Earth Microbiome Project primer pairs 515F (46) and 806RB (47). For fungi, the ITS1 region was targeted using primer pairs ITS1F (48) and ITS2 (49). High-fidelity, hot-start polymerase mastermixes were used for the PCR reaction with iProof (Bio-Rad Laboratories, USA) for 16S and Phusion (ThermoFisher Scientific Inc., USA) for ITS. Internal testing showed that these polymerases amplified each respective gene more efficiently. In the first PCR, the targeted loci (16S, ITS) were amplified under the following thermocycling conditions using 3-5 ng of DNA: For 16S, polymerase activation at 98°C for 30 s, followed by 20 cycles of 98°C for 10 s, 55°C for 15 s, 72°C for 10 s, and a final extension at 72°C for 7 min; and for ITS, polymerase activation at 98°C for 30 s, followed by 20 cycles of 98°C for 10 s, 53°C for 15 s, 72°C for 10 s, and a final extension at 72°C for 7 min. PCR products were assessed using gel electrophoresis and successfully amplified samples were cleaned using AMPure SPRI beads (Beckman Coulter, USA). In the second PCR step, 1 μl of the cleaned products was used as templates and amplified using a second set of primers that included P7/P5 overhangs, barcodes, and Illumina adapters (**Supplementary Figure 1**) under the following thermocycling conditions: polymerase activation at 98°C for 30 s, followed by 14 cycles of 98°C for 10 s, 52°C for 15 s, 72°C for 10 s, and final extension at 72°C for 7 min. PCR products were assessed using gel electrophoresis, and cleaned using SPRI beads as above. Barcoded amplicon libraries were quantified using a Qubit 3 Fluorometer (ThermoFisher Scientific Inc., USA). Each sample for a gene library (16S, ITS) was combined at equimolar concentration, including negative controls according to Nguyen et al. (45). Gene libraries were then combined at a ratio of 3:7 (by mass) ITS to 16S, spiked with 11.75% PhiX, and sequenced together on a single lane of Illumina (MiSeq) PE250 at the University of California, Davis Genome Center. Sequence data was deposited in the Sequence Read Archive under BioProject #PRJNA551045.

Bioinformatics

Sequence data processing and quality control were performed using the QIIME2 v2018.11 workflow and available plugins (50). Raw sequences were demultiplexed and primers and adapters removed. For 16S sequences, ends of sequences with base quality of $q < 25$ were truncated, and reads were paired and denoised using the DADA2 plugin (51). DADA2 Amplicon Sequence Variants (ASVs) were used for further analyses. For ITS sequences, conservative regions (18S and 5.8S) that flanked

TABLE 1 | Chemical properties of soils fertilized with urea or compost, compared to an unfertilized control ($n = 3$).

Treatment	pH	Total N	Total C	Ca^{2+}	K^{+}	Mg^{2+}	Na^{+}	CEC
		(mg g^{-1})		(cmol^{+} kg^{-1})				
Control	7.8 ± 0.04	1.85 ± 0.01^{b}	20.2 ± 0.10	23.4 ± 0.58	0.11 ± 0.006^{b}	2.32 ± 0.05^{b}	0.27 ± 0.011^{b}	26.1 ± 0.58
Urea	7.8 ± 0.03	1.82 ± 0.04^{b}	20.7 ± 1.43	23.1 ± 0.80	0.09 ± 0.014^{b}	2.34 ± 0.10^{b}	0.27 ± 0.011^{b}	25.8 ± 0.81
Compost	7.8 ± 0.03	2.44 ± 0.08^{a}	28.3 ± 3.93	22.0 ± 0.71	0.31 ± 0.075^{a}	2.80 ± 0.08^{a}	0.48 ± 0.036^{a}	25.6 ± 0.72

Three subsamples of soil from each pot were collected, composited, and analyzed at the end of the sixth crop cycle. Letters indicate statistical significance at $p < 0.05$.

the ITS1 gene were removed using ITSXpress (52), followed by pairing and denoising as described above. This step was essential to accurately classify ITS sequences as verified using our mock community data. Furthermore, DADA2 ASVs overinflated fungal mock community richness (multiple OTUs per species) so it was necessary to further cluster these OTUs into 97% similarity OTUs using open reference clustering via the VSEARCH plugin (53). This clustering was essential to accurately recover the expected mock community diversity in the dataset (45). Hereafter both 16S DADA2 ASVs and ITS 97% clustered OTUs will be referred to simply as OTUs. The Naïve Bayes Classifier was used to classify OTUs using the Greengenes Database specifically trimmed for the primer pairs 515F-806R (gg-13-8-99-515-806-nb, (54) for prokaryotic 16S, and the UNITE database version 7.2 (55) for fungal ITS. For 16S, sequences classified as "unassigned", "mitochondria", or "chloroplast" were removed. For ITS, only sequences that aligned with at least 70% of reference sequences were kept. Internal validation showed that sequences that do not currently match to 70% of the UNITE v7.2 database are typically non-fungal. Sequences labeled as "unassigned", "rhizaria, "Protista", and "Metazoa" were removed. Negative PCR controls showed a maximum occurrence of 3 OTUs for fungi and 6 OTUs for bacteria and archaea. As a quality control measure, these maximum occurrence numbers from the negative control were then used as minimum occurrence thresholds for the rest of the data. As a result, only OTUs that had more than 7 sequences were kept for the 16S dataset and only OTUs that had more than 4 sequences were kept for ITS. The 16S dataset was rarefied to 2831 sequences, and the ITS dataset was rarefied to 514 sequences. Sampling saturation was variable for datasets (**Supplementary Figure 2**). Trophic guild data for fungi was identified using FUNGuild (56).

Statistical Analyses

Statistical analyses were conducted in R version 3.5.1 (57) and QIIME2 version 2018.11 (50). A repeated measures approach was used to compare changes in above and below plant biomass as well as microbial community richness and diversity as response variables to the different fertilizer treatments and control. A generalized least squares (GLS) model was chosen due to its flexibility when dealing with observations across time (58), which is common for repeated measures experiments with autocorrelated residuals (59, 60). The GLS model $y_i = X_i\beta + \varepsilon_i$, $\varepsilon_i \sim(V_i)$, was created using the "lme" function in the package *nlme* (58), where y_i denotes the outcome variable for the i-th group (our response), X_i is the design matrix for the fixed effects β (Treatment + Time; Treatment & Time interactions) and V_i, the variance-covariance matrix for the error terms. V_i was specified to CorAR1, which confined errors within each individual replicate (pot). For the biomass data, a box-cox transformation was performed to meet the assumption of normality and equal variance. An Analysis of Variance (ANOVA) was performed using the "anova" function in base R. Pairwise comparisons of least-squares means were calculated for sample groupings by both treatment and time. These were calculated using functions "lsmeans" and "cld" via the *lsmeans* and *emmeans* (61, 62) packages. Least-squared mean comparisons were averaged over the levels of treatment using the Satterthwaite method (63) at a confidence level of 0.95. For the biomass data only, the "glht" function in the *multcomp* package was used (64). Pairwise comparisons were corrected for multiple comparisons with the Tukey p-adjustment.

Sequence reads obtained through high-throughput sequencing are compositional (65). Hence, in our community composition analysis, we followed protocol as suggested for compositional datasets. The first steps of data wrangling were conducted in R using the *vegan* package (66). First, unrarefied abundance matrices were normalized using the function "decostand" followed by the Hellinger square root method (67). We found this was sufficient in balancing our data. Next, Euclidean distances were created using the function "vegdist" on the transformed data matrices. We chose Euclidean distances as our metric for dissimilarity because of their appropriateness in Eigenvector-type analyses such as Principal Components Analysis (68–70), which we used for visualization. Community composition was compared using the function "adonis2", which conducted permutational analysis of variances (PERMANOVAs) on the Euclidean distances as grouped by treatment. These PERMANOVAs were performed at the first crop cycle (1) and then included subsequent crop cycle accumulations (crop cycles 1–2, 1–3, 1–4, 1–5, and 1–6). This allowed us to compare how samples grouped in "species-space" at different points throughout the study. Comparisons were conducted using both an un-nested approach that looked at interactive effects and a nested approach that limited errors within "strata" of time within each crop cycle. Pairwise comparisons of treatment groupings were performed using the Wilks statistic with FDR corrections for multiple testing (59) via the function "pairwise.perm.manova" from the package *RVAideMemoire* version 0.9–72 (71). Results were visualized using *ggplot2* (72) and *sjPlot* (73).

To determine how different fertilizer types affected the abundance of any specific OTU, we conducted an Analysis

of Composition of Microbiomes [ANCOM, (74)], using the "Composition" plug-in for the QIIME2 platform. ANCOM is a robust analysis tool that uses compositional constraints to reduce false discoveries and can improve identification of differentially abundant microbes in complex datasets (75). Differences in relative abundances across treatments were compared at each crop cycle for both fungi and prokaryotes. To differentiate changes that could be confounded by time, relative abundances of OTUs were also compared across crop cycles. This occurred for crop cycles (1–6) for fungi, and because of computational power constraints even at the supercomputer level, we minimized analysis of prokaryotes at intervals of crop cycles (1–4 and 4–6). In addition, we performed a similar test, "log2FoldChange", for changes in the microbiome using the package DESeq2 (76) to detect significant changes in OTU relative abundance between urea and compost treatments for cycle 1 and cycle 6.

Network Construction

All microbial association networks were created using the R package *SPIECEASI* (77). The SPIECEASI method was chosen because it was developed for compositional data analysis and assumes taxon-taxon associations scale linearly with the number of measured taxa. It also features greater precision when compared to other methods such as CoNet, gCoda, SparCC, and Spearman network inferences (34). Best-practices in microbial association network construction require large sample sizes to increase the precision of networks (34, 77). Although the sample size in our study was small ($n = 3$ for each treatment), we found that time across the 6 cropping cycles, although significant, had a much smaller effect in shaping microbial community structure when compared to the effect of the fertilization treatments. The effect of time across cropping cycles had an R^2 value ranging from 0.022 to 0.067 for fungi and 0.024 to 0.062 for bacteria & archaea, as compared to the effect of fertilizer type with R^2 ranging from 0.298 to 0.387 for fungi and 0.041 to 0.117 for bacteria & archaea (**Table 2**). Because time across cropping cycles explained a much smaller portion of the variation compared to treatment, we combined samples from all crop cycles (C1–C6) for each fertilizer treatment, which allowed for network inference with a pseudo $n = 18$ (3 pots x 6 cycles). Using these combined data, we created prokaryote networks for bacteria & archaea alone, fungi alone, and a combined network of bacteria, archaea, and fungi. We recognize that the prokaryotic network is technically a cross-domain network, but for ease of discussion, will refer to the combined network as the cross-domain network henceforth.

To reduce the likelihood of false positive associations within networks, OTU counts were first filtered for minimum occurrence across ∼33% of samples using the R package *Phyloseq* (78). OTUs with 0 abundance were filtered out of any individual sample, but their sums across the whole dataset were kept so as not to change the overall sample counts. SPIECEASI networks were then assembled via the "spiec.easi" function using the Meinshausen and Bühlmann or "MB" method (79) with a pulsar parameter threshold of 0.05 and lambda minimum ratio of $1e^{-2}$ following basic user guidelines (77, 80). The function "multi.spiec.easi" was used for creating cross-amplicon networks under the same parameters. Resulting networks were visualized in Cytoscape V-3.7.1 (81) and network topological properties were assessed using NetworkAnalyzer (82). Subnetworks were created by selecting individual hub OTUs from the CytoScape interface, selecting nearest-neighbors of those OTUs, and then creating a new network from the combined selections.

Network hub taxa were selected based on node degree (the number of edges linked to that node) and closeness centrality (how close a node is to all other nodes) as measurements of overall network connectivity using NetworkAnalyzer in Cytoscape. Currently, there is no consensus for what determines statistically significant network hub taxa, although previous studies determined good candidates by selecting those with higher node degree, betweenness centrality, or closeness centrality based on normal distribution fit with $p < 0.1$ and correlation cut-offs (83, 84). The SPIECEASI method of network inference is a conservative method that although results in fewer connections than other inference tools, can help to reduce false positives (77, 80). Therefore, we took a simpler approach for determination of hub taxa that did not dismiss non-outliers by choosing the top 5 OTUs that contained the highest degree and closeness centrality for each treatment. We limited our network

TABLE 2 | PERMANOVA comparison of community differences across cropping cycles for prokaryotes and fungi.

Prokaryotes			Fungi		
Time			Time		
Crop cycles	**R squared**	***P*-value**	**Crop cycles**	**R Squared**	***P*-value**
2	0.06163	0.025	2	0.067	0.103
3	0.04326	0.001	3	0.044	0.117
4	0.03612	0.001	4	0.022	0.267
5	0.02845	0.001	5	0.03153	0.001
6	0.02417	0.001	6	0.034	0.021
Fertilizer type (unblocked)			**Fertilizer type (unblocked)**		
Crop cycles	**R squared**	***P*-value**	**Crop cycles**	**R squared**	***P*-value**
1	0.2492	0.626	1	0.37536	0.045
2	0.11696	0.446	2	0.32728	0.001
3	0.07904	0.06	3	0.306	0.001
4	0.05978	0.014	4	0.387	0.001
5	0.04896	0.002	5	0.30217	0.001
6	0.04133	0.001	6	0.29788	0.001
Fertilizer blocked by time			**Fertilizer blocked by time**		
Crop cycles	**R squared**	***P*-value**	**Crop cycles**	**R squared**	***P*-value**
2	0.116	0.635	2	0.327	0.002
3	0.079	0.066	3	0.306	0.001
4	0.0597	0.006	4	0.386	0.001
5	0.0489	0.002	5	0.302	0.001
6	0.0413	0.003	6	0.297	0.001

Comparisons were constraint by treatment and time. All analyses were compared to the first crop cycle as a basepoint to show how community shifts relative to this point.

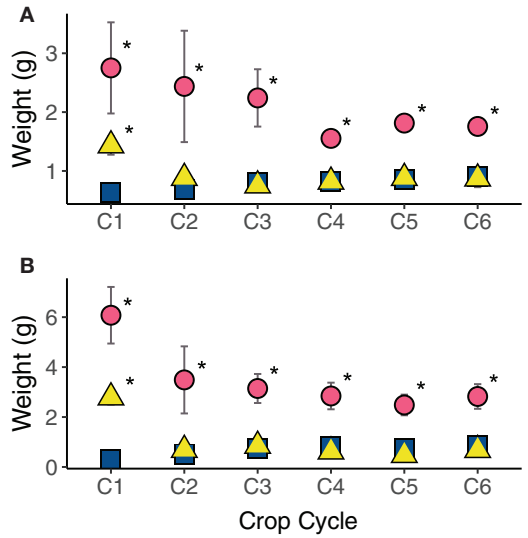


FIGURE 1 | Plant growth response **(A)** aboveground and **(B)** belowground to compost (circles) and urea (triangles) fertilization as compared to unfertilized controls (squares) over 6 crop cycles. Asterisks (*) denote significant treatment differences within each cropping cycles compared to the control. Error bars indicates standard error.

hub taxa analysis to only the cross-domain networks as these were the most comprehensive network inferences.

RESULTS

Plant Growth, Soil Chemistry, and Microbial Community Response to Fertilization

At the end of the experiment, fertilization treatments did not significantly affect soil pH, which averaged 7.8 across all treatments (**Table 1**). There was a significant treatment effect on soil total N ($p = 0.0002$) and a marginally significant effect on soil carbon ($p = 0.0696$). Compost increased concentrations of soil nitrogen and carbon relative to both urea and control. There was no significant treatment effect on cation exchange capacity by the end of the sixth crop cycle. Calcium was the dominant cation across all treatments. Compost significantly increased concentrations of potassium ($p = 0.0031$), magnesium ($p = 0.0069$), and sodium ($p = 0.0005$).

Plant growth responded differently to treatments in the six consecutive cropping cycles following a single initial application of urea or compost (**Figure 1**). When compared to the control, compost fertilization significantly increased both aboveground and belowground plant biomass for all crop cycles ($p < 0.01$). Urea fertilization, in contrast, only significantly increased above and belowground plant biomass in the first crop cycle ($p < 0.001$). When the treatments were compared to each other, compost had greater biomass relative to the urea treatment for crop cycles 2–6 ($p < 0.001$).

Fertilizing soils with either compost or urea affected microbial richness and diversity differently (**Figure 2**). Fertilizer type did not affect prokaryote richness ($p = 0.850$), Shannon's Index ($p = 0.984$) nor Faith's Phylogenetic Diversity ($p = 0.635$). These metrics remained stable across the six cropping cycles ($p > 0.4$). These patterns were somewhat different for fungi where fertilization did not affect overall richness ($p = 0.326$) and Shannon's Index ($p = 0.628$) when compared to unfertilized soils, but over the six cropping cycles richness ($p = 0.001$) and diversity ($p = 0.004$) significantly decreased. Fungal richness and diversity were highest immediately following fertilization of field-collected soil prior to first sowing of seeds, then continued on a decreasing trend until the sixth cycle. These effects were observed for both urea and compost fertilizer sources.

We compared changes in microbial communities of different fertilizers across the six cropping cycles relative to the first cycle and found that fertilizer type significantly changed soil microbial community composition, but these effects were latent in prokaryotic communities (**Figure 3**, **Tables 2**, **3**). Fungal communities in compost-fetilized soil differed significantly from unfertilized controls ($p < 0.013$) and urea ($p < 0.013$), but did not differ between urea-fertilized and unfertilized soils ($p > 0.327$) (**Table 3**). The variation in the data explained (R^2) across the six cycles ranged from 0.297 to 0.386. Prokaryotic communities did not respond significantly to fertilization until the third crop cycle ($p = 0.025$) with increasing statistical significance until the sixth cycle. Similar to the fungal communities, prokaryotic communities from compost-fertilized soils differed from unfertilized control ($p < 0.048$) and urea ($p < 0.036$), while communities in control and urea-fertilized soils were not statistically different ($p > 0.27$). The variation in the data explained (R^2) across the six cycles ranged from 0.249 at the first crop cycle, and consistently decreasing to 0.041 in cycle 6.

Fertilizer type significantly changed the relative abundance of certain individual fungal OTUs based on ANCOM analysis, but trends were more prominent for fungi than for prokaryotes across the 6 cycles (**Supplementary Table 1**). Two fungal OTUs, *Thermomyces lanuginosus* and a *Myceliophthora* sp. were more abundant in compost-fertilized soils. *Thermomyces lanuginosus* was more abundant for crop cycles 1 (w-score = 142), and crop cycle 6 (w = 146), while *Myceliophthora* sp. was more abundant in the second (w = 283), third (w = 245), fourth (w = 227), and fifth (w = 205) crop cycles. A *Pezizaceae* sp. was more abundant in the control and urea treated soils, but only at crop cycle 4. Only one bacterial OTU, an unidentified *Acidomicrobiales*, was more abundant in the control treatment at the first crop cycle (w = 3952). No differences in the relative abundance of prokaryotic OTUs were detected across all crop cycles.

Fold change analysis of OTU relative abundance in urea vs. compost treatments showed that prokaryotic taxa responded more strongly in crop cycle 1, whereas fungi responded more strongly in crop cycle 6. We detected 214 OTUs within 42 taxa (mostly at the genus level but some could be not assigned) that responded either strongly to urea or compost fertilization (**Figure 4**). Of these, OTUs of *Actinomadura, Catellatospora, Iamia, Phytohabitans, Parasegitibacter*, unidentified *Chloroflexi*, *Nitrospira, Gemmata, Planctomyces, Arthrospira, Dechloromonas, Devosia, Luteibacter, Massilia, Tepidimonas, Opitutus*, and *Prosthecobacter* significantly increased in abundance in urea-fertilized soils. In contrast,

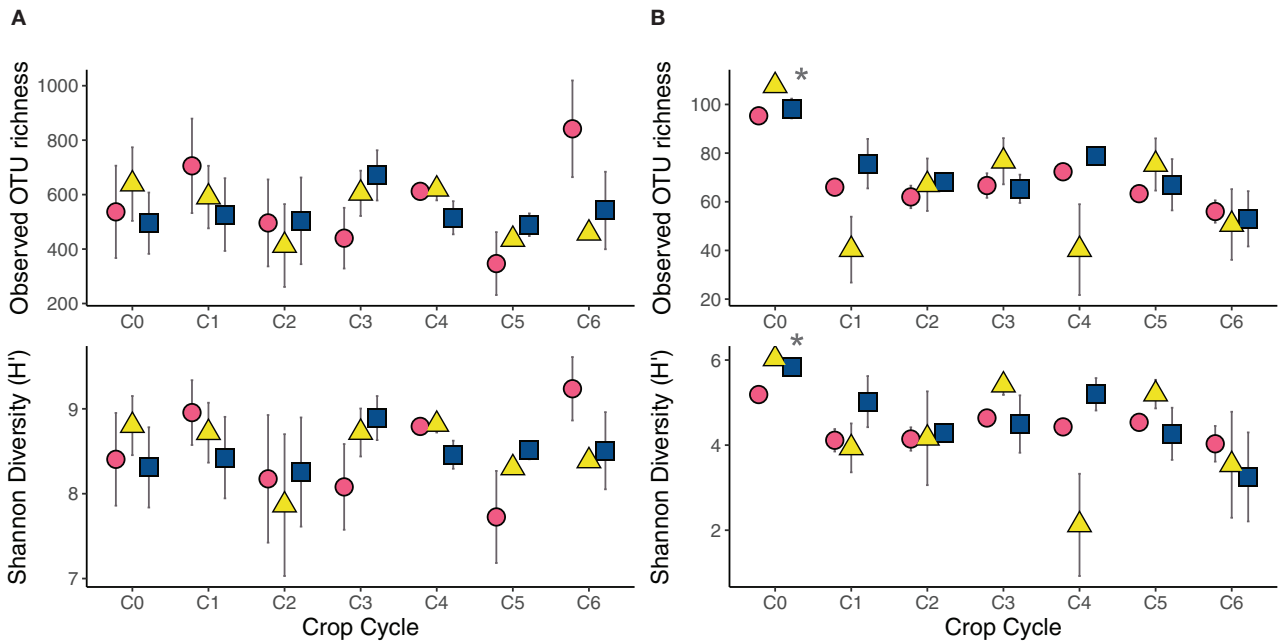


FIGURE 2 | Observed OTUs and Shannon's Diversity index measuring the community responses of **(A)** prokaryotes and **(B)** fungi to fertilizer treatments over six crop cycles, including soils prior to planting (C0). Circles indicate compost treatment, triangles indicate urea treatment, and squares indicate controls. Asterisks (*) denote significant treatment differences across all crop cycles. Error bars indicates standard error.

TABLE 3 | PERMANOVA and pairwise comparisons of community changes across treatments.

Crop cycle	PERMANOVA		Pairwise comparison *p*-values		
	R^2	*p*-value	Compost vs. control	Compost vs. urea	Urea vs. control
Fungi					
1	0.375	0.052	0.3	0.3	0.4
2	0.327	0.001	0.013	0.013	0.545
3	0.306	0.001	0.0015	0.0015	0.334
4	0.386	0.001	0.0015	0.0015	0.679
5	0.306	0.001	0.0015	0.0015	0.334
6	0.297	0.001	0.0015	0.0015	0.327
Bacteria and Archaea					
1	0.249	0.589	0.8	0.8	0.8
2	0.116	0.464	0.79	0.79	0.84
3	0.079	0.025	0.034	0.024	0.815
4	0.597	0.018	0.048	0.036	0.721
5	0.0489	0.001	0.0075	0.006	0.534
6	0.0413	0.001	0.012	0.006	0.27

Pairwise comparisons used Euclidean distances using Wilk's statistics with FDR p-valuecorrection. All analyses were compared to the first sampling as a basepoint to show how community shifts relative to this point.

Kibdelosporangium, Mycobacterium, Rubrobacter, Streptomyces, Flavisolibacter, an unidentified *Nitrospiraceae*, unidentified *Pirellulaceae*, *Geobacter, Hyphomicrobium*, and *Reyranella* significantly increased in abundance in compost-fertilized soils. Other genera contain OTUs that show different preferences for the different types of fertilizers. No differences in the relative abundance of prokaryotic OTUs were detected at crop cycle 6.

We found only one OTU of fungi, *Thermomyces lanuginosus*, that significantly favored compost at crop cycle 1, but six OTUs at crop cycle 6. These were *Acremonium dichromosporum, T. lanuginosus*, a *Myceliophthora* sp., and three unidentified *Ascomycota* in the orders *Sordariales* and *Eurotiales*. General visual comparisons of all OTUs aggregated at the phylum level across all 6 cycles can be found in **Supplementary Figures 3, 4**.

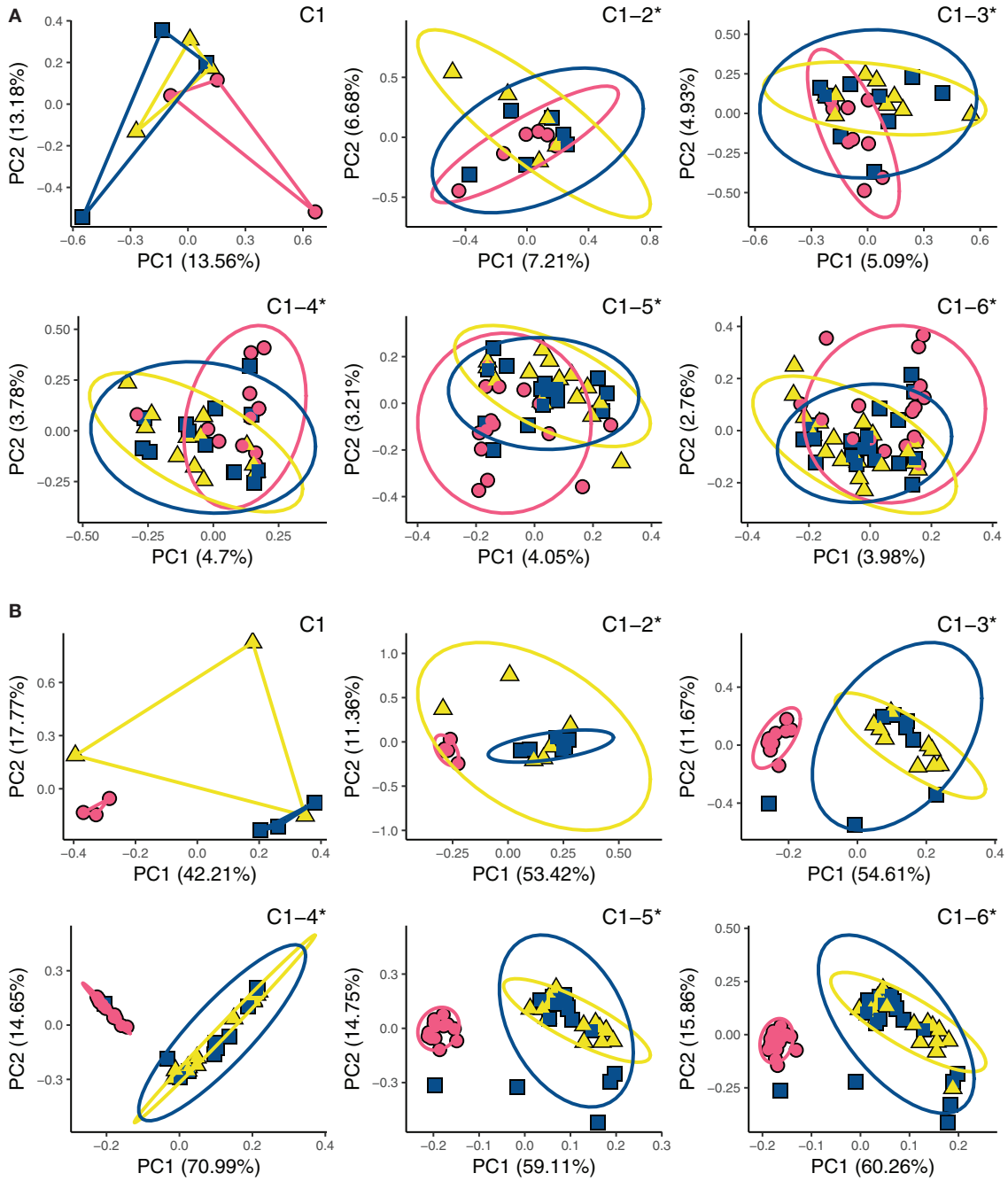


FIGURE 3 | Principal component analysis (PCA) ordination showing **(A)** prokaryotic and **(B)** fungal community similarity. Each panel shows an accumulation of samples, starting with crop cycle 1 (C1) and ending at cycle 6 (C1-6). Asterisks (*) denote PERMANOVA significant treatment differences across crop cycles.

Soil Network Complexity

Soils treated with fertilizers altered network topography for the individual prokaryotic network, the fungal network, and the cross-domain network (**Figure 5**). Network complexity, as measured by the number of nodes and edges, increased when compost was added to the soil, and decreased when urea was added, relative to the control. For the prokaryotic network, the control contained 134 nodes and 150 edges, the compost-fertilized network was higher with 141 nodes and 217 edges, and the urea-fertilized network was fewer with 117 nodes and 154 edges. We observed a similar trend for the fungal networks. The control network had 14 nodes and seven edges, the compost-fertilized network was higher with 44 nodes and 31 edges. The urea-fertilized network, however, had 18 edges and 11 nodes,

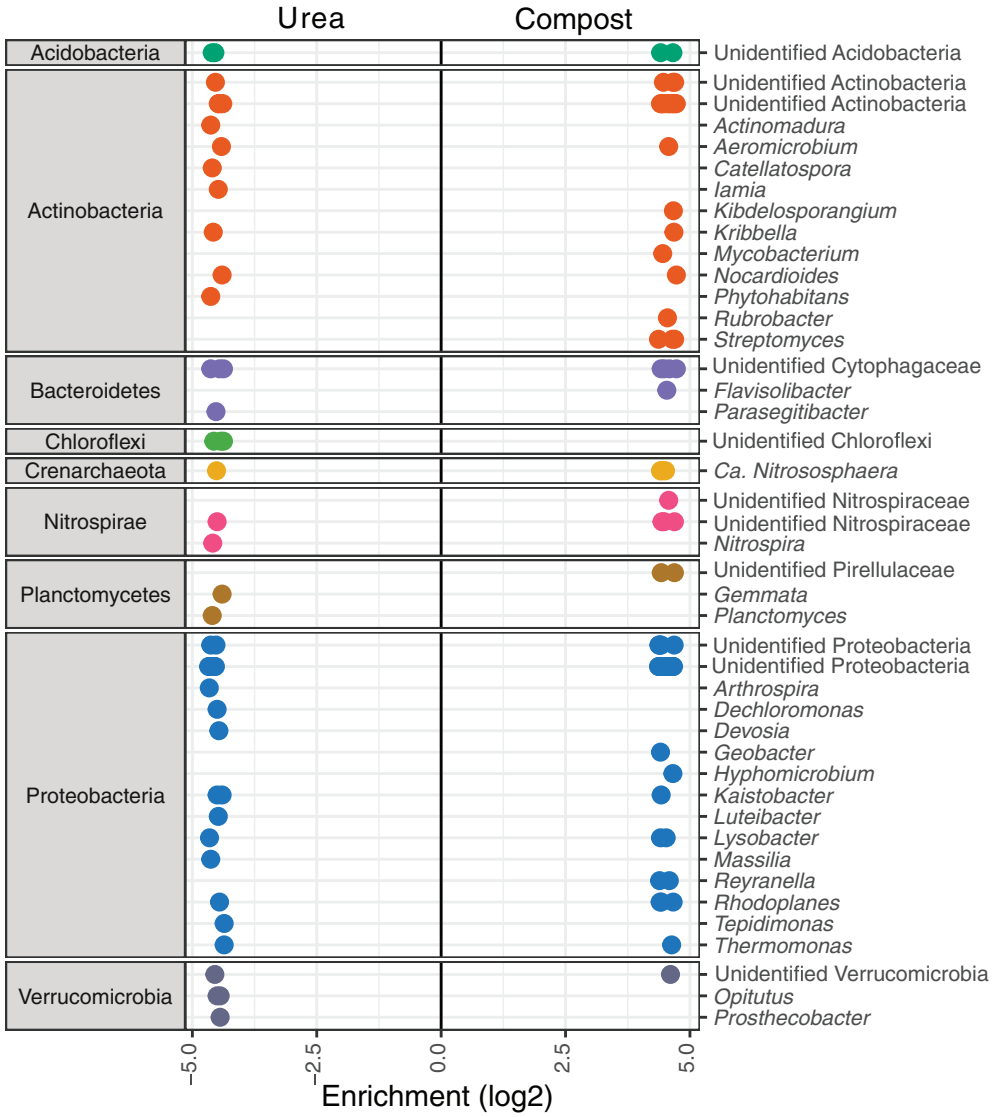


FIGURE 4 | Significant increases in relative abundance of bacterial OTUs in urea and compost-fertilized soils at crop cycle 1. OTUs are grouped by Phylum and identified to genus when possible. Each genus may contain multiple OTUs, represented by each dot on the line. When a genus cannot be identified although they were classified as different genera based on sequence homology, the identity of the most positively identified taxonomic level is provided (e.g., *Actinobacteria*). Positive $\log_2$ fold changes indicate preferences toward compost and negative $\log_2$ fold changes indicate preferences toward urea.

both of which were higher than the control. The cross-domain networks followed this trend as well where the control network had 229 nodes and 372 edges, the compost-fertilized network had the highest number of 248 nodes and 408 edges, and the urea network had the lowest number of 207 nodes and 307 edges.

Within the networks, fertilizer type had a strong effect on network hub taxa. Not only did fertilizer type affect which OTUs became hub taxa, but it affected how they were connected in the overall networks (**Figure 6**). The compost-fertilized network contained three bacterial (a *Nostocaceae* sp., *Steroidobacter* sp., *Bradyrhizobiaceae* sp.) and two fungal (*Lasidioploidia lignicola*, and *Tetracladium furcatum)* hub taxa. The unfertilized control network contained three bacterial (a *Nostocaceae* sp., *Rhizobiales* sp., *Micrococales* sp.) and two fungal (*Hypocreales* sp., and *Aspergillus purpureus*) hub taxa. The urea-fertilized network contained one bacterial taxon (a *Solirubrobacteriales* sp.) and four fungal (Ascomycota sp., *Aspergillus* sp., *Trechispora* sp., and an unidentified fungus) hub taxa. Only one OTU, a member of the *Nostocaceae*, was present as a hub taxon in both the unfertilized soil and compost-fertilized network, but not the urea-fertilized network. Each hub taxon in the compost-fertilized network was directly connected to other hub taxa, and indirectly connected to 2 or more other hub taxa. In stark contrast, the hub taxa in the unfertilized network shared only indirect connections to other hub taxa, and averaged less than two connections to other hub taxa. Hub taxa in the urea-fertilized network similarly had fewer direct and indirect connections, including

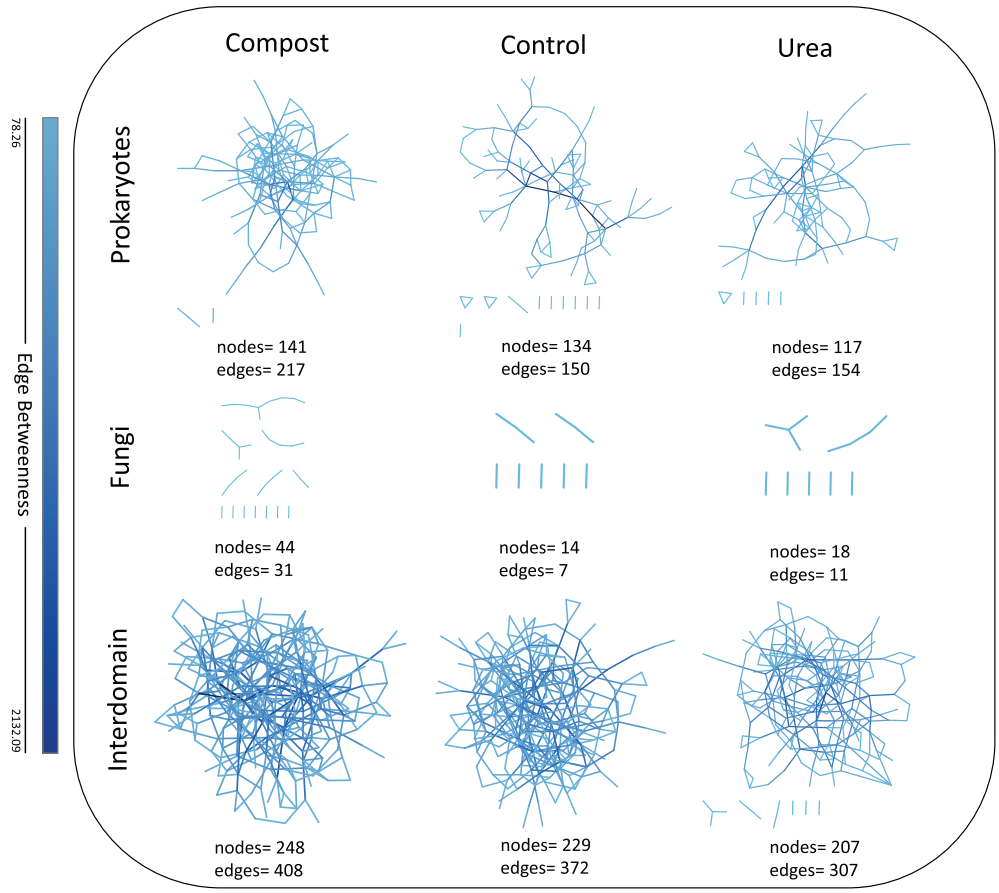


FIGURE 5 | Visual and quantitative measures of association networks for prokaryotes, fungi, and cross-domain across compost, urea, and control. Complexity of networks are represented here by nodes and edges, which may be inferred as predicted interaction, either positive or negative, and are colored by a measurement of edge betweenness, a component of network centrality (85).

one hub taxa that was not connected to any other hub taxa by nearest neighbors.

A comparison of OTUs found in the ANCOM analysis with those from the hub taxa analysis showed that the taxon *Thermomyces lanuginosus* (significantly more abundant in compost-fertilized soil) was directly connected to a hub taxon, a *Steroidobacter* sp. (**Supplementary Figure 5**). The *Myceliophthora* sp. was found in a separate node cluster and was neither a hub taxon nor connected to one (data not shown). No other taxa from the ANCOM analysis were found to interact closely with network hub taxa of any treatment group. The same *T. lanuginosus* and *Myceliophthora* sp. were also detected in the fold change analysis, favoring compost-fertilized soil.

DISCUSSION

In this study, we provided direct evidence that a single application of a synthetic N source or a more complex N compost prior to planting can have different effects on plant growth, soil microbial composition and network complexity, but not microbial richness and diversity. High N composts can be an effective plant fertilizer in tropical soils (86, 87) especially in the nutrient-poor Oxisols used in this experiment. Compost imparted greater residual effects on plant growth than urea throughout the experiment. These trends were expected since urea is rapidly hydrolyzed by soil bacteria and fungi to provide a readily available N source (i.e., NH_4^+) at the beginning of the first growth cycle (88, 89). This NH_4^+ can be taken up by plant roots, consumed by microorganism, lost through ammonia volatilization (17) or be converted by ammonia oxidizing bacteria or archaea to nitrate (16). The nitrate is either taken up by plant roots, immobilized by microorganisms, denitrified, or leached out of the soil system with percolating water (90). The labile N pool in compost is subjected to the same fates, but compost also contains N sources associated with more complex organic compounds that mineralize N more slowly through microbial-mediated transformations (27, 91, 92). In this sense, high N organic compost not only provides a rapidly available nutrient pool upon application but also provides a slowly available pool of organic

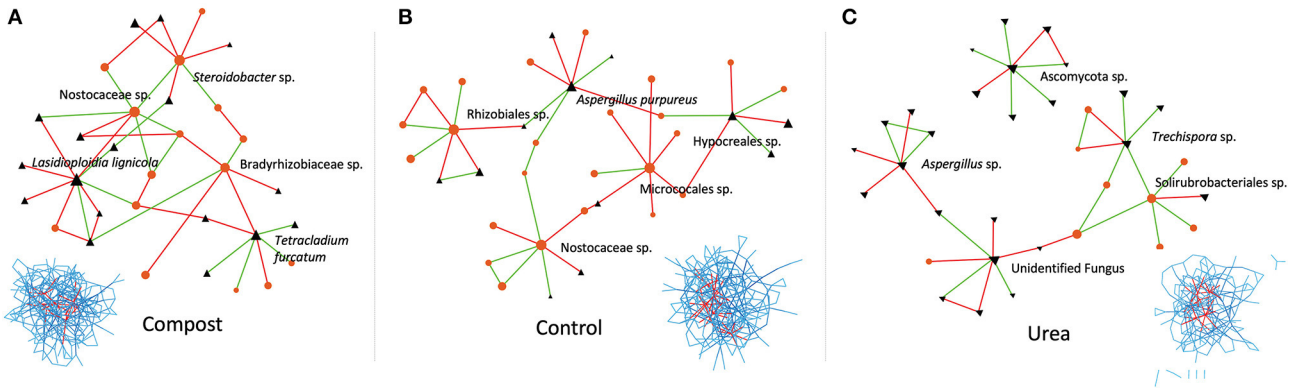


FIGURE 6 | Cross-domain subnetworks showing hub taxa and their interactions across **(A)** compost, **(B)** control, and **(C)** Urea. Bacterial nodes are represented by orange circles, and fungal nodes are represented by black triangles. Inset shows the location of the subnetworks (red) within the full cross-domain network (blue). Bacterial nodes are indicated by orange circles and fungal nodes are indicated by black triangles. Edges between nodes represented a positive association (green) or negative association (red).

nitrogen, including the many additional benefits of substrates rich in organic matter that continues to support plant growth in subsequent cycles.

The relatively low to no plant response to urea fertilization contradicts expectations given that urea is also known to be an effective seasonal N fertilizer in agricultural systems in the tropics and elsewhere (93). Considering that urea is rapidly hydrolyzed, we would not expect residual effects of urea on crop growth or soil total nitrogen across multiple subsequent crop cycles, and this is reflected in our results. However, we would expect that there would be less of a difference in plant biomass between the two fertilizer types since they were applied at an equivalent available N basis. The observed significant differences between plant biomass may be explained through volatilization of a substantial portion of urea-N that reduces plant nitrogen use efficiency and thus plant growth (17). An alternative explanation is that plant growth was limited by deficiencies in other essential nutrients, such as phosphorus or potassium, especially considering that Oxisols tend to have low capacity to retain and supply nutrients (94). Compost, on the other hand, supplies the plants with more than just N (e.g., P, K, etc…), thus potentially alleviating multiple macro- and micronutrient deficiencies. While the compost treatment did not alter cation exchange capacity of the soil, as base saturation was dominated by calcium, it did increase concentrations of potassium, magnesium, and sodium.

Although a single application of different fertilizer types can have significant effects on plant growth, it did not affect the overall microbial richness and diversity. This finding was unexpected in the soils fertilized with compost given that composted materials often contain a diversity of microorganisms (95) thereby having the potential to increase a soil's richness and diversity. However, Pérez-Piqueres et al. (27) showed that community responses may be dependent upon the nature of the compost in combination with soil types. In addition, changes in physicochemical properties favoring microbial proliferation might be a more important factor for the soil community response to compost fertilization than the compost-borne organisms themselves (96). Urea fertilization also did not decrease microbial richness and diversity. These results suggest that a longer time period of urea fertilizer applications is perhaps necessary to observe the effects of decreased bacterial diversity (18, 97). Repeated planting through time did not change prokaryotic diversity but did have an effect on fungi. These measures were highest in the field-collected soil before active radish growth, suggesting a possible selection process in the rhizosphere as an effect of the active radish growth (98), in part through the production of carbon and energy rich compounds, and bioactive phytochemicals (99).

This study provides evidence that the type of soil fertilizer can affect microbial community composition differently. The concept that organic fertilizers can have a strong impact on soil microbial communities is reflected in previous literature investigating microbial community shifts under long term organic vs. conventional management systems (27, 96). In the present study, even single applications of compost caused detectable continued community shifts relative to the first sampling point for the duration of this 227-day study. Conversely, the microbial community did not significantly shift in response to a single application of urea fertilizer, although we did detect significant shifts in certain genera. Previous research suggests that negative impacts on the community can occur due to the repeated use of synthetic N fertilization over long periods of time (18, 97, 100); however, short-term applications, like in this study, may not be enough to inhibit resident microbial composition (18). This work reflects our current understanding of how disturbance events (such as those caused by fertilization, especially compost), can significantly shift microbial communities, and that the legacy effect left by roots grown in the previous cycle is not likely the strongest driver of soil microbial communities (101). The contrast in microbial community shifts between compost and urea highlights the need to better understand the mechanisms of how each of these fertilizers may affect bacteria and fungi differently. This is especially important if we were to more

effectively apply as a means to reduce plant pathogens or improving soil health and fertility in the tropics (102, 103).

To gain a more nuanced understanding of how different members of the microbial community respond to the urea or compost treatment as opposed to whole community analyses discussed above, we applied ANCOM and DESeq analyses that identify significantly associated with experimental variables. Of the genera that favored urea-fertilized soils, increases in the genus *Nitrospira*, and OTUs of the phylum *Chloroflexi* are consistent with the increases in ammonium nitrogen found in the urea-fertilized soils. *Nitrospira* contribute to nitrogen cycling by oxidizing nitrite to nitrate, and certain members of the group can completely denitrify ammonia to nitrate (104). *Chloroflexi* are often found associated with nitrogen rich environments like treatment plants that are designed to remove nitrogen (105). The absence of *Streptomyces* in urea-fertilized soils but its significantly higher abundance in compost-fertilized soil suggests a preference for this genus for organic matter rather than simply nitrogen alone. Apart from these specific examples, most of the genera showing significant increases have members that prefer either of these substrates (**Figure 4**). Further work using more sensitive methods will be required to make stronger inferences into the preference of each of these taxa and how they contribute to nitrogen cycling in soil.

We found several fungal OTUs to be significantly associated with the compost treatment. Of these, *T. lanuginosus* and *Myceliopthora* sp. are thermophilic, compost-dwelling saprotrophic fungi (106). They are likely inhabitants of the compost and introduced into the study soil system via the compost fertilizer. It is possible that these organisms could be contributing to an improved role in decomposition and nutrient cycling in the soil system, or that they are displacing native soil microbes with the same ecological niche. *Thermomyces lanuginosus* has mostly been observed as a saprotroph, although it has been recorded as a potential opportunistic human pathogen (107), and although pathogenicity of this organism was not confirmed in this study, it is important to consider the health and ecosystem risk of organisms introduced to soil via compost fertilizer (108–110). The mechanisms for how individual OTUs, such as the two found here, might successfully establish themselves in a soil system was not part of this study [although see Gravuer and Scow (28) for interesting insights]. However, mechanisms behind rhizosphere competency are currently of great scientific and economic interest because of the growing popularity of microbes as biofertilizers. Identifying organisms that are rhizosphere competent and what factors might influence rhizosphere competency are key to developing more effective agricultural products (111).

The choice of fertilizer influenced network complexity (and thus network stability and robustness) in both individual and cross-domain networks. Complex organic fertilizers such as compost resulted in higher network complexity, similar to Schmid et al. (31) who found that long-term amendments of manure or straw to agricultural soils increased bacterial network complexity. Other studies investigating single-domain networks have shown very clear differences in network topology when comparing stark habitat differences like the rhizosphere vs. bulk soil (29) or when comparing different microbiome reactions to stressful conditions like extreme drought (32). Here we showed that networks were more complex in compost-fertilized soils as compared to the control or urea (**Figure 5**). The less complex networks in urea relative to the control suggests that simple sources of fertilizers may not be able to support robust networks across multiple planting cycles. Indeed, the amount of total C & N at cycle 6 (**Table 1**) remained generally higher in compost, lending further support to the idea that more complex fertilizer sources that slowly release nutrients may be able to support stronger networks of microbes over time. Cross-domain networks provide a more comprehensive understanding of the complexity of soil communities than individual-domain networks. Using cross-domain network interactions such as those between prokaryotes and fungi can provide a greater understanding of beneficial, antagonistic, and associative interactions of the microbes in the soil system (35, 37, 84). This study revealed greater connectivity in cross-domain networks compared to individual-domain networks, and from these network associations, we can create and test hypotheses about how these organisms might interact. For example, the OTU *Nostocaceae* sp. was less connected in the urea network compared to the compost where it acted as a hub taxon (**Figure 6A**). It is known that members of the bacterial family *Nostocaceae* photosynthesize, and in some cases can fix nitrogen (112). As a possible N contributor in this soil system, *Nostocaceae* could be important in helping to provide a source of plant N in an agricultural system. Future research is needed to investigate whether urea negatively affects potentially beneficial hub taxa in soil. Similarly, the OTU *Nostocaceae* sp. has a negative association with another hub taxon, *Lasiodiplodia lignicola,* in the compost network (**Figure 6A**). Many *Lasiodiplodia* are recognized plant and animal pathogens (113). This interaction between a possibly beneficial microbe acting antagonistically toward a potential plant pathogen could be further explored. Whether these hub taxa behave as keystone species, whose removal could cause the collapse of an ecosystem, or that they are lever species, which can steer ecosystems toward specific community types (114), by directly inhibiting or facilitating the growth of other microbes and thus affecting overall the interconnectedness of communities (115) pose an interesting set of questions to be tested in soil community ecology.

Beyond generating hypotheses for the interactions among different members of a microbiome, cross-domain networks provide a different dimension within which to quantitatively measure the microbiome interactions. For instance, Shi et al. (29) showed that rhizosphere microbiome network complexity increased with time relative to the bulk soil that had relatively consistent and weak network complexity. In the present study, network complexity can be used as a tool to quantitatively measure a shift in microbiome in response to a soil fertilizer. This has relevance in many areas of microbial community ecology since it is still not yet trivial to quantitatively compare shifts in microbial community composition across different studies. Under the framework of network complexity, such community shifts may be comparable across studies to provide a quantitative measure that can translate to ecosystem function.

Development of such methods would be a leap forward in microbial ecology.

CONCLUSION

In this study, we provided multiple lines of evidence obtained through multiple types of analyses that partially supported the hypothesis that complex N fertilizers when applied to soils have a detectable impact on plant growth, soil microbial diversity, community structure, network topography, and change network hub taxa as compared to urea, a simpler N fertilizer. Unlike urea, a single application of compost, a complex N-fertilizer, increased plant growth throughout the duration of the experiment, selected for a subset of fungal OTUs, and shifted overall microbial community structure. Fertilization with compost also altered overall microbial network topography by increasing network and hub taxa connectivity, especially across domains. Network analysis can play an important role in detecting cross-domain interactions that might be important drivers of microbial interactions in the soil, and network complexity could be developed into a tool that allows the research community to quantitatively compare microbial shifts across studies. Although our study was limited by the number of soils tested, the type of fertilizers used, source inoculum identity in the compost, and under controlled conditions, we showed that even a single amendment of a complex N-fertilizer source such as compost can have prolonged impact on soil nitrogen concentration, plant growth, as well as soil microbial community assembly under low-input settings. Future experiments using a broader set of soils and wider range of fertilizers under field setting will provide stronger support for the concepts highlighted here.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA551045; https://github.com/nnguyenlab/N-fertilizer-microbes, GitHub.

AUTHOR CONTRIBUTIONS

SH, RR, and NN conceived the research and setup the experiment. SH performed the experimental work and drafted the manuscript. SH and TM analyzed the data. NN, RR, and TM edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the USDA National Institute of Food and Agriculture, Hatch project 8042H, managed by the College of Tropical Agriculture and Human Resources, the Foundational and Applied Science Program, grant no. 2020-67020-31173/project accession no. 1022247, from the U.S. Department of Agriculture, National Institute of Food and Agriculture, and in part by the U.S. Department of Energy Office of Science Office of Biological and Environmental Research Genomic Science program under award DE-SC0020163, subawarded to NN at UH Manoa.

ACKNOWLEDGMENTS

We thank Michael Kantar for invaluable help with statistical analyses, Laura Tipton for assistance setting up the cross-kingdom network analyses, Ricky Lewis for DESeq analysis pipeline, Kate Porterfield for her initial work in preparing and designing the chambered pot system, as well as Naomi Jun, Jonathan Fisk, and Ishwora Dhungana for their assistance with some of the greenhouse work. We thank the three reviewers for providing detailed constructive comments that helped to improve the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsoil.2022.749212/full#supplementary-material

REFERENCES

1. Bharti V, Dotaniya M, Shukla S, Yadav V. Managing soil fertility through microbes: prospects, challenges and future strategies BT-agro-environmental sustainability. In: *Agro-Environmental Sustainability*. Cham: Springer (2017), p. 81–111.
2. Kibblewhite MG, Ritz K, Swift MJ. Soil health in agricultural systems. *Philos Trans R Soc Lond B Biol Sci.* (2008) 363:685–701. doi: 10.1098/rstb.2007.2178
3. Allen DE, Singh BP, Dalal RC. Soil Health Indicators Under Climate Change: A Review of Current Knowledge. In: Singh BP. Cowie AL, Chan CC, Singh BP, Cowie AL, Chan YK, editors. *Soil Health and Climate Change* Soil Biology. Berlin, Heidelberg. (2011) p. 25–45. doi: 10.1007/978-3-642-20256-8_2
4. Fierer N, Jackson RB. The diversity biogeography of soil bacterial communities. *Proc Natl Acad Sci U S A.* (2006)103:626. doi: 10.1073/pnas.0507535103
5. Li J, Li Y, Xiang-dong Y, Jian-jun Z, Zhi-an L, Bing-qiang Z. Microbial community structure and functional metabolic diversity are associated with organic carbon availability in an agricultural soil. *J Integr Agric.* (2015) 14:2500–11. doi: 10.1016/S2095-3119(15)61229-1
6. Fierer N. Embracing the unknown: disentangling the complexities of the soil microbiome. *Nat Rev Microbiol.* (2017) 15:579–90. doi: 10.1038/nrmicro.2017.87
7. Nakatsu CH, Carmosini N, Baldwin B, Beasley F, Kourtev P, Konopka A. Soil microbial community responses to additions of organic carbon substrates and heavy metals (Pb and Cr). *Appl Environ Microbiol.* (2005) 71:7679–89. doi: 10.1128/AEM.71.12.7679-7689.2005
8. Stark CH, Condron LM, O'Callaghan M, Stewart A, Di HJ. Differences in soil enzyme activities, microbial community structure and short-term nitrogen mineralisation resulting from farm management history and organic matter amendments. *Soil Biol Biochem.* (2008) 40:1352–63. doi: 10.1016/j.soilbio.2007.09.025

9. Geisseler D, Scow KM. Long-term effects of mineral fertilizers on soil microorganisms – a review. *Soil Biol Biochem.* (2014) 75:54–63. doi: 10.1016/j.soilbio.2014.03.023
10. Chaudhary DR, Gautam RK, Ghosh A, Chikara J, Jha B. Effect of nitrogen management on soil microbial community and enzymatic activities in Jatropha curcas L. Plantation. *CLEAN - Soil, Air, Water*. (2015) 43:1058–65. doi: 10.1002/clen.201400357
11. Bünemann EK, Bongiorno G, Bai Z, Creamer RE, De Deyn G, de Goede R, et al. Soil quality – a critical review. *Soil Biol Biochem.* (2018) 120:105–25. doi: 10.1016/j.soilbio.2018.01.030
12. Heffer P, Prud'homme M. Fertilizer outlook 2015–2019. In: *83rd IFA annual conference*. Istanbul Turkey. (2015) p. 25–27.
13. Chin W, Kroontje W. Urea Hydrolysis Subsequent Loss of Ammonia. *Soil Sci Soc Am J*. (1963) 27:316–8. doi: 10.2136/sssaj1963.03615995002700030030x
14. Sigurdarson JJ, Svane S, Karring H. The molecular processes of urea hydrolysis in relation to ammonia emissions from agriculture. *Rev Environ Sci Biotechnol.* (2018) 17:241–58. doi: 10.1007/s11157-018-9466-1
15. Witte C-P. Urea metabolism in plants. *Plant Sci.* (2011) 180:431–8. doi: 10.1016/j.plantsci.2010.11.010
16. Zhu S, Vivanco JM, Manter DK. Nitrogen fertilizer rate affects root exudation, the rhizosphere microbiome and nitrogen-use-efficiency of maize. *Appl Soil Ecol.* (2016) 107:324–33. doi: 10.1016/j.apsoil.2016.07.009
17. Sun R, Li W, Hu C, Liu B. Long-term urea fertilization alters the composition and increases the abundance of soil ureolytic bacterial communities in an upland soil. *FEMS Microbiol Ecol.* (2019) 95:fiz044. doi: 10.1093/femsec/fiz044
18. Fauci MF, Dick RP. Soil microbial dynamics: short- long-term effects of inorganic organic nitrogen. *Soil Sci Soc Am J*. (1994) 58:801–6. doi: 10.2136/sssaj1994.03615995005800030023x
19. Edmeades DC. The long-term effects of manures and fertilisers on soil productivity and quality: a review. *Nutr Cycl Agroecosystems.* (2003) 66:165–80. doi: 10.1023/A:1023999816690
20. Gong W, Yan X, Wang J, Hu T, Gong Y. Long-term applications of chemical and organic fertilizers on plant-available nitrogen pools and nitrogen management index. *Biol Fertil Soils.* (2011) 47:767. doi: 10.1007/s00374-011-0585-x
21. Gardner JB, Drinkwater LE. The fate of nitrogen in grain cropping systems: a meta-analysis of ^{15}N field experiments. *Ecol Appl.* (2009) 19:2167–84. doi: 10.1890/08-1122.1
22. Birkhofer K, Bezemer TM, Bloem J, Bonkowski M, Christensen S, Dubois D, et al. Long-term organic farming fosters below and aboveground biota: Implications for soil quality, biological control and productivity. *Soil Biol Biochem.* (2008) 40:2297–308. doi: 10.1016/j.soilbio.2008.05.007
23. van Bruggen AHC, Semenov AM. In search of biological indicators for soil health and disease suppression. *Appl Soil Ecol.* (2000) 15:13–24. doi: 10.1016/S0929-1393(00)00068-8
24. Mazzola M. Assessment management of soil microbial community structure for disease suppression. *Annu Rev Phytopathol*. (2004) 42:35–59. doi: 10.1146/annurev.phyto.42.040803.140408
25. Steiner C, Glaser B, Teixeira WG, Lehmann J, Blum WEH, Zech W. Nitrogen retention and plant uptake on a highly weathered central Amazonian Ferralsol amended with compost and charcoal. *J Plant Nutr Soil Sci.* (2008) 171:893–9. doi: 10.1002/jpln.200625199
26. Rivero C, Chirenje T, Ma LQ, Martinez G. Influence of compost on soil organic matter quality under tropical conditions. *Geoderma.* (2004) 123:355–61. doi: 10.1016/j.geoderma.2004.03.002
27. Pérez-Piqueres A, Edel-Hermann V, Alabouvette C, Steinberg C. Response of soil microbial communities to compost amendments. *Soil Biol Biochem.* (2006) 38:460–70. doi: 10.1016/j.soilbio.2005.05.025
28. Gravuer K, Scow KM. Invader-resident relatedness and soil management history shape patterns of invasion of compost microbial populations into agricultural soils. *Appl Soil Ecol.* (2021) 158:103795. doi: 10.1016/j.apsoil.2020.103795
29. Shi S, Nuccio EE, Shi ZJ, He Z, Zhou J, Firestone MK. The interconnected rhizosphere: High network complexity dominates rhizosphere assemblages. *Ecol Lett*. (2016) 19:926–36. doi: 10.1111/ele.12630
30. Wei Z, Hu X, Li X, Zhang Y, Jiang L, Li J, et al. The rhizospheric microbial community structure and diversity of deciduous and evergreen forests in Taihu Lake area, China. *PLoS ONE.* (2017) 12:e0174411. doi: 10.1371/journal.pone.0174411
31. Schmid CAO, Schröder P, Armbruster M, Schloter M. Organic amendments in a long-term field trial—consequences for the bulk soil bacterial community as revealed by network analysis. *Microb Ecol.* (2018) 76:226–39. doi: 10.1007/s00248-017-1110-z
32. de Vries FT, Griffiths RI, Bailey M, Craig H, Girlanda M, Gweon HS, et al. Soil bacterial networks are less stable under drought than fungal networks. *Nat Commun.* (2018) 9:3033. doi: 10.1038/s41467-018-05516-7
33. Wang H, Wei Z, Mei L, Gu J, Yin S, Faust K, et al. Combined use of network inference tools identifies ecologically meaningful bacterial associations in a paddy soil. *Soil Biol Biochem.* (2017) 105:227–35. doi: 10.1016/j.soilbio.2016.11.029
34. Röttjers L, Faust K. From hairballs to hypotheses–biological insights from microbial networks. *FEMS Microbiol Rev.* (2018) 42:761–80. doi: 10.1093/femsre/fuy030
35. Tipton L, Müller CL, Kurtz ZD, Huang L, Kleerup E, Morris A, et al. Fungi stabilize connectivity in the lung and skin microbial ecosystems. *Microbiome.* (2018) 6:12. doi: 10.1186/s40168-017-0393-0
36. Mundra S, Kjønaas OJ, Morgado LN, Krabberød AK, Ransedokken Y, Kauserud H. Soil depth matters: Shift in composition and inter-kingdom co-occurrence patterns of microorganisms in forest soils. *FEMS Microbiol Ecol.* (2021) 97:fiab022. doi: 10.1093/femsec/fiab022
37. Yuan MM, Kakouridis A, Starr E, Nguyen N, Shi S, Zhou J, et al. Fungal-bacterial cooccurrence patterns differ between arbuscular mycorrhizal fungi and nonmycorrhizal fungi across soil niches. *MBio.* (2021) 12:1–19. doi: 10.1128/mBio.03509-20
38. Deenik J, McClellan AT. Soils of Hawai'i. *Soil Crop Manag.* (2007) 20:1–12.
39. Grayston SJ, Wang S, Campbell CD, Edwards AC. Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biol Biochem.* (1998) 30:369–78. doi: 10.1016/S0038-0717(97)00124-7
40. Berg G, Smalla K. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol Ecol.* (2009) 68:1–13. doi: 10.1111/j.1574-6941.2009.00654.x
41. Ryals R, McNicol G, Porder S, Kramer S. Greenhouse gas fluxes from human waste management pathways in Haiti. *J Clean Prod.* (2019) 226:106–13. doi: 10.1016/j.jclepro.2019.04.079
42. McNicol G, Jeliazovski J, François JJ, Kramer S, Ryals R. Climate change mitigation potential in sanitation via off-site composting of human waste. *Nat Clim Chang.* (2020) 10:545–9. doi: 10.1038/s41558-020-0782-4
43. Rigby H, Clarke BO, Pritchard DL, Meehan B, Beshah F, Smith SR, et al. A critical review of nitrogen mineralization in biosolids-amended soil, the associated fertilizer value for crop production and potential for emissions to the environment. *Sci Total Environ.* (2016) 541:1310–38. doi: 10.1016/j.scitotenv.2015.08.089
44. Lavkulich L. *Exchangeable cations and total exchange capacity by the ammonium acetate method at pH 7.0*. Canadian Society of Soil Science, Ottawa, Ontario, Canada (1981).
45. Nguyen NH, Smith D, Peay KG, Kennedy PG. Parsing ecological signal from noise in next generation amplicon sequencing. *New Phytol.* (2015) 205:1389–93. doi: 10.1111/nph.12923
46. Parada AE, Needham DM, Fuhrman JA. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol.* (2016) 18:1403–14. doi: 10.1111/1462-2920.13023
47. Apprill A, McNally S, Parsons R, Weber L. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat Microb Ecol.* (2015) 75:129–37. doi: 10.3354/ame01753
48. White T, Bruns TD, Lee S, Taylor J. Amplification direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, editor. *PCR protocols: a guide to methods and applications*. San Diego. (1990) p 315–322. doi: 10.1016/B978-0-12-372180-8.50042-1
49. Gardes M, Bruns TD. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae

and rusts. *Mol Ecol.* (1993) 2:113–8. doi: 10.1111/j.1365-294X.1993.tb00005.x
50. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol.* (2019) 37:852–7. doi: 10.1038/s41587-019-0209-9
51. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High resolution sample inference from Illumina amplicon data. *Nat Methods.* (2016) 13:581–3. doi: 10.1038/nmeth.3869
52. Rivers AR, Weber KC, Gardner TG, Liu S, Armstrong SD. ITSxpress: Software to rapidly trim internally transcribed spacer sequences with quality scores for marker gene analysis [version 1; peer review: 2 approved]. *F1000 Res.* (2018) 7:1418. doi: 10.12688/f1000research.15704.1
53. Rognes T, Flouri T, Nichols B, Quince C, Mah,é F. VSEARCH: a versatile open source tool for metagenomics. *PeerJ (San Fr. CA).* (2016) 4:e2584. doi: 10.7717/peerj.2584
54. DeSantis TZ, Stone CE, Murray SR, Moberg JP, Andersen GL. Rapid quantification and taxonomic classification of environmental DNA from both prokaryotic and eukaryotic origins using a microarray. *FEMS Microbiol Ecol.* (2005) 245:271–8. doi: 10.1016/j.femsle.2005.03.016
55. UNITE Community. *UNITE QIIME release. Version 01.12.2017*. (2017). Available online at: https://unite.ut.ee/repository.php
56. Nguyen NH, Song Z, Bates ST, Branco S, Tedersoo L, Menke J, et al. FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecol.* (2016) 20:241–8. doi: 10.1016/j.funeco.2015.06.006
57. R Core Team. *R: A Language and Environment for Statistical Computing*. (2018). Available online at: https://www.r-project.org/.
58. Pinheiro J, Bates D. *Mixed-Effects Models in S and S-PLUS*. New York, NY: Springer (2000). Available online at: http://www.springer.com/us/book/9780387989570
59. Hand DJ, Taylor CC. *Multivariate analysis of variance and repeated measures: a practical approach for behavioural scientists*. Boca Raton, FL. (1987). doi: 10.1007/978-94-009-3143-5
60. Schober P, Vetter TR. Repeated measures designs and analysis of longitudinal data: If at first you do not succeed-try, try again. *Anesth Analg.* (2018) 127:569–75. doi: 10.1213/ANE.0000000000003511
61. Lenth R. *lsmeans: Least-Squares Means*. 2.30–0. (2018). Available online at: https://cran.r-project.org/package=lsmeans.
62. Lenth R, Singmann H, Love J, Buerkner P, Herve M. *emmeans: Estimated Marginal Means, aka Least-Squares Means*. 1.3.3. (2019). Available online at: https://cran.r-project.org/package=emmeans.
63. Westfall PH, Tobias RD, Wolfinger RD. *Multiple comparisons and multiple tests using SAS*. SAS Institute. (2011).
64. Hothorn T, Bretz F, Westfall P. Simultaneous Inference in General Parametric Models. *Biometrical J.* (2008) 50:346–63. doi: 10.1002/bimj.200810425
65. Gloor GB, Macklaim JM, Pawlowsky-Glahn V, Egozcue JJ. Microbiome datasets are compositional: And this is not optional. *Front Microbiol.* (2017) 8:1–6. doi: 10.3389/fmicb.2017.02224
66. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. vegan: Community Ecology Package. (2017). Available online at: https://cran.r-project.org/package=vegan.
67. Legendre P, Gallagher ED. Ecologically meaningful transformations for ordination of species data. *Oecologia.* (2001) 129:271–80. doi: 10.1007/s004420100716
68. Anderson MJ, Willis TJ. Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. *Ecol.* (2003) 84:511–25. doi: 10.1890/0012-9658(2003)084[0511:CAOPCA]2.0.CO;2
69. Ramette A. Multivariate analyses in microbial ecology. *FEMS Microbiol Ecol.* (2007) 62:142–60. doi: 10.1111/j.1574-6941.2007.00375.x
70. Oksanen J. Multivariate analysis of ecological communities in R: vegan tutorial. *R Packag version.* (2011) 1:1–43.
71. Hervé M. *RVAideMemoire: Testing and Plotting Procedures for Biostatistics*. 0.9-75. (2020). Available online at: https://cran.r-project.org/package=RVAideMemoire.
72. Wickham H, Chang W, Henry L, Pedersen TL, Takahashi K, Wilke C, et al. *ggplot2: Create Elegant Data Visualisations Using the Grammar of Graphics*. 3.1.0. (2018). Available online at: https://cran.r-project.org/package=ggplot2.
73. Lüdecke D, Schwemmer C. *sjPlot: Data Visualization for Statistics in Social Science*. 2.6.2. (2018). Available online at: https://cran.r-project.org/package=sjPlot.
74. Mandal S, Van Treuren W, White RA, Eggesbø M, Knight R, Peddada SD. Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb Ecol Health Dis.* (2015) 26:27663. doi: 10.3402/mehd.v26.27663
75. Jiang L, Amir A, Morton JT, Heller R, Arias-Castro E, Knight R. Discrete false-discovery rate improves identification of differentially abundant microbes. *mSystems.* (2017) 2:e00092–17. doi: 10.1128/mSystems.00092-17
76. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* (2014) 15:550. doi: 10.1186/s13059-014-0550-8
77. Kurtz ZD, Müller CL, Miraldi ER, Littman DR, Blaser MJ, Bonneau RA. Sparse and compositionally robust inference of microbial eological networks. *PLOS Comput Biol.* (2015) 11:e1004226. doi: 10.1371/journal.pcbi.1004226
78. McMurdie PJ, Holmes S. phyloseq: An R ackage for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE.* (2013) 8:e61217. doi: 10.1371/journal.pone.0061217
79. Meinshausen N, Buhlmann P. High-dimensional graphs and variable selection with the Lasso. *Ann Stat.* (2006) 34:1436–62. doi: 10.1214/009053606000000281
80. Kurtz Z. Sparse inversE covariance estimation for ecological association and statistical inference: zdk123/SpiecEasi. (2019). Available online at: https://github.com/zdk123/SpiecEasi.
81. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res.* (2003) 13:2498–504. doi: 10.1101/gr.1239303
82. Assenov Y, Ramírez F, Schelhorn S-E, Lengauer T, Albrecht M. Computing topological parameters of biological networks. *Bioinformatics.* (2008) 24:282–4. doi: 10.1093/bioinformatics/btm554
83. Fierer N, Leff JW, Adams BJ, Nielsen UN, Bates ST, Lauber CL, et al. Cross-biome metagenomic analyses of soil microbial communities their functional attributes. *Proc Natl Acad Sci - PNAS.* (2012) 109:21390–5. doi: 10.1073/pnas.1215210110
84. Yurgel SN, Douglas GM, Dusault A, Percival D, Langille MGI. Dissecting Community Structure in Wild Blueberry Root and Soil Microbiome. *Front Microbiol.* (2018) 9:1187. doi: 10.3389/fmicb.2018.01187
85. Yoon J, Blumer A, Lee K. An algorithm for modularity analysis of directed and weighted biological networks based on edge-betweenness centrality. *Bioinformatics.* (2006) 22:3106–8. doi: 10.1093/bioinformatics/btl533
86. Ouédraogo E, Mando A, Zombr,é NP. Use of compost to improve soil properties and crop productivity under low input agricultural system in West Africa. *Agric. Ecosyst. Environ.* (2001) 84:259–266. doi: 10.1016/S0167-8809(00)00246-2
87. Soumar,é M, Tack FMG, Verloo MG. Characterisation of Malian and Belgian solid waste composts with respect to fertility and suitability for land application. *Waste Manag.* (2003) 23:517–522. doi: 10.1016/S0956-053X(03)00067-9
88. Littman ML. An Improved Method for Detection of Urea Hydrolysis by Fungi. *J Infect Dis.* (1957) 101:51–61. doi: 10.1093/infdis/101.1.51
89. Lloyd AB, Sheaffe MJ. Urease activity in soils. *Plant Soil.* (1973) 39:71–80. doi: 10.1007/BF00018046
90. Allison FE. The fate of nitrogen applied to soils. *Advances in Agronomy (Elsevier).* (1966) 18:219–58. doi: 10.1016/S0065-2113(08)60651-3
91. Tognetti C, Mazzarino MJ, Laos F. Compost of municipal organic waste: Effects of different management practices on degradability and nutrient release capacity. *Soil Biol Biochem.* (2008) 40:2290–6. doi: 10.1016/j.soilbio.2008.05.006
92. Duong TTT, Verma SL, Penfold C, Marschner P. Nutrient release from composts into the surrounding soil. *Geoderma.* (2013) 195–6:42–7. doi: 10.1016/j.geoderma.2012.11.010

93. Glibert PM, Harrison J, Heil C, Seitzinger S. Escalating worldwide use of urea: a global change contributing to coastal eutrophication. *Biogeochemistry.* (2006) 77:441–63. doi: 10.1007/s10533-005-3070-5
94. Subbarao GV, Sahrawat KL, Nakahara K, Ishikawa T, Kishii M, Rao IM, et al. Biological nitrification inhibition—a novel strategy to regulate nitrification in agricultural systems. *Adv Agron.* (2012) 114:249–302. doi: 10.1016/B978-0-12-394275-3.00001-8
95. Antunes LP, Martins LF, Pereira RV, Thomas AM, Barbosa D, Lemos LN, et al. Microbial community structure and dynamics in thermophilic composting viewed through metagenomics and metatranscriptomics. *Sci Rep.* (2016) 6:38915. doi: 10.1038/srep38915
96. Saison C, Degrange V, Oliver R, Millard P, Commeaux C, Montange D, et al. Alteration and resilience of the soil microbial community following compost amendment: effects of compost level and compost-borne microbial community. *Environ Microbiol.* (2006) 8:247–57. doi: 10.1111/j.1462-2920.2005.00892.x
97. Zhang N, Wan S, Li L, Bi J, Zhao M, Ma K. Impacts of urea N addition on soil microbial community in a semi-arid temperate steppe in northern China. *Plant Soil.* (2008) 311:19–28. doi: 10.1007/s11104-008-9650-0
98. Broeckling CD, Broz AK, Bergelson J, Manter DK, Vivanco JM. Root exudates regulate soil fungal community composition and diversity. *Appl Environ Microbiol.* (2008) 74:738–44. doi: 10.1128/AEM.02188-07
99. Ellouze W, Esmaeili Taheri A, Bainard LD, Yang C, Bazghaleh N, Navarro-Borrell A, et al. Soil fungal resources in annual cropping systems and their potential for management. *Biomed Res Int.* (2014) 2014:1–15. doi: 10.1155/2014/531824
100. Treseder KK. Nitrogen additions and microbial biomass: a meta-analysis of ecosystem studies. *Ecol Lett.* (2008) 11:1111–20. doi: 10.1111/j.1461-0248.2008.01230.x
101. Jongen R, Hannula SE, De Long JR, Heinen R, Huberty M, Steinauer K, et al. Plant community legacy effects on nutrient cycling, fungal decomposer communities and decomposition in a temperate grassland. *Soil Biol Biochem.* (2021) 163:108450. doi: 10.1016/j.soilbio.2021.108450
102. Abawi GS, Widmer TL. Impact of soil health management practices on soilborne pathogens, nematodes and root diseases of vegetable crops. *Appl Soil Ecol.* (2000) 15:37–47. doi: 10.1016/S0929-1393(00)00070-6
103. Chaparro JM, Sheflin AM, Manter DK, Vivanco JM. Manipulating the soil microbiome to increase soil health and plant fertility. *Biol Fertil Soils.* (2012) 48:489–99. doi: 10.1007/s00374-012-0691-4
104. Daims H, Lebedeva EV, Pjevac P, Han P, Herbold C, Albertsen M, et al. Complete nitrification by *Nitrospira* bacteria. *Nature.* (2015) 528:504–9. doi: 10.1038/nature16461
105. Speirs LBM, Rice DTF, Petrovski S, Seviour RJ. The phylogeny, biodiversity, and ecology of the chloroflexi in activated sludge. *Front Microbiol.* (2019) 10:2015. doi: 10.3389/fmicb.2019.02015
106. Cooney DG, Emerson R. *Thermophilic Fungi: An Account of Their Biology, Activities, and Classification*. University of California, Los Angeles, United States (1964).
107. Howard DH. *Pathogenic Fungi in Humans and Animals*. Baton Rouge: CRC Press. (2002). doi: 10.1201/9780203909102
108. Déportes I, Benoit-Guyod J-L, Zmirou D. Hazard to man and the environment posed by the use of urban waste compost: a review. *Sci Total Environ.* (1995) 172:197–222. doi: 10.1016/0048-9697(95)04808-1
109. Noble R. Risks and benefits of soil amendment with composts in relation to plant pathogens. *Australas plant Pathol.* (2011) 40:157–67. doi: 10.1007/s13313-010-0025-7
110. Komnitsas K, Zaharaki D. Assessment of human and ecosystem risk due to agricultural waste compost application on soils: a review. *Environ Forensics.* (2014) 15:312–28. doi: 10.1080/15275922.2014.950775
111. Nadeem SM, Naveed M, Zahir ZA, Asghar HN. Plant-microbe interactions for sustainable agriculture: fundamentals and recent advances. In: Arora NK, editor. *Plant Microbe Symbiosis - Fundamentals and Advances*. New Delhi: Springer India (2013). p. 51–103. doi: 10.1007/978-81-322-1287-4_2
112. Komárek J. A polyphasic approach for the taxonomy of cyanobacteria: principles and applications. *Eur J Phycol.* (2016) 51:346–53. doi: 10.1080/09670262.2016.1163738
113. Mandyam KG, Jumpponen A. Mutualism-parasitism paradigm synthesized from results of root-endophyte models. *Front Microbiol.* (2014) 5:776. doi: 10.3389/fmicb.2014.00776
114. Gibson TE, Bashan A, Cao H-T, Weiss ST, Liu Y-Y. On the origins and control of community types in the human microbiome. *PLoS Comput Biol.* (2016) 12:e1004688. doi: 10.1371/journal.pcbi.1004688
115. Agler MT, Ruhe J, Kroll S, Morhenn C, Kim S-T, Weigel D, et al. Microbial hub taxa link host and abiotic factors to plant microbiome variation. *PLoS Biol.* (2016) 14:e1002352. doi: 10.1371/journal.pbio.1002352

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Soil Science

ORIGINAL RESEARCH
published: 30 June 2022
doi: 10.3389/fsoil.2022.818862

Increasing Biodiversity and Land-Use Efficiency Through Pea (*Pisum aestivum*)-Canola (*Brassica napus*) Intercropping (Peaola)

Isaac J. Madsen[1]*, *Janice M. Parks*[2], *Maren L. Friesen*[2] and *Robert E. Clark*[3]

[1] Department of Crop and Soil Sciences, Washington State University, Pullman, WA, United States, [2] Department of Plant Pathology, Washington State University, Pullman, WA, United States, [3] Department of Entomology, Washington State University, Pullman, WA, United States

Intercropping is an ancient agricultural management practice quickly re-gaining interest in mechanized agricultural systems. Mechanized management practices have led to a decreased biodiversity at the macro- and micro-fauna levels. These agricultural practices have also resulted in the degradation of soil and long-term inefficiencies in land, water, and nutrients. The inland Pacific Northwest (iPNW) of the United States of America is a wheat-dominated cropping system. The integration of winter and spring legumes and oilseeds has improved the biodiversity and nutrient-use efficiency of the cropping systems. This article examines the feasibility of pea-canola (peaola) intercropping in dryland production systems of the iPNW. In two site years, small plot peaola trials were established near Davenport, WA. Overall, the land equivalence ratio (LER) of peaola was found to be 1.46, showing an increase in efficiency of the system. Increasing the N fertilizer application rates did not affect peaola yield, indicating that peaola has low demand for N inputs. The effects of peaola on insects and bacterial diversity were examined on replicated large scale strip trials. Peaola was found to have significantly greater numbers of beneficial insects than the monoculture controls. There were no significant differences between the diversity of the soil bacterial communities found in peaola vs. pea and canola monocultures. However, we found that the strict core soil bacterial microbiome of peaola was larger than the monocultures and included core members from both the canola and pea soil microbiomes. In conclusion, the widespread adoption of peaola would likely increase the biodiversity and increase the land use efficiency of dryland production systems in the iPNW.

Keywords: intercrop, peaola, canola, pea (field), soil microbiome

OPEN ACCESS

Edited by:
Patricia Dorr De Quadros, University of Waterloo, Canada

Reviewed by:
Esmaeil Rezaei-Chiyaneh, Urmia University, Iran
Sajjad Raza, Nanjing University of Information Science and Technology, China

***Correspondence:**
Isaac J. Madsen
isaac_madsen@wsu.edu

Specialty section:
This article was submitted to Soil Biology, Ecosystems and Biodiversity, a section of the journal Frontiers in Soil Science

Received: *20 November 2021*
Accepted: *20 April 2022*
Published: *30 June 2022*

Citation:
Madsen IJ, Parks JM, Friesen ML and Clark RE (2022) Increasing Biodiversity and Land-Use Efficiency Through Pea (Pisum aestivum)-Canola (Brassica napus) Intercropping (Peaola). Front. Soil Sci. 2:818862. doi: 10.3389/fsoil.2022.818862

INTRODUCTION

Most industrial agriculture systems are monocultures with the only feasible option for increased diversity being crop rotation. Subsistence agriculture on the other hand has long relied on multispecies systems (1). These multispecies systems prohibit the use of chemicals and are not easily adapted to mechanization and the economies of scale prevalent in large-scale industrial agriculture. To incorporate intercropping into large-scale industrial systems, the feasibility should be considered as well as the ecological benefits. Oilseed-legume intercrops have been shown to be compatible with large-scale adoption, primarily due to

complementary plant architectures and the fact that intercropping broad-leafed plants lends itself to grassy weed control (2). One such oilseed-legume intercropping system of particular interest in the inland Pacific Northwest (iPNW) is pea-canola intercrops (peaola). For the purposes of this study, the iPNW region is defined as the areas of Oregon, Washington, and Idaho east of the Cascade Mountain range in the United States of America.

Peas (*Pisum aestivum*) and canola (*Brassica napus*) are both grown in the iPNW and are grown as fall- or spring-seeded crops (3). Dryland production systems are dominated by wheat production, and like canola and peas, winter and spring wheats are both produced in the region. The iPNW has a strong precipitation gradient, with precipitation increasing from west to east across the dryland production region. The dry western part of the iPNW is dominated by winter wheat—fallow cropping sequences, while in the wet eastern portion, annual cropping is common in a winter wheat—spring wheat—spring legume rotation (4). Winter peas and canola have both been used to extend the cropping sequence in the grain fallow rotation by replacing every other wheat crop (4). In the annual cropping zone, the spring legume part of the rotation is frequently replaced with spring canola due to price and herbicide options. The use of group 1 herbicides to control grassy weeds, which can be difficult in wheat production systems, is allowed for use in both winter and spring canola (5).

Canola has been shown to be a useful tool for improving the water and nutrient efficiency as a nutrient catch crop and increasing the water infiltration when grown in rotation (6–8). The increase in efficiency is most likely due to the deep-rooting nature of canola plants. The increase in infiltration is thought to be caused by canola having tap roots as compared with the fibrous roots of wheat (8). Despite these benefits, canola has also been shown to reduce the yield of the subsequent wheat crop in some instances due to the inability of canola, as a Brassica, to form symbiotic relationship with arbuscular mycorrhizal fungi (AMF) (9, 10). In contrast, peas offer the advantage of being able to form symbiotic relationships with AMF and nitrogen (N)-fixing bacteria that are housed in root structures known as nodules. As such, peas can introduce more biologically available N to this system since N is released when nodules decompose and AMF can mineralize N from decomposing plant matter through improving the activity of soil enzymes (11–13). As canola does not form relationships with these microorganisms under normal circumstances, these sources of essential plant nutrients could become available to canola or the following crops in this intercropping system. Therefore, it is likely that peas bring a cascading suite of bacteria and fungi that do not normally form relationships with canola.

Due to the ability of legumes to host symbiotic bacteria that conduct biological N fixation, they have frequently been included in intercropping systems. A number of studies that assessed the effects of N rate on peaola productivity have been conducted (14). Most of these trials were conducted in Canada on spring canola and show a mixed effect of N land equivalence ratio (LER) and the relative yields of peas and canola. In some instances, LER has been shown to decline with increasing N while the relative yield of canola increases (15). The spring-crop-dominated systems of Canada are significantly different from the winter-wheat-dominated production region of the iPNW however.

In addition to the questions regarding N rate, intercropping systems should improve production through increasing the ecological diversity, increasing the resilience, and increasing the resource-use efficiency (16). In theory, the combination of peas and canola will result in greater resource-use efficiency due to their differing microbial interactions and subsequent increase in microbial diversity. This has been observed in studies on other intercropping systems (17–21). Peaola may also offer an increase in the adaptive capacity of a crop, as peas and canola can fill different ecological niches across the landscape. Additionally, both canola and peas come to maturation in slightly offset timelines with flowering occurring at different times, thereby reducing the overall vulnerability to acute environmental stress and pests. Aphid pests and seed predators impact both canola and pea (22, 23). Offset times of flowering and production can lead to lower rates of brassica pest outbreaks in either member of a polyculture (24). In terms of broad ecological mechanisms, doubling the species number on a single piece of ground at the macro (plant) scale may have cascading effects on various interacting communities such as pests, beneficial insects, and soil microbial communities (25, 26).

This study has three principal objectives. The first objective was to assess the LER of winter and spring peaola in the iPNW. The second was to assess the effect of N fertilizer rate on the relative yields of peas and canola as well as LER in winter peaola. The third objective was to assess the changes in insects and soil microbial communities in peaola vs. the corresponding monocultures. We hypothesize (2) that both winter and spring peaola intercropping in the iPNW will outperform the respective

TABLE 1 | Significance of year, cropping system, and *N* rate on yield and LER at Davenport small plot trials.

Year	Cropping system	*N* rate Kg ha-1	Canola yield Kg ha-1	Pea yield Kg ha-1	LER
2020	Canola	67	2,198	0	1.00
2020	Pea	0	0	2,752	1.00
2020	Peaola	0	2,029	2,011	1.65
2020	Peaola	34	1,704	1,667	1.38
2020	Peaola	67	991	2,698	1.43
2021	Canola	67	933	0	1.00
2021	Pea	0	0	85	1.00
2021	Peaola	0	649	84	1.68
2021	Peaola	34	1,071	63	1.89
2021	Peaola	67	541	71	1.42
Year			***	***	.
Cropping system			***	***	***
N rate			NS	NS	NS
Year X cropping system			NS	***	NS
Year × *N* rate			NS	NS	NS

$p < 0.0001 = ^{***}$, $p < 0.05 = .$

monocultures as measured by LER; (3) that increasing N rate in peaola will not increase the overall productivity of the system; (4) that the microbial communities associated with the peaola cropping systems will develop a microbial community distinct from both the pea and the canola monocultures; and (5) that the peaola cropping system will have more beneficial insects and less pests than the monoculture controls.

METHODS

Yield, Land Equivalence Ratio, and N Response

The small plot (1.7 × 9 m) trials were conducted near Davenport, WA, and were seeded and harvested with small plot research equipment. The small plot experiment was laid out using a randomized complete block design with four plot replicates per treatment combination. The two controls were monoculture canola with 67 kg N ha^{-1} and monoculture peas with 0 kg N ha^{-1}. The three treatments were peaola at different N rates (0, 33, and 67 kg N ha^{-1}). N applications were made using urea-ammonium nitrate and streamed on using a CO_2 backpack sprayer in the spring. During the 2020 growing season, these applications were made just prior to precipitation. However, in the 2021 growing season, there was little to no spring precipitation and the fertilizer was simply applied in March.

A Fabro double disk no-till drill was used to seed the plots into no-till winter wheat chemical fallow. The winter pea variety Goldenwood (ProGene Plant Research, LLC) was used in both the monoculture and the intercropping plots, while Plurax (Rubisco Seeds) was used as the canola variety of choice. Both Goldenwood and Plurax have been successfully grown in the iPNW. The peas and canola were planted in the same row at the same time for both the 2020 and the 2021 cropping years. Typically, peas are planted later in the fall than canola in Eastern Washington. However, in this study, the planting date was a compromise between peas and canola with a late August planting date. Grassy weed herbicide applications were made in the spring of 2020 and 2021. The whole plot yield was sampled, and the peas and canola were separated using an M-2B clipper mill from A. T. Ferrell & Company Bluffton, Indiana.

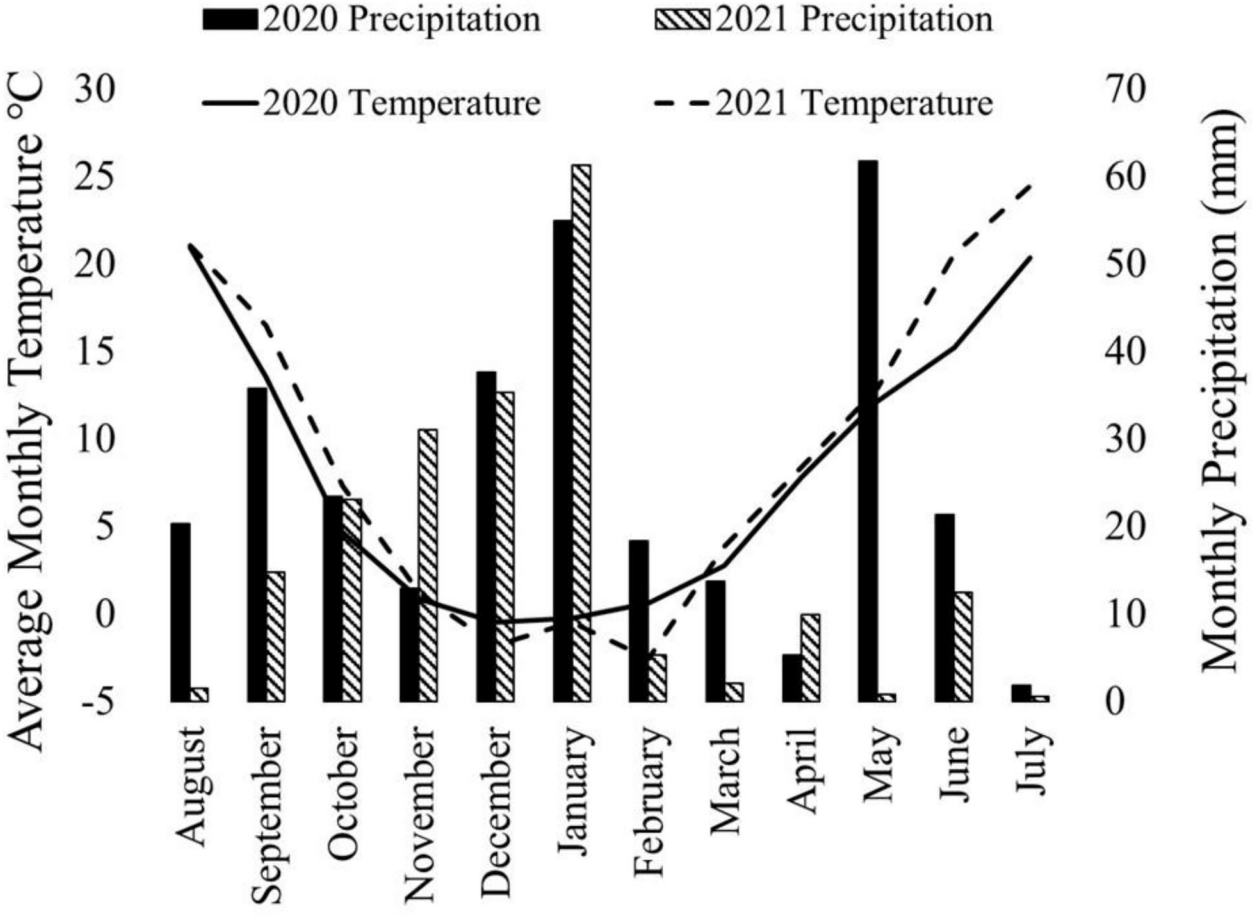


FIGURE 1 | Precipitation and temperature on a month-by-month basis for the 2019–2020 growing season and the 2020–2021 growing season. Spring (March, April, and May) precipitation was substantially higher in 2020 than 2021. Additionally, June and July average temperatures were warmer in 2021 than in 2020. This chart was developed using data from WSU AgWeatherNet (https://weather.wsu.edu/).

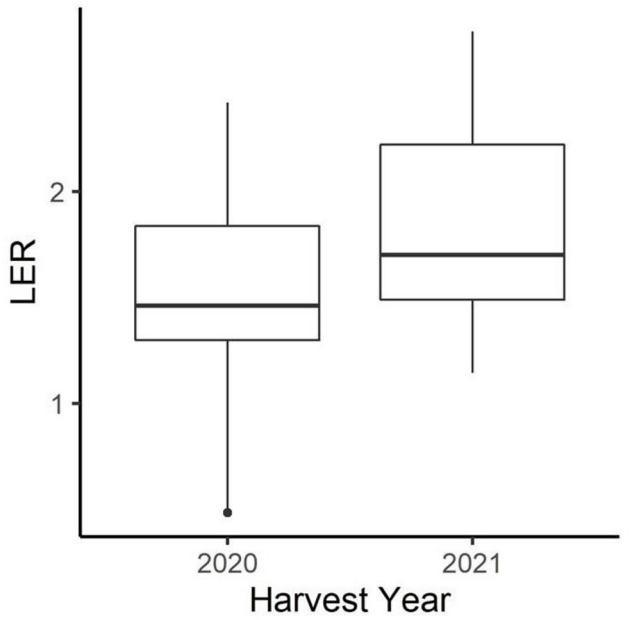


FIGURE 2 | Distribution of peaola LER across all treatments in 2020 and 2021. The year 2021, which had much lower yields, had a higher average LER across all peaola treatments.

The large-scale replicated spring peaola strip trials (11 × 61 m) were established on 9 April 2020 near Colfax, WA. The large-scale strip trials included 4 replicates of canola monoculture, pea monoculture, and peaola. Placement of replicates was randomized. The strips were direct seeded into stubble from the previous year's winter wheat crop using a no-till Cross Slot drill. A winter pea variety (Goldenwood from ProGene Plant Research, LLC) was used, as there was a concern that early and aggressive growth of spring peas would outcompete the early stages of canola growth, The spring-type canola was a Clearfield canola variety from DynaGro 200 CL. Fertilizers were applied in furrow at planting with 101 kg N ha^{-1} applied to the canola monoculture, 51 kg N ha^{-1} applied to the peaola, and 0 kg N ha^{-1} applied to the monoculture peas. Beyond (imazomox) and select (clethodim) herbicides were applied in late May. The strips were harvested on 14 September of 2020 and weighed using a weigh wagon. As the harvested pea-canola mix was dumped into the weigh wagon, a small amount (∼1 kg) was sampled from the grain stream using a polyvinyl chloride (PVC) pipe. The peas and canola were separated using an M-2B clipper mill from A. T. Ferrell & Company Bluffton, Indiana. The peas and canola were then weighed individually and were applied to the overall grain yield which was used to calculate the relative pea and canola yield on a per hectare basis.

Land equivalence ratio was calculated using Equation 1, where *ICp* and *ICc* were the intercropping pea and canola

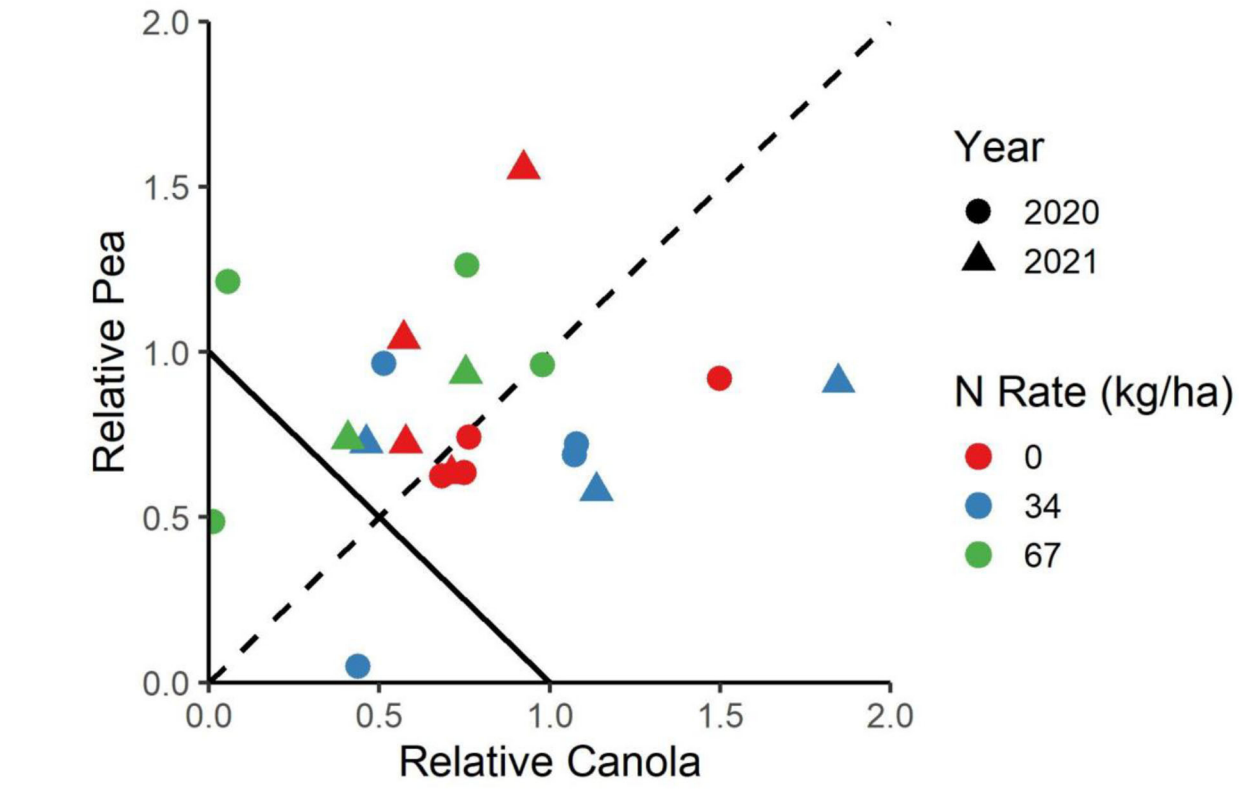


FIGURE 3 | The relative yield of canola and peas in the canola system. The negative-sloped line from (0.1) to (1.0) indicates the LER of the monocultures, while the dashed line indicates to which degree the peas or canola are favored.

yield, respectively, and *Mp* and *Mc* were the monoculture pea and canola yield, respectively. *Mp* and *Mc* were calculated by calculating the mean of all four replicates for peas and canola, respectively (27). The LER for each individual peaola plot was calculated using the same *Mp* and *Mc* within each year.

$$LER = \frac{ICp}{Mp} + \frac{ICc}{MCc} \quad (1)$$

Insect Class and Abundance

Insect samples were collected at the Colfax location in the spring of 2020 as the small plot experiments in Davenport were not considered suitably large enough to conduct an adequate insect sampling. The large-scale strips were oriented roughly north to south lengthwise. The insect samples were taken 10 m from the north end of the strips and 3 m east side of the plot to ensure a uniform sampling location between plots. The insects were identified and categorized into a functional group. The functional groups were pollinators (Hymenoptera in the Apoidea superfamily and Diptera in the Syrphid family), parasites (Hymenoptera in the families Braconidae and Ichneumonidae), predators [Araneae (spiders) and Coleoptera in the family Coccinellidae (ladybeetles)], and herbivores (Hemiptera in the families Aphidae, Miridae, and Pendatomidae, all larval Lepidoptera, and Coleoptera in the family Curculionidae). For analyses, these pollinators, parasites, and predators were classified broadly as beneficial arthropods, while the herbivores were classified as pest arthropods.

TABLE 2 | Spring canola yield at Colfax location.

Cropping system	*N* rate Kg ha-1	Canola yield Kg ha-1	Pea yield Kg ha-1	LER
Peaola	50	805	489	1.37
Canola	101	778	–	1
Pea	0	–	1,427	1
Cropping system		NS	***	NS

$p < 0.0001 = ^{***}$.

Soil Microbial Community Analysis

Microbial Soil Sample Collection

Soil samples were collected on 14 July 2020 from the large-scale strip trials located near Colfax, WA, to a depth of 10 cm. This corresponded with early flowering of canola. Three samples were taken within each replication (four canola monoculture, four pea monoculture, and four peaola) toward the middle of the plot, resulting in twelve samples per treatment. Once collected, the samples were put in a cooler and transported to WSU where they were kept at −20°C until DNA extraction.

DNA Extraction and Sequencing

The DNA was extracted using a Kingfisher DNA extraction machine following the Earth Microbiome Project's protocol for the QIAGEN® MagAttract® PowerSoil® DNA KF Kit. A no-soil blank was added to each extraction plate to control for cross-contamination. A high-sensitivity dsDNA quantification was performed using a Qubit following the manufacturer's protocol. The amplification of the 16S V4 region was done using the primers 515F: 5′-GTGCCAGCMGCCGCGGTAA-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′ using the Thermo Scientific DreamTaq DNA Polymerase following the manufacturer's instructions. The thermocycler program was denaturation at 95°C for 3 min; 30 cycles of 95°C for 45 s, 50°C for 60 s, and 72°C for 90 s; final elongation at 72°C for 10 min; and

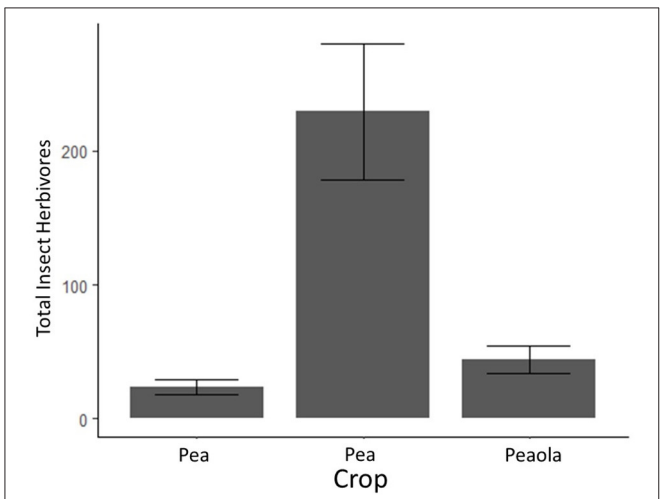


FIGURE 4 | Average counts of beneficial insects (and estimated standard errors) based on 2020 field survey. The bars with error bars that do not overlap are significantly different. Output estimates from negative binomial generalized linear mixed model.

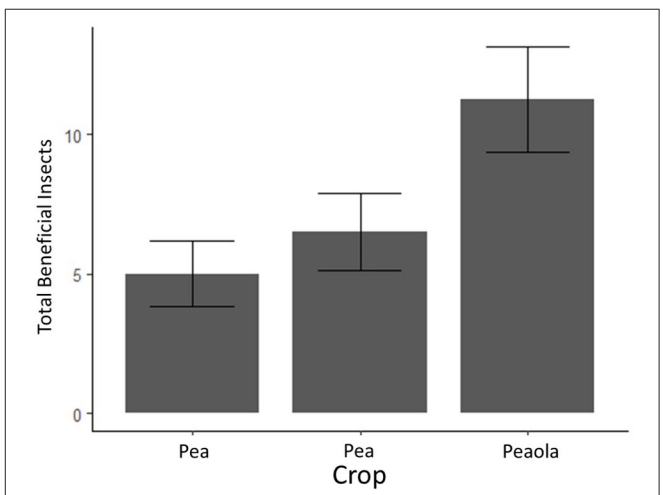


FIGURE 5 | Average counts of insect herbivores (and estimated standard errors) based on 2020 field survey. The bars with error bars that do not overlap are significantly different. Output estimates from negative binomial generalized linear mixed model.

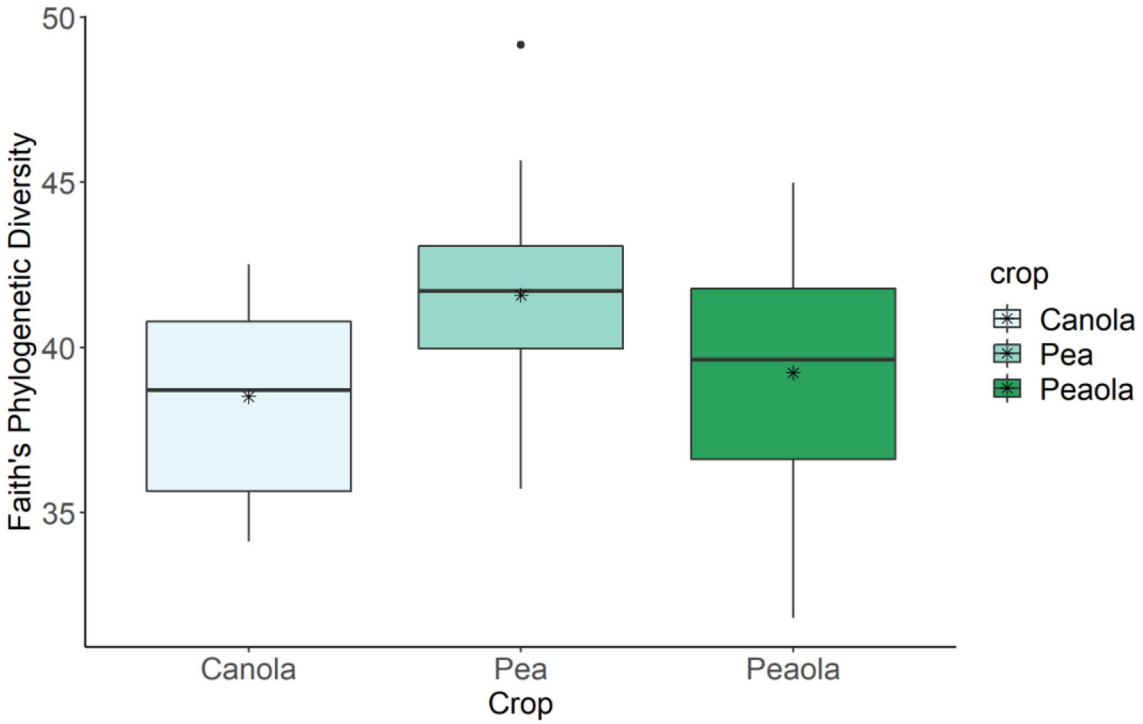


FIGURE 6 | Results of Faith's Phylogenetic Diversity index for canola, pea, and peaola soils. The microbial communities in the monoculture pea soil are trending toward being richer than the monoculture canola soil as determined by the Faith's Phylogenetic Diversity index (Kruskal–Wallis Test, n1 = 12, n2 = 12, $H = 5.60$, $P = 0.0537$). No significant difference or trend was found in the community richness of the peaola soil and pea monoculture soil (Kruskal–Wallis Test, n1 = 12, n2 = 12, $H = 1.47$, $P = 0.3380$) and canola monoculture soil (Kruskal–Wallis Test, n1 = 12, n2 = 12, $H = 0.653$, $P = 0.4189$) as determined by the Faith's Phylogenetic Diversity index. * is the mean.

an infinite hold at 15°C. An agarose gel electrophoresis was performed to confirm the presence of correctly sized amplicons at ∼300 bp.

The DNA was sent to Michigan State University's Research Technology Support Facility for an Illumina Amplicon sequencing of the 16S V4 region on the MiSeq v2 Standard platform, resulting in 250-bp paired end reads. The ZymoBIOMICS Microbial Community Standard II (Log Distribution) was included in place of our extraction negative. A negative control was added by the sequencing center.

Soil Biology Data Analysis

The sequences were analyzed using the QIIME2 version 2021.8 on WSU's Kamiak High Performance Computing Cluster. The bacteria were classified using "qiime feature-classifier classify-sklearn" with the Silva 138 99% OTUs from 515F/806R classifier found on the QIIME2 data resources page. The mitochondria and chloroplasts were filtered out before analyzing the diversity metrics. The samples were analyzed at a depth of 10,201 in QIIME2 to determine the diversity of the microbial communities. The analysis of our alpha-rarefaction plot proved to be sufficient in showing the full diversity of our samples. Identification of the bacterial core microbiome was done using "qiime feature-table core-features" with the mitochondria and chloroplasts filtered out. We chose to use the strict bacterial core microbiome with core members being present in 100% of the tested samples. Boxplots were made using the raw data generated by the QIIME2 version 2021.8 using the R version 4.0.3 in the RStudio version 1.2.5001. *P*-values were adjusted using the Benjamini–Hochberg FDR correction.

RESULTS

Yield, Land Equivalence Ratio, and N Response

Both canola and pea yields were significantly higher in 2020 than in 2021 (**Table 1**). The 2020–2021 growing season was an unusually dry growing season compared with the 2019–2020 growing season. The greatest difference was in the spring, precipitations in March, May, and June in 2020 were higher than in 2021 (**Figure 1**). In addition to 2021 being a drought year, the last few weeks of June were abnormally hot, resulting in stress during flowering for the winter peas. The year also had a significant effect on the LER ($p < 0.05$; **Figure 2**), and there was a significant interaction between year and the cropping system on pea yield ($p < 0.0001$).

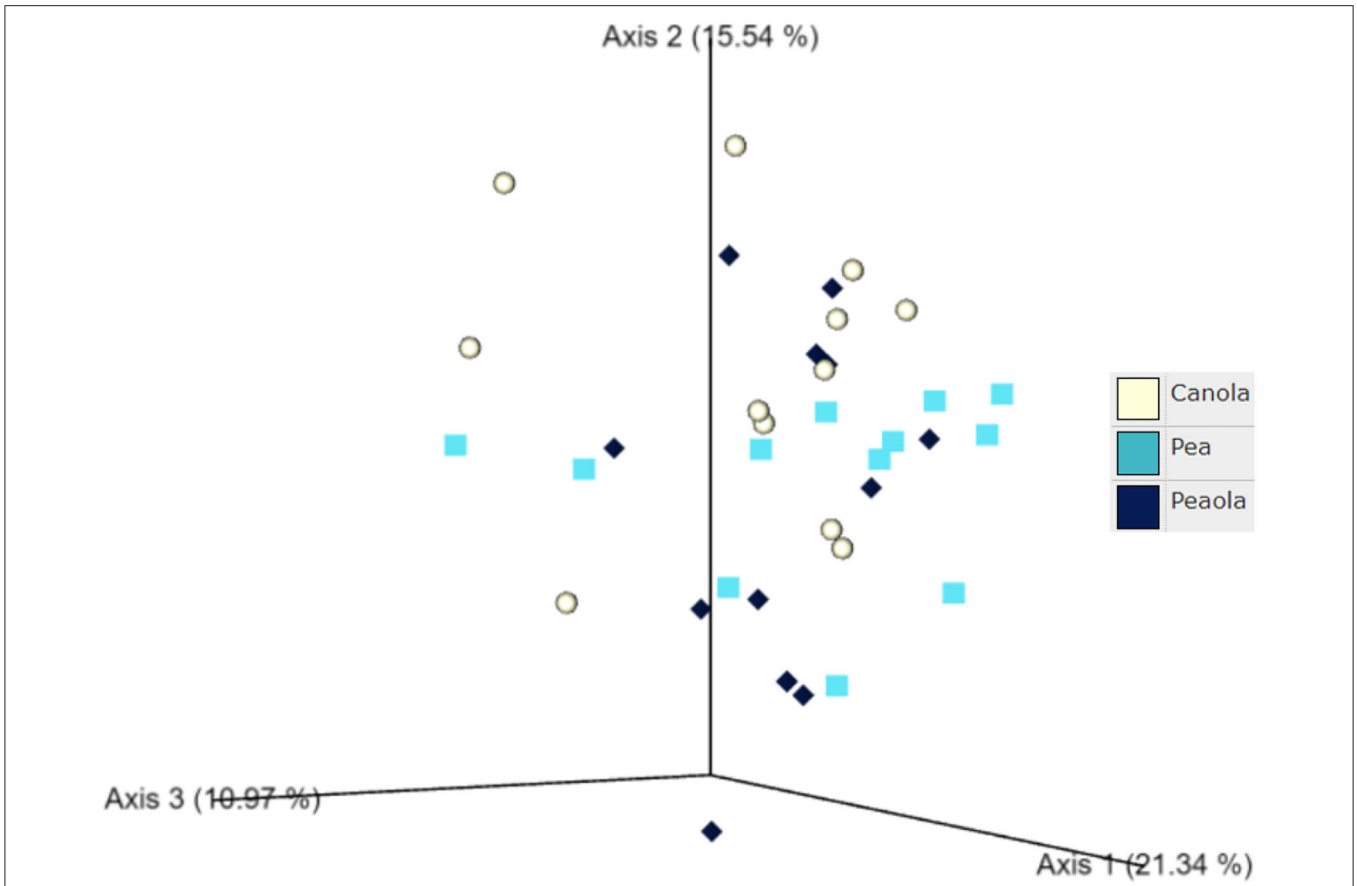


FIGURE 7 | Ordination showing the results of the Weighted UniFrac Distance comparison performed between the different cropping systems. A trend was found toward there being a difference in the community composition of the pea and canola monoculture soils (PERMANOVA, $F = 2.06$, $P = 0.0705$) and the peaola and canola monoculture soils (PERMANOVA, $F = 1.72$, $P = 0.0705$). No significant difference or trend was found between the pea monoculture and peaola soils (PERMANOVA, $F = 1.06$, $P = 0.3500$).

The cropping system was shown to have a significant main effect on the canola yield, the pea yield, and the LER (**Table 1**). The average LER across both years and locations was 1.63 for the peaola compared to the normalized value of 1 for the monocultures. The N fertilization rate was not shown to have a significant effect on canola yield, pea yield, or LER in either year. The relative yields of both peas and canola were calculated as components of the LER (**Figure 3**).

At the strip trial near Colfax, the LER of the peaola (1.37) was not significantly different from the LER of the monocultures (**Table 2**). The average yield of the canola was not significantly different between the intercropped (805 kg ha^{-1}) to the monoculture (778 kg ha^{-1}) strips. However, the yield of peas was significantly reduced in the intercropped (489 kg ha^{-1}) when compared to the monoculture pea yields (1,427 kg ha^{-1}).

Insect Class and Abundance

Herbivores (mostly pea aphids) were significantly higher in pea-only plots ($p < 0.001$, GLMM, **Figure 4**). Beneficial insects, including pollinators, parasitoid wasps, and ladybugs, were significantly higher in peaola trials compared to either peas or canola ($p = 0.0107$, GLMM, **Figure 5**). Consequently, even though peaola contained peas and was located at the same site, the intercropping strategy greatly reduced the threat of pea aphids. This was likely driven by the presence of more beneficial insects in peaola, including two primary biocontrol agents for aphids (wasps and ladybugs).

Soil Microbial Community Analysis

The analysis of our microbial community standard revealed that we were able to detect the included bacteria at their appropriate abundance down to bacteria present at a relative abundance of 0.089%. The measures of α-diversity-Shannon diversity index, Observed Features, and Evenness-did not show any significant differences ($p < 0.05$) or trends ($0.05 \leq p \leq 0.1$) between pea monoculture, canola monoculture, and peaola soils. It was found that the microbial communities in the monoculture pea soil are trending toward being richer than the monoculture canola soil as

determined by the Faith's Phylogenetic Diversity index (Kruskal–Wallis Test, n1 = 12, n2 = 12, $H = 5.60$, $p = 0.0537$; **Figure 6**). However, no significant differences or trends were found in the community richness of the peaola soil and pea monoculture soil (Kruskal–Wallis Test, n1 = 12, n2 = 12, $H = 1.47$, $P = 0.3380$) and canola monoculture soil (Kruskal–Wallis Test, n1 = 12, n2 = 12, $H = 0.653$, $P = 0.4189$) as determined by the Faith's Phylogenetic Diversity index (**Figure 6**).

The measures of β-diversity–Jaccard distance, Bray-Curtis distance, and unweighted UniFrac distance–did not show any significant differences ($P < 0.05$) or trends ($0.05 \leq P \leq 0.1$) between pea monoculture, canola monoculture, and peaola soils. Using the weighted UniFrac distance, we did find a trend toward there being a difference in the community composition between pea and canola monoculture soils (PERMANOVA, $F = 2.06$, $P = 0.0705$), and between peaola and canola monoculture soils (PERMANOVA, $F = 1.72$, $P = 0.0705$). No significant difference or trend was found between the pea monoculture and peaola soils (PERMANOVA, $F = 1.06$, $P = 0.3500$; **Figure 7**).

When looking at the makeup of the strict bacterial core microbiome, we found that the peaola core microbiomes consisted of 34 members, the pea core microbiome consisted of 23 members, and the canola core microbiome consisted of 29 members (**Table 3**). Overall, there were 13 bacteria that were shared across all three core microbiomes. Out of the canola core microbiome, 8 additional bacteria were shared with peaola, and out of the pea core microbiome, 3 additional bacteria were shared with peaola. Peaola had 10 core members that were not shared with either the pea or canola core microbiomes.

TABLE 3 | Strict bacterial core microbiomes for canola, pea, and peaola.

Canola core microbiome	Peaola core microbiome	Pea core microbiome
Acidobacteriaceae (Subgroup 1)	*Acidobacteriaceae* (Subgroup 1)	*Acidiphilium*
Acidobacteriales	*Acidiphilium*	Acidobacteriales
Acidobacteriales	Acidobacteriales	Acidobacteriales
Acidothermus	Acidobacteriales	*Acidothermus*
Acidothermus	*Acidothermus*	*Actinoplanes*
Blastococcus	*Acidothermus*	*Blastococcus*
Blastococcus	*Blastococcus*	*Candidatus* Solibacter
Bryobacter	Burkholderiales *SC-I-84*	*Cellulomonas*
Burkholderia-Caballeronia-Paraburkholderia	*Burkholderia-Caballeronia-Paraburkholderia*	*Chitinophagaceae*
Candidatus Solibacter	*Catenulispora sp.*	*Comamonadaceae*
Caulobacteraceae	*Caulobacteraceae*	*Conexibacter*
Comamonadaceae	*Cellulomonas*	Gaiellales
Frankiales	*Comamonadaceae*	Gaiellales
Gaiellales	*Comamonadaceae*	*Haliangium*
Gaiellales	*Conexibacter*	*Nocardioides*
Gaiellales	*Conexibacter*	*Phenylobacterium*
Haliangium	Gaiellales *Ellin6517*	Polyangiales *Blrii41*
Kutzneria	Gaiellales	*Porphyrobacter*
Mycobacterium	Gaiellales	*Sphingomonas*
Phenylobacterium	*Granulicella paludicola*	Uncultured Acidobacteria
Polyangiales *Blrii41*	*Haliangium*	Uncultured Acidobacteriales
Porphyrobacter	*Micropepsaceae*	*WPS-2*
Solirubrobacterales *67–14*	*Micropepsaceae*	*Xanthobacteraceae*
Sphingomonas	*Mycobacterium*	–
Sphingomonas	*Pedosphaeraceae Ellin516*	–
Uncultured Acidobacteria	Polyangiales *Blrii41*	–
Uncultured *Rhodospirillaceae*	*Porphyrobacter*	–
Uncultured *Steroidobacter*	*Rhodanobacter*	–
Xanthobacteraceae	Solirubrobacterales *67–14*	–
–	*Sphingomonas*	–
–	Uncultured Acidobacteria	–
–	Uncultured *Rhodospirillaceae*	–
–	Uncultured *Steroidobacter*	–
–	*Xanthobacteraceae*	–

The yellow shading signifies sharing between the canola and peaola core microbiomes. The green shading signifies sharing between the pea and peaola core microbiomes. The blue shading signifies sharing between all three of the core microbiomes.

DISCUSSION

Yield, Land Equivalence Ratio, and N Response

During the 2020–2021 cropping season, the location of the winter peaola trials experienced extreme meteorological drought when compared with the 2019–2020 cropping season (**Figure 8**). The drought may be responsible for the overall reductions in yield between 2020 and 2021 harvests. In addition to the lack of precipitation, record-breaking temperatures were recorded during the last few weeks of June 2021 (28). The heat wave was coincidental with the flowering of the peas and may be partially responsible for the crop failure of the peas. Heat at flowering is known to reduce yield in both peas and canola (29). While yields for both peas and canola were reduced, the LER remained relatively stable increasing slightly from 2020 to 2021 (**Figure 2**). The increase in LER from 2020 to 2021 indicates that the intercropping may be less vulnerable to extreme weather events than the corresponding monocultures. In fact, if LER is used as the measurement of choice, the peaola systems may be slightly antifragile when compared to the monoculture cropping systems. The stability of LER over time has previously been noted as a feature of intercropping systems and appears to be a feature of peaola systems in the iPNW (2).

The peaola system did not appear to benefit from increasing the rates of synthetic N fertilizer in either 2020 or 2021. In

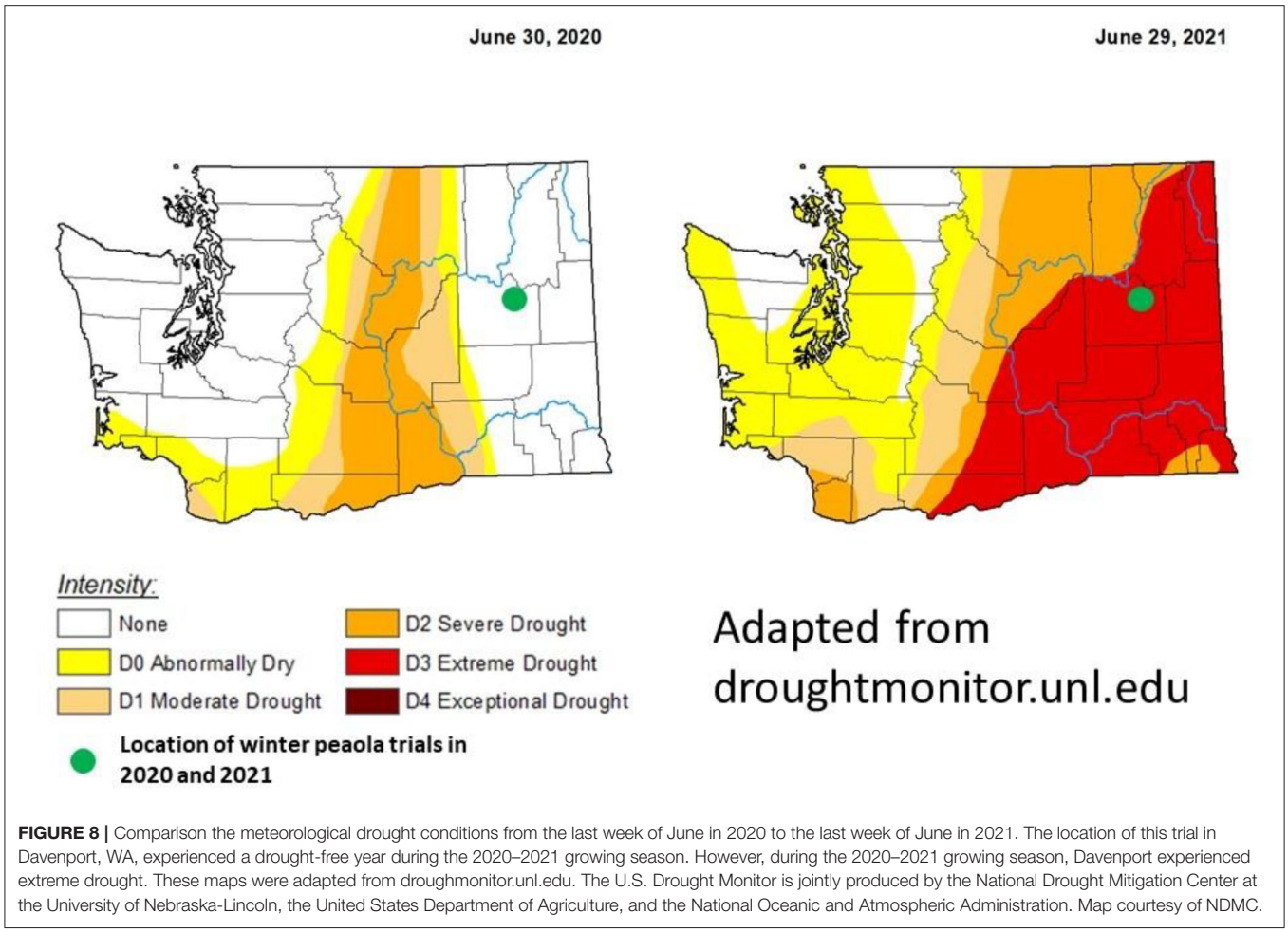


FIGURE 8 | Comparison the meteorological drought conditions from the last week of June in 2020 to the last week of June in 2021. The location of this trial in Davenport, WA, experienced a drought-free year during the 2020–2021 growing season. However, during the 2020–2021 growing season, Davenport experienced extreme drought. These maps were adapted from droughmonitor.unl.edu. The U.S. Drought Monitor is jointly produced by the National Drought Mitigation Center at the University of Nebraska-Lincoln, the United States Department of Agriculture, and the National Oceanic and Atmospheric Administration. Map courtesy of NDMC.

a review of legume-oilseed intercropping, Dowling et al. (14) included five studies which assessed the effect of N rate on LER of peaola intercropping systems and found that increasing N rate reduced LER in some instances. In some instances, increasing N rate has been shown to increase the relative yield of the canola while the LER of peaola decreases (15). However, the yield and LER data presented here showed that the N rate has no effect on LER, canola, and pea yield in the peaola treatments (**Table 1**).

The lack of a response to increasing N rate should not be interpreted as conclusive evidence that peaola negates the need for N fertilization. A positive crop response to fertilizer inputs is dependent on the fertilizer being a limiting factor in production. As noted above, in 2021, the crop yield was most likely limited by extreme weather events rather than N supply. However, the lack of a positive effect from N fertilizer in 2019–2020 requires further explanation. Previous studies conducted in the region have shown that monoculture winter canola does not always respond to increasing N applications in a manner that would be expected (30). The lack of response of canola to N in the iPNW may be due to deep soils, unaccounted for mineralization, and/or canola being an exceptional nutrient scavenger (30). This could be the reason why we did not see a response of canola to N fertilizer within the peaola cropping system in 2020. While these results indicate that peaola production would benefit relatively little from synthetic N additions, further research is required to conclusively demonstrate that peaola yield is not positively impacted by N inputs.

Future research may choose to address the potential for transfer of N from peas to canola to be able to determine if plant-plant-microbe interactions are responsible for the increased LER with decreased synthetic N inputs of peaola. Such research would likely require the use of stable isotopes. Regardless of whether the N is transferred from the peas to the canola during the peaola cropping year, incorporating peaola as opposed to monoculture canola should provide rotational N, thereby reducing the dependence of the entire cropping systems on synthetic N. The reduced need for synthetic N inputs in the peaola system will serve to increase the adaptive capacity of the overall cropping system to the economic and supply chain stress, which may impact the availability and cost of synthetic fertilizers.

The relative yield of peas to canola showed that the winter peaola systems did not strongly favor either pea or canola yield (**Figure 3**). The relative yields are calculated based on the monoculture checks and do not represent the yield of canola in relation to the pea yield. The relative yield allows for an analysis

of which species might be favored with a particular intercrop, which may have important implications on the economics of the system as legume and oilseed prices may move independently of each other. In a review of six peaola data sets, Fletcher et al. (2) found that in dramatically overyielding peaola crops, peas were favored over canola. However, the data presented here align with most of the peaola studies reviewed in that the systems has an overall LER of 1.63 and does not strongly favor either peas or canola (2).

Insect Class and Abundance

The abundance of insects by class was shown to shift based on intercropping at Colfax in 2020. Peaola was shown to have significantly greater numbers of beneficial insects among the monoculture systems. Meanwhile, both the canola and the peaola had significantly lower herbivores than the pea monoculture. Whether or not these shifts in populations result in higher economic thresholds for pest insects in peaola over peas cannot be determined from these data. However, future work should look at developing the economic thresholds for insecticide applications in peaola as compared to canola and peas.

Soil Microbial Community Analysis

Considering that we saw no significant differences between the diversity of the peaola soil bacterial community and the soil bacterial communities of pea and canola, it can be concluded that intercropping did not increase the diversity of the soil bacterial community. This is not surprising, however, since in previous studies done in other intercropping systems, only slight changes were observed in the soil bacterial community (17, 20). Despite the lack of significant differences in the diversity of the soil bacterial communities, we did see differences in the composition of the strict bacterial core microbiome. The peaola core microbiome appears to be influenced by both the pea and canola core microbiomes, as it consists of members from both. In addition, it also appears that the peaola core microbiome contains members not observed in the canola and pea core microbiomes, suggesting it could be producing a soil environment unique from what is created by canola and pea. This is supported by the findings of another study that found that the root exudates of intercropped plants differed from when they were grown individually (31). In addition, this provides evidence that under the peaola intercropping system, canola is potentially interacting with microorganisms that it does not normally associate with in monoculture.

To begin to test the hypothesis that canola can interact with microorganisms that are not normally available to it in monoculture, we will need to determine how the rhizosphere and root microbiomes are changing. In studies that have focused on how intercropping impacts the diversity of the bacterial community in the rhizosphere and root microbiomes, it has been found that they experience an increase in their diversity compared to their monoculture counterparts (17, 18, 20). This is likely due to the fact that the plant rhizosphere and root systems are more selective environments than the soil is. Therefore, it will be important for future work to investigate how the rhizosphere and root microbiomes are changing under this intercropping system to fully understand how the microbial community and function in peaola are impacted.

CONCLUSIONS

Peaola is a promising production strategy for the iPNW and other regions dominated by large-scale mechanized monoculture agriculture (2, 14). Peaola appears to consistently outyield the monoculture production systems on a land unit basis and does not appear to benefit from the addition of synthetic N. In a drought year (2021), the efficiency of peaola compared to the monocultures on a land basis exceeded that of peaola on a "typical" year (2020). Additionally, the peaola was found to have a different strict bacterial core microbiome and insect populations than either of the existing production systems. The effects of the shifts in the strict bacterial core microbiome are not fully understood at this time and should be explored further in the future. The increase in beneficial insects compared with the control may result in decreased insecticide applications through an increase in beneficial insects. Future research should be conducted to better understand the effects of the peaola cropping system on the function of the microbial community, the potential for reduced insecticide inputs, and the movement of N through the peaola system.

While not originally set forth as an objective, one of the most important findings from this trial is the role of peaola in apparent resistance to drought and heat stress. The data from 2021 highlight that in a year with drought and heat stress where one crop fails (peas), an intercropping scheme can provide a more productive system. Since extreme weather events cannot be easily predicted, planting intercrops can be considered a means of increasing adaptive capacity of the system or insuring potential loss. Previous research conducted on sunflower-soybean intercrops increasing moisture was shown to increase the LER (32). Future research should include the introduction of artificial drought and flooding to test the adaptive capacity of the peaola in comparison to the monoculture production system across a range of climate conditions. Continued research of this nature will serve to better understand the adaptive capacity of peaola under a wide range of environmental conditions.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI repository, accession number PRJNA832758, https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA832758.

AUTHOR CONTRIBUTIONS

IM designed and carried out the field trials as they relate to field agronomy, wrote the first draft of the manuscript, analyzed the yield, and LER data. MF and JP were responsible for

designing and carrying out the microbial sampling plan as well as analyzing the microbial data and writing the sections regarding the soil microbiology. RC was responsible for carrying out the entomological sampling conducting the data analysis on the entomological data. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported in part by the WSU Center for Sustaining Agriculture and Natural Resources BIOAg Program (USDA-NIFA #2020-67013-30864 and USDA-NIFA-SARE #GW21-228).

REFERENCES

1. Bybee-Finley KA, Ryan MR. Advancing intercropping research and practices in industrialized agricultural landscapes. *Agriculture*. (2018) 8:80. doi: 10.3390/agriculture8060080
2. Fletcher AL, Kirkegaard JA, Peoples MB, Robertson MJ, Whish J, Swan AD. Prospects to utilise intercrops and crop variety mixtures in mechanised, rain-fed, temperate cropping systems. In: *Crop and Pasture Science, Vol. 67*. CSIRO (2016). p. 1252–67. doi: 10.1071/CP16211
3. Maaz T, Wulfhorst JD, McCracken V, Kirkegaard J, Huggins DR, Roth I, et al. Economic, policy, and social trends and challenges of introducing oilseed and pulse crops into dryland wheat cropping systems. *Agric Ecosyst Environ.* (2018) 253:177–94. doi: 10.1016/j.agee.2017.03.018
4. Kruger C, Allen E, Abatzogou J, Rajagopalan K, Kirby E. *Advances in Dryland Farming in the Inland Pacific Northwest*. Washington State University Extension (2017). Available online at: http://pubs.cahnrs.wsu.edu/publications/pubs/em108/
5. Schillinger WF. New winter crops and rotations for the Pacific Northwest low-precipitation drylands. *Agron J.* (2020) 112:3335–49. doi: 10.1002/agj2.20354
6. Maaz T, Pan W, Hammac W. Influence of soil nitrogen and water supply on canola nitrogen use efficiency. *Agron J.* (2016) 108:2099–109. doi: 10.2134/agronj2016.01.0008
7. Schillinger WF, Paulitz TC. Canola versus wheat rotation effects on subsequent wheat yield. *Field Crops Res.* (2018) 223:26–32. doi: 10.1016/j.fcr.2018.04.002
8. Williams JD, Reardon CK, Wuest SB, Long DS. Soil water infiltration after oilseed crop introduction into a Pacific Northwest winter wheat-fallow rotation. *J Soil Water Conserv.* (2020) 75:739–45. doi: 10.2489/jswc.2020.00165
9. Hansen JC, Schillinger WF, Sullivan TS, Paulitz TC. Rhizosphere microbial communities of canola and wheat at six paired field sites. *Appl Soil Ecol.* (2018) 130:185–93. doi: 10.1016/j.apsoil.2018.06.012
10. Hansen JC, Schillinger WF, Sullivan TS, Paulitz TC. Soil microbial biomass and fungi reduced with canola introduced into long-term monoculture wheat rotations. *Front Microbiol.* (2019) 10:1488. doi: 10.3389/fmicb.2019.01488
11. Agboola A, Fayemi A. Fixation and excretion of nitrogen by tropical legumes. *Agron J.* (1972) 64:409–12. doi: 10.2134/agronj1972.00021962006400040001x
12. Qin M, Zhang Q, Pan J, Jiang S, Liu Y, Bahadur A, et al. Effect of arbuscular mycorrhizal fungi on soil enzyme activity is coupled with increased plant biomass. *Euro J Soil Sci*. (2019) 71:84–92. doi: 10.1111/ejss.12815
13. Verzeaux J, Hirel B, Dubois F, Lea P, Tétu T. Agricultural practices to improve nitrogen use efficiency through the use of arbuscular mycorrhizae: basic and agronomic aspects. *Plant Sci.* (2017) 264:48–56. doi: 10.1016/j.plantsci.2017.08.004
14. Dowling A, Sadras OV, Roberts P, Doolette A, Zhou Y, Denton MD. Legume-oilseed intercropping in mechanised broadacre agriculture – a review. *Field Crops Res*. (2021) 26:107980. doi: 10.1016/j.fcr.2020.107980
15. VanKoughnet B. On-Farm evaluation of peaola intercropping. In: *Manitoba Pulse and Soybean Growers*. Agri Skills Inc. (2016). Available online at: https://manitobapulse.ca/wp-content/uploads/2018/02/On-Farm-Evaluation-of-Peola-2016.pdf
16. Verbruggen E, Kiers E. Evolutionary ecology of mycorrhizal functional diversity in agricultural systems: AMF in agriculture. *Evol Applic.* (2010) 3:547–60. doi: 10.1111/j.1752-4571.2010.00145.x
17. Bargaz A, Noyce G, Fulthorpe R, Carlsson G, Furze J, Jensen E, et al. Species interactions enhance root allocation, microbial diversity and P acquisition in intercropped wheat and soybean under P deficiency. *Appl Soil Ecol.* (2017) 120:179–88. doi: 10.1016/j.apsoil.2017.08.011
18. Cao X, Luo J, Wang X, Chen Z, Liu G, Khan M, et al. Responses of soil bacterial community and Cd phytoextraction to a sedum alfredii-oilseed rape (*Brassica napus* L. and *Brassica juncea* L.) intercropping system. *Sci Total Environ.* (2020) 723:138152. doi: 10.1016/j.scitotenv.2020.138152
19. Granzow S, Kaiser K, Wemheuer B, Pfeiffer B, Daniel R, Vidal S, et al. The effects of cropping regimes on fungal and bacterial communities of wheat and faba bean in a greenhouse pot experiment differ between plant species and compartment. *Front Microbiol.* (2017) 8:902. doi: 10.3389/fmicb.2017.00902
20. Mwakilili A, Mwaikono K, Herrera S, Midega C, Magingo F, Alsanius B, et al. Long-term maize-Desmodium intercropping shifts structure and composition of soil microbiome with stronger impact on fungal communities. *Plant Soil*. (2021) 467, 437–50. doi: 10.21203/rs.3.rs-221776/v1
21. Zheng B, Zhang X, Chen P, Du Q, Zhou Y, Yang H, et al. Improving maize's N uptake and N use efficiency by strengthening roots' absorption capacity when intercropped with legumes. *PeerJ*. (2021) 9:e11658. doi: 10.7717/peerj.11658
22. Weiss MJ, McLeod P, Schatz BG, Hanson BK. Potential for insecticidal management of flea beetle (*Coleoptera: Chrysomelidae*) on canola. *J Econ Entomol.* (1991) 84:1597–603. doi: 10.1093/jee/84.5.1597
23. Buntin DG, Raymer PL. Pest status of aphids and other insects in winter canola in Georgia. *J Econ Entomol.* (1994) 87:1097–104. doi: 10.1093/jee/87.4.1097
24. Silva JHC, Saldanha AV, Carvalho PMR, Machado CFM, Flausino BF, Antonio AC. The interspecific variation of plant traits in brassicas engenders stronger aphid suppression than the intraspecific variation of single plant trait. *J Pest Sci*. (2022) 9:723–34. doi: 10.1007/s10340-021-01421-z
25. Jones GA, Gillett JL. Intercropping with sunflowers to attract beneficial insects in organic agriculture. *Flen.* (2005) 88:91–6. doi: 10.1653/0015-4040(2005)088[0091:IWSTAB]2.0.CO;2
26. Ponti L, Altieri MA, Gutierrez AP. Effects of crop diversification levels and fertilization regimes on abundance of *Brevicoryne brassicae* (L.) and its parasitization by *Diaeretiella rapae* (M'Intosh) in broccoli. *Agric Forest Ent.* (2007) 9:209–14. doi: 10.1111/j.1461-9563.2007.00330.x
27. Mead R, Willey RW. The concept of a 'land equivalent ratio' and advantages in yields from intercropping. *Exp Agric.* (1980) 16:217–28. doi: 10.1017/S0014479700010978
28. Ansah EO, Walsh OS. Impact of 2021 drought in the Pacific Northwest. *Crops Soils.* (2021) 54:46–9. doi: 10.1002/crso.20145
29. Mohapatra C, Chand R, Tiwari JK, Singh AK. Effect of heat stress during flowering and pod formation in pea (*Pisum sativum* physiology L). *Mol Biol Plants.* (2020) 26:1119–25. doi: 10.1007/s12298-020-00803-4
30. Porter MJ, Pan WL, Schillinger WF, Madsen IJ, Sowers KE, Tao H. Winter canola response to soil and fertilizer nitrogen in semiarid mediterranean conditions. *Agron J.* (2020) 112:801–14. doi: 10.1002/agj2.20119
31. Badri D, De-la-Peña C, Lei Z, Manter D, Chaparro J, Guimarães R, et al. Root secreted metabolites and proteins are involved in the early events of plant-plant recognition prior to competition. *PLoS ONE.* (2012) 7:e46640. doi: 10.1371/journal.pone.0046640
32. Andrade JF, Cerrudo A, Rizzalli RH, Monzon JP. Sunflower-soybean intercrop productivity under different water conditions and sowing

managements. *Agron J.* (2012) 104:1049–55. doi: 10.2134/agronj2012.0051

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Soil Science

TYPE Original Research
PUBLISHED 02 August 2022
DOI 10.3389/fsoil.2022.869136

OPEN ACCESS

EDITED BY
Nigel Victor Gale,
University of Toronto, Canada

REVIEWED BY
Carlos Garcia,
Spanish National Research Council (CSIC), Spain
Stephani Ann Yarwood
University of Maryland, United States
Paulo Roger Lopes Alves,
Universidade Federal da Fronteira Sul, Brazil
Leandro Nascimento Lemos,
National Laboratory for Scientific Computing (LNCC), Brazil

*CORRESPONDENCE
Jeanette M. Norton
jeanette.norton@usu.edu

SPECIALTY SECTION
This article was submitted to Soil Biology, Ecosystems and Biodiversity, a section of the journal Frontiers in Soil Science

RECEIVED 03 February 2022
ACCEPTED 28 June 2022
PUBLISHED 02 August 2022

CITATION
Ouyang Y, Reeve JR and Norton JM (2022) The quality of organic amendments affects soil microbiome and nitrogen-cycling bacteria in an organic farming system.
Front. Soil Sci. 2:869136.
doi: 10.3389/fsoil.2022.869136

The quality of organic amendments affects soil microbiome and nitrogen-cycling bacteria in an organic farming system

Yang Ouyang[1,2], Jennifer R. Reeve[1] and Jeanette M. Norton[1]*

[1]Department of Plants, Soils and Climate, Utah State University, Logan, UT, United States, [2]Department of Microbiology and Plant Biology, Institute for Environmental Genomics, University of Oklahoma, Norman, OK, United States

Organic amendments are applied in organic farming systems to provide nutrients for crop uptake and to improve soil health. Compost is often favored over fresh manure for food safety reasons, while fresh manure can be a valuable source of readily available nitrogen (N). However, the potential for fresh versus composted manure to differentially affect soil microbial and N-cycling functional communities over multiple seasons remains unknown. We compared the effect of composted vs. fresh cattle manure on soil microbial communities using taxonomic and functional approaches. Soils were collected from field plots with three organic N treatments: control (no amendment), composted manure (compost, 224 kg/ha total N), and fresh manure (manure, 224 kg/ha total N) in an organic production system. Illumina amplicon sequencing was used to comprehensively assess the bacterial community (16S rRNA genes), fungal community (ITS), ureolytic community (*ureC*), chitinolytic community (*chiA*), bacterial ammonia oxidizers (AOB *amoA*), and nitrite oxidizers (*Nitrospira nxrB*). The results showed that both compost and manure treatment significantly changed the soil microbial communities. Manure had a stronger effect than compost on soil bacterial and fungal community composition, as well as on the ureolytic and chitinolytic communities, while compost treated soils had higher microbial richness than manure treated soils. Both taxonomic and functional approaches showed that the microbial community was more responsive to fresh manure than to compost. Manure treated soil also had more complex microbial interactions than compost treated soil. The abundance and community composition of N-cycling functional groups often played more limited roles than soil chemical properties (soil organic carbon, extractable organic carbon, and pH) in driving N-cycling processes. Results from our study may guide strategies for the management of organic amendments in organic farming systems and provide insights into the linkages between soil microbial communities and soil function.

KEYWORDS

organic nitrogen management, compost, manure, microbial community network analysis, ureC, chiA, amoA, nxrB

Introduction

Organic amendments are widely used in organic farming systems to supply nutrients for crop uptake and to improve soil health. Compost is often favored over fresh manure for food safety reasons as well as for ease of transportation and application due to its reduced moisture content and lower vitality of weed seeds. However, fresh manure can be a valuable source of readily available N in organic farming systems and hence is often valued by farmers especially when applied to nutrient demanding crops such as corn. The release of nutrients from organic amendments is largely mediated by soil microbes and their enzymes. A number of studies have found that compost and/or manure have altered soil microbial communities (1–5), while others have shown no or little effect (6, 7). This variability in findings is likely due to the type, rate and frequency of the compost and manure that were applied (5, 8). However, few studies have directly compared the effect of fresh manure versus compost side by side, especially with the same total nitrogen (N) application rate, on the soil microbial communities in organic farming systems over multiple years. For the same total N application rate, manure often has higher N availability, but less overall material is applied than with compost. Differences between manure and compost in carbon (C) and N content and their availability may result in contrasting impacts on soil microbial communities. Improving management of organic amendments for a desirable response in soil microbiomes and their nutrient cycling processes is a long-term goal for sustainable agriculture.

The transformation of organic polymers to biologically available N forms is largely controlled by N mineralization and nitrification. N mineralization converts organic polymers to monomers or ammonium (9), while nitrification transforms ammonium to nitrite or nitrate (10). Numerous microbial enzymes are involved in N mineralization (11). Previous studies have consistently showed that organic amendments increased the activities of soil enzymes involved in N mineralization (reviewed in Luo et al., 2018 (12)). However, few studies have examined the effect of organic amendments on the diversity of genes encoding these key N mineralization enzymes (2, 13, 14). Several studies have assessed the effect of organic fertilization on the communities of nitrifiers, but the results are controversial (15–18). This is probably due to the differences in rates, quality, and duration of organic fertilization. The current study examines the effect of fresh steer manure versus composted cattle manure on select N mineralizers and nitrifiers.

Microbial co-occurrence networks provide critical insights into microbial associations (19–21). Network analysis has been widely used to examine the response of microbial associations to agricultural management and to identify putative keystone species (22–25). Several studies have revealed that organic fertilization increased the complexity of microbial interaction compared with mineral fertilization (22, 26). Compared with bacteria and fungi, it is less understood how different soil N-cycling functional groups interact and respond to the quality of organic fertilizers. Our study seeks to use an existing organic long-term rotational experiment with a robust experimental design to examine microbial community changes in response to organic fertilizer amendments.

The use of manures and composts is common on organic farms yet knowledge gaps in their microbial characteristics remain for their safe and sustainable use (27). Previous research at our site indicated fresh manure released more available N for plant uptake while composted manure increased soil organic C, with no differences in soil enzymatic activity involved in nutrient cycling detected (28). In this study, we measure bacterial and fungal community composition in soils treated with fresh manure and composted manure at the same total N application rate. We expected that soil microbial community compositions and their co-occurrence networks would be different between manure and compost treated soils. To complement our previous study, which demonstrated organic N fertilizers increased soil functions and functional gene abundances (28). we examined the community composition using four functional genes involved in N mineralization and nitrification. We hypothesized that the community compositions of these N-cycling functional groups would be changed by organic N fertilizers and that the community composition might play a role in prediction of the corresponding microbial process rates.

Methods

Site history and soil sampling

The field site and experiment design is a certified organic farming systems trial that was started in 2007 and has been previously described (28). In brief, the experiment consisted of three different rotations incorporating three different cover crops with or without fresh manure or composted manure in a completely randomized block with split split plot design. Cover crop (buckwheat (*Fagopyrum esculentum*, Moench), millet (*Pennisetum glaucum* L.), or black turtle bean (*Phaseolus vulgaris* L.)) was the main plot factor, crop rotation the sub plot factor and fertility treatment (compost, manure, nothing as control) the sub-sub plot factor, each with three replicates. In this study, we used the most intensive rotation: field corn (in 2007), potatoes (2008), dry beans (2009), sweet corn (2011), potatoes (2012), cover crops (2013), dry beans (2014), sweet corn (in 2015). In total, 27 plots were selected with three organic N amendment treatments, three cover crop treatments, and three replicates. A cover crop mixture of

winter wheat (*Triticum aestivum* L), and hairy vetch (*Vicia villosa* L.) was grown every year across all treatments over the winter and mowed and incorporated immediately prior to applying composted or fresh manure. Organic fertilizers were applied and immediately incorporated two weeks before planting in mid-June. Organic fertilizers were not applied to cover crops in 2013 and beans in 2014. For each fertilization, field plots received applications of 224 kg/ha total N either in the form of a commercial steer manure compost (compost), fresh cattle manure (manure) or no amendment (control). The basic characteristics of the compost and manure were previously described (28). Each year, manure was selected with a higher total N content than the compost so that manure plots received less overall material for the same total N application rate. The C: N ratio of the compost (1:12 to 1:16) was higher than the manure three years out of six but otherwise similar (see Supplementary Figure 1. for rotations and treatment timing).

Soil samples (0-15 cm) were collected in late Sep 2011 (after corn harvest), in Jul 2015 (six weeks after fertilization), and in early Oct 2015 (after corn harvest). Six soil cores were randomly taken from each plot, composited and thoroughly mixed, and about 10 g sample of soil was stored at -80°C immediately after soils were brought to the laboratory. Soil chemical and microbiological properties were measured and described in Ouyang *et al.* (28). In this study, we used Illumina amplicon sequencing to evaluate the overall prokaryotic and fungal communities in soils sampled in Sep 2011 and Oct 2015. Illumina amplicon sequencing was performed for four N-cycling genes in soils sampled in Jul 2015 (See Supplementary Figure 1).

Illumina sequencing and data processing for 16S rRNA genes and fungal ITS

Soil DNA was extracted using a MoBio PowerSoil DNA isolation kit (MoBio Laboratories Inc, Carlsbad, USA). DNA extracts were quantified by using the Quant-iT™ PicoGreen dsDNA BR Assay Kit (Invitrogen) according to the manufacturer's protocol. The variable V4-V5 region of the 16S ribosomal rRNA gene (16S) was amplified with 515F-Y (GTGYCAGCMGCCGCGGTAA) and 926R (CCGYCAAT TYMTTTRAGTTT) universal primers for the bacterial and archaeal community (29). The fungal internal transcribed spacer (ITS) was amplified with the primer pair ITS9F (GAACGCAGCRAAIIGYGA) and ITS948R (TCCTCCGC TTATTGATATGC). The amplicon sequencing was performed on an Illumina MiSeq instrument (Illumina Inc., San Diego, CA, USA). The Illumina raw reads were processed using a custom pipeline developed at the Joint Genome Institute (JGI) (https://bitbucket.org/berkeleylab/jgi_itagger). Briefly, raw reads were first quality-filtered, and then the high quality sequences were clustered into amplicon sequence variants (ASV) based on zero-radius operational taxonomic units (OTU) using the USEARCH pipeline (30). Singletons were removed. Taxonomies were assigned to each ASV using the RDP Classifier with a confidence threshold of 0.60 (31). Maximum-likelihood phylogenetic trees were constructed from representative sequences using FastTree with default parameters (32). All data files were then organized using R package *phyloseq* (33). Sequence data can be accessed from JGI data portal at https://data.jgi.doe.gov/ and searching for Utah Georgia Soil iTags (plate 1 and plate 2 are available).

Illumina sequencing and data processing for four N-cycling genes

Amplicon sequencing of four N-cycling genes was accomplished for soils sampled in July 2015 (six weeks after fertilization). Four N-cycling genes were *ureC* (encoding urease), *chiA* (encoding chitinase), bacterial *amoA* (encoding ammonia monooxygenase) and *Nitrospira nxrB* (encoding nitrite oxidoreductase). The primer sets and library preparation of Miseq sequencing were the same as our previous studies (2, 16). We tried to design new archaeal *amoA* primers with amplicon length less than 500bp, but the primer sets failed to amplify archaeal *amoA* in our soils. The sequencing data was processed using RDPTools (34) and USEARCH (30). Briefly, raw forward and reverse reads were merged using the USEARCH workflow (30). High quality sequences were extracted from merged reads in each sample using the RDP SeqFilters with a read Q score cutoff of 20 (34). Chimera sequences were detected and removed using UCHIME (35). The obtained sequences were further processed using the FrameBot tool (36) to fix frame shifts. Sequences were dereplicated and clustered at 90% nucleotide similarity using USEARCH. Singletons were removed. To obtain the phylum-level classification of representative sequences for *ureC* and *chiA*, the taxonomy from the closet matches to the protein reference downloaded from the FunGene were used (37). If the percent identity to the reference sequences was less than 80%, we defined the phylum as unclassified. A maximum-likelihood phylogenetic tree was constructed from representative sequences using FastTree with default parameters (32). OTU table and taxonomy files were further organized for diversity analysis using R package *phyloseq* (33). The Sequence Read Archive for the amplicon sequencing is available through Bioproject PRJNA804976 at https://www.ncbi.nlm.nih.gov//bioproject/PRJNA804976.

Network analysis

We aimed to examine the effect of organic fertilizers on microbial network properties. Three networks were constructed for each organic N treatment. Cover crop treatments and samples in 2011 and 2015 were pooled together in each organic N treatment. Therefore, there were 18 replicates in

each N treatment. To ensure network reliability, mitigate temporal variance, and compare organic fertilizer, only bacterial and fungal ASVs detected in all 54 samples were used for each network construction. Finally, 854 bacterial and 25 fungal ASVs were used for network construction. The networks were built using the pipeline of Molecular Ecological Network Analyses (MENA) (http://ieg4.rccc.ou.edu/mena/) (38). Briefly, pairwise similarities of ASVs were calculated based on Spearman correlation coefficients. The random matrix theory was applied to identify the similarity threshold before network construction (19, 38). Network topological features were calculated by the MENA pipeline, including the total node number, total link number, average degree, average cluster coefficient, average path distance, and modularity. Network modules were identified by the fast-greedy modularity optimization. Detailed definitions of these network features were described previously (39, 40). To randomize network in each organic N treatment, a total 100 randomly rewired networks were generated (19). To test if there were differences in network topological characteristics among the three organic N treatments, one-way ANOVA was performed by using averages and standard deviations of their corresponding 100 random networks. Putative keystone taxa were also identified based on network hubs, module hubs, and connectors (38). All networks were visualized using Gephi (41).

To evaluate the effect of organic N fertilizers on the network properties of N-cycling genes, OTUs presented in all soil samples were obtained to build three networks for each organic N treatment. There were 595 OTUs, 40 OTUs, ten OTUs, and seven OTUs for *ureC*, *chiA*, *amoA*, and *nxrB*, respectively. Nine replicates regardless of the cover crop treatments were pooled together to construct the network in each organic N treatment, since cover crop type had no effect on the microbial communities of N-cycling functional groups. The same procedure as above was used to generate networks. All networks with a focus on the linkage among four N-cycling genes were visualized using Cytoscape (42).

Statistical analysis

All statistical analyses were conducted in R software (https://www.R-project.org). For 16S and ITS sequencing data, we had a very high sequencing depth. The retained high-quality sequences were randomly resampled to a depth of 141,248 and 108,584 reads per sample for 16S and ITS, respectively. For four N-cycling genes, the retained high-quality sequences were randomly resampled to a depth of 20000, 6000, 540, and 1400 reads per sample for ureC, chiA, bacterial amoA, and Nitrospira nxrB, respectively. The rarefaction curves showed that these normalized reads were sufficient to capture the diversity of these four N-cycling genes. Alpha diversity and beta diversity of the microbial communities were then calculated. Nonmetric multidimensional scaling (NMDS) and PERMANOVA were conducted to visualize and assess the Weighted UniFrac distance matrices using the R package vegan version 2.5-6 (43). Canonic correspondence analysis (CCA) was used to evaluate relationships between chemical properties and soil structural and functional microbial communities. Soil chemical properties included soil organic carbon (SOC), total N, pH, extractable organic C (EOC), extractable organic N (EON), amino acids, ammonium, and nitrate. Forward selection of soil chemical properties was performed to determine a parsimonious set of explanatory variables. To evaluate the effect of organic N fertilization on the relative abundances of ASVs or OTUs, the fold changes of ASVs or OTUs in compost or manure versus the control was calculated using the R package DESeq2 (44). The p values were adjusted using the Benjamini-Hochberg correlation method (44). The analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) were used to characterize the statistical significance of the differences among organic N treatments.

Several analyses were performed to evaluate the relative importance of soil chemical properties and functional genes in controlling the rates of selected soil N-cycling processes in soil. Four selected N-cycling processes were urease activity (i.e. urea hydrolysis), N-acetyl β-glucosaminidase (NAGase) activity, octyne-sensitive nitrification potential (nitrification potential contributed by AOB, NP_{AOB}), and potential nitrite oxidation (PNO). Firstly, the Mantel test was used to evaluate the relationships between the community composition of N-cycling functional groups and their corresponding process rates. Secondly, Random Forest analysis was used to explore the importance of each soil chemical parameter and the abundance and community of N-cycling functional groups for explaining selected N-cycling process rates. We used NMDS1 to represent the community composition of N-cycling functional groups. The Random Forest analysis was conducted using the R package *randomForest*. The significance of each predictor on N-cycling process rates was assessed with the R package *rfPermute*. Lastly, structural equation modeling (SEM) was used to evaluate the direct or indirect effects of soil chemical properties and the abundance and diversity of N-cycling groups on selected N-cycling process rates. To simplify the SEM, only three soil chemical factors (SOC, EOC, and pH) were included in the model, since the Random Forest analysis showed that these three factors were all significant for predicting the four N-cycling process rates. We first generated a full model that included all reasonable pathways, and then sequentially eliminated nonsignificant pathways. The $\chi 2$ test and the root mean square error of approximation were used to evaluate the fit of our model. The SEM analysis was performed using the R package *lavaan*.

Results

Similar to the results for soil basic properties and soil function in our previous study (Supplementary Table 1; 28),

we found that cover crop type had no effect on the compositions of bacterial and fungal communities, and selected N-cycling functional groups (Supplementary Tables 2, 3). Therefore, we focused on the effects of organic N treatment only for this study.

Bacterial and fungal community compositions

Both organic N treatment and year significantly changed soil bacterial (R^2_N = 0.21, $p < 0.001$; R^2_{year}=0.30, $p < 0.001$) and fungal (R^2_N = 0.14, $p < 0.001$; R^2_{year}=0.10, $p < 0.001$) community composition (Figure 1 and Supplementary Table 2). Pairwise comparison indicated that the three organic N treatments were different from each other in the soil bacterial and fungal community composition. Overall, manure had a stronger impact than compost on the soil bacterial and fungal community composition, based on their dissimilar distances to the control soil. Organic N treatment ($p < 0.001$) and year ($p < 0.001$) also significantly influenced the alpha diversity of the soil bacterial community (Supplementary Figure 2A). Specifically, compost treatment increased richness, Chao1, and Shannon diversity in both 2011 and 2015. For the soil fungal community, compost treatment increased the alpha diversity in 2011, but organic N treatment had no effect on the alpha diversity in 2015 (Supplementary Figure 2B).

Responsive bacterial and fungal taxa to organic N fertilizers

Organic N fertilizers significantly changed many bacterial taxa. Five of 12 abundant bacterial phyla (> 1%) were significantly changed by organic N treatment. Organic N fertilizers increased *Proteobacteria* and *Bacteroidetes*, but decreased *Acidobacteria*, *Chloroflexi*, and *Gemmatimonadetes* in both 2011 and 2015 (Figure 2A). Overall, these five responsive phyla showed no difference between compost and manure treatments. Only *Chloroflexi* in the manure treatment was significantly lower than the compost treatment in 2015. Numerous bacterial ASVs were enriched (16.19%) or depleted (12.68%) by organic N fertilizers (Figure 2B). Most of these responsive ASVs belonged to *Proteobacteria*, *Bacteroidetes*, *Planctomycetes*, *Chloroflexi*, *Actinobacteria*, and *Acidobacteria*. Manure treated soil had more numbers of responsive ASVs than compost treated soil in both 2011 and 2015 (Supplementary Figure 3A). In both compost and manure treated soils, there were higher numbers of responsive ASVs in 2015 than in 2011. Many responsive ASVs were shared between compost and manure treated soils (50-61%), or between 2011 and 2015 (36-42%). Both the numbers and $\log_2$-fold change values of responsive ASVs responded more positively than negatively to compost and manure treatment (Supplementary Figure 3B).

Compared with bacteria, fungal taxa were less responsive to organic N fertilizers. At the phylum level, we found that *Ascomycota* and *Zygomycota* were significantly affected by organic N treatment (Supplementary Figure 4A). Specifically, manure treatment significantly decreased *Ascomycota* in 2011, and compost treatment significantly increased *Zygomycota* in 2015 compared with control. There were also fewer fungal responsive ASVs than bacterial responsive ASVs (Supplementary Figure 4B). In 2011, there were only 24 and 35 responsive ASVs in compost and manure treatments, respectively. In 2015, there were 45 and 57 responsive ASVs in compost and manure treatments, respectively. Most of these responsive ASVs were enriched by organic N treatment.

Microbial network

Using bacterial and fungal ASVs present in all soil samples, we built networks for each organic N treatment. These three networks were non-random and scale-free with power-law R^2 ranging from 0.84 to 0.88 (Table 1). Compared with the control, networks in compost and manure treatments had higher total nodes, total links, numbers of modules, and average degree (Table 1 and Figure 3). Based on 100 randomized networks, average path distance was lowest in the manure treatment, medium in compost treatment and highest in control treatment ($p < 0.001$). Compost and manure treatments also had significant higher average clustering coefficients than the control ($p < 0.001$). We also calculated the percentage of negative correlations and found that compost and manure treatments had higher negative interactions than the control (Table 1).

Putative keystone taxa were also identified by the network analysis (Supplementary Table 4). We only identified one connector in the control network, belonging to *Verrucomicrobia*. There were six connectors and five module hubs in the compost network. Most of these connectors and module hubs were in *Acidobacteria*. In the manure network, there were 22 connectors and three module hubs, mainly belonging to *Protobacteria*, *Acidobacteria*, and *Planctomycetes*. Module hubs and connectors were largely from bacteria, and we identified only one fungal connector (*Chytridiomycota*) in the compost network. The relative abundance of module hubs and connectors ranged from 0.02% to 0.9%. Notably, most of module hubs and connectors were unclassified at the genus level. We also evaluated the response of module hubs and connectors to organic N fertilization using the $\log_2$-fold change approach, but no clear pattern was identified.

Community compositions of N-cycling functional groups

Four N-cycling genes were sequenced for soils sampled in July 2015, which was about six weeks after compost and manure

10.3389/fsoil.2022.869136

TABLE 1 Topological features of empirical networks and random networks for combined bacterial and fungal communities.

Network features	Control	Compost	Manure
Empirical networks			
R squared of power-law	0.87	0.88	0.84
Total nodes	118	333	319
Total links	142	852	947
No. of modules	19	45	50
Average degree (avgK)	2.19	5.12	5.94
Average path distance (GD)	6.2 c	4.3 b	3.74 a
Average clustering coefficient (avgCC)	0.21 a	0.30 b	0.34 b
Modularity	0.79	0.54	0.48
Random networks			
Average path distance (GD)	4.71 ± 0.19	3.83 ± 0.07	3.49 ± 0.08
Average clustering coefficient (avgCC)	0.03 ± 0.01	0.18 ± 0.01	0.22 ± 0.01
Modularity	0.67 ± 0.01	0.50 ± 0.01	0.42 ± 0.01
Negative interactions	32.88%	40.79%	42.50%

Lowercase letters indicate significant differences among organic N treatments ($p < 0.05$).

application. Based on 90% nucleotide cutoff, there were 7001, 3658, 34, and 91 OTUs for *ureC*, *chiA*, bacterial *amoA*, and *Nitrospira nxrB*, respectively. Organic amendments significantly changed the community composition for N-cycling functional groups as indicated by *ureC*, *chiA* and AOB *amoA* but not for *nxrB* amplicon sequencing, (Figure 4; Supplementary Table 3). Pairwise PERMANOVA further showed that the community compositions of *ureC* ($p<0.001$) and *chiA* ($p=0.018$) were significantly different between manure and compost treatments. The CCA indicated that SOC, nitrate, EOC, and pH were often significantly associated with the community composition of N-cycling functional guilds (Supplementary Figure 5). We also built networks for N-cycling functional groups in each N treatment (Supplementary Figure 6 and Supplementary Table 5). Overall, compost treatment had the most complex network, mainly due to the higher interaction in the ureolytic community. Interestingly, both compost and manure treatments increased links among the four different N-cycling functional groups compared with control.

Organic N fertilizers also changed the N-cycling functional groups at both phylum and OTU level. In the ureolytic community, the majority of OTUs were assigned to *Proteobacteria* and *Actinobacteria* (Supplementary Figure 7A). *Proteobacteria* and *Verrucomicrobia* were increased, but *Nitrospirae* were decreased by compost and manure treatments. There was no difference between compost and manure treatments at the phylum level. Using the $\log_2$ fold-change approach, we identified 707 and 858 responsive OTUs in the compost and manure treatments (475 OTUs were shared), respectively (Supplementary Figure 7B). Most of these responsive OTUs were from *Proteobacteria* (Supplementary Figure 7C). In the chitinolytic community, the majority of OTUs were assigned to *Actinobacteria* or unclassified (Supplementary Figure 8). Compared with control and manure treatments, compost treatment significantly increased *Actinobacteria*. We also identified more responsive OTUs in the manure treatment (103 OTUs) than compost treatment (65 OTUs). In the AOB community, we identified three and seven responsive OTUs in the compost and manure treatments, respectively. The *Nitrospira* community was less responsive to organic N treatment at the OTU level, compared with other N cycling groups. We only detected three responsive OTUs (one in compost treatment and two in manure treatment).

Several approaches were used to evaluate the relative importance of soil geochemical properties and functional genes on soil processes. First, the Mantel test indicated that the community composition of three N-cycling functional groups were significantly correlated with its corresponding process (R_{ureC}=0.60, $p < 0.001$; $R_{chiA} = 0.27$, $p = 0.002$; $R_{AOB} = 0.28$, $p = 0.001$) but not that of nxrB;($R_{nxrB} = 0.04$, $p = 0.26$). However, the Random Forest analysis indicated that SOC, pH, EOC, and nitrate pools played significant roles in the N-cycling processes (Table 2). The abundance and community composition of N-cycling functional groups were often not important in explaining the N-cycling processes. We only found that the *ureolytic* community was significantly associated with urease activity. Similarly, the SEM analysis showed that EOC and pH often directly impacted the abundance of N-cycling genes and N-cycling processes (Figure 5). We found that the AOB community significantly influenced the activity of AOB ($p = 0.02$) while the abundance and community composition of the other three N-cycling functional groups often played limited roles in explaining their associated N-cycling processes.

Discussion

Organic amendments improve nutrient cycling and soil health in organic farming systems (45, 46). In this study, we

10.3389/fsoil.2022.869136

TABLE 2 Random forest mean predictor importance (percentage of increase of mean square error) of soil chemical properties and the abundance and community of N-cycling functional groups as drivers of their corresponding N process rates.

Predictors	Urease	NAGase	NP_{AOB}	PNO
SOC	3.76**	6.50*	2.51**	4.59*
Total N	0.70	3.52	0.33	1.78
pH	3.35*	7.13**	2.22**	9.20**
EOC	4.70**	10.64**	2.56**	7.81**
EON	1.46	5.21*	2.11**	2.75
Amino acid	-0.28	0.7	-0.06	-0.44
Ammonium	-0.19	-0.06	0.06	-0.34
Nitrate	6.00**	4.34*	1.62*	15.72***
Abundance	0.66	0.19	0.53	0.14
Community	6.03**	1.52	0.03	-0.07

Four selected N-cycling processes are urease, β-glucosaminidase (NAGase), octyne-sensitive nitrification potential (nitrification potential contributed by AOB, NP_{AOB}), and potential nitrite oxidation (PNO). NMDS1 is used to represent the community composition of N-cycling functional groups. Percentage increases in the MSE (mean squared error) of variables are used to estimate the importance of these predictors, and higher MSE% values imply more important predictors. Asterisks highlight significant p values (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). Abbreviation: SOC, soil organic C; EOC, extractable organic C; EON, extractable organic N.

compare the effect of organic amendments with different qualities (e.g. composted vs. fresh manure) on soil function and microbial communities in an organic farming systems. Our current and previous studies have comprehensively examined soil chemical properties, soil enzyme activities and process rates, soil bacterial and fungal communities, and the abundance and diversity of N-cycling genes under organic amendments with different qualities (28). We found that organic amendments improved these soil chemical and microbial properties, compared with the control soil. Compost and manure treatments often had similar enzyme activities and abundance of N-cycling genes, while manure treatment had higher N transformation rates than compost (28). This current study further demonstrates that manure treatment had a stronger effect than compost treatment on bacterial and fungal communities, as well as on ureolytic and chitinolytic communities. Although the compost treated soils had higher microbial richness than manure treated soils, both taxonomic and functional microbial groups were more responsive to manure than compost. Manure treated soils also had more complex microbial interactions than compost treated soils. Overall, our study suggests that manure treatment has a stronger influence than compost treatment on structural and functional community composition in this organic farming system.

Organic fertilization affected bacterial and fungal community compositions

Interestingly, we found that compost and manure treatments exerted a contrasting impact on the alpha and beta diversity of

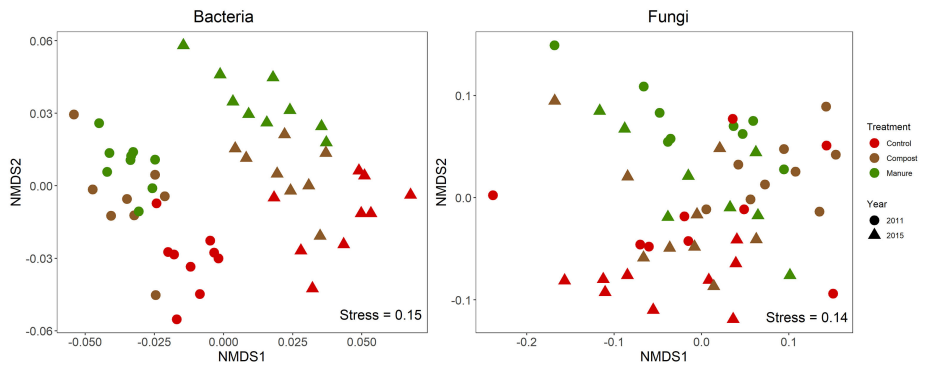


FIGURE 1
Nonmetric multidimensional scaling (NMDS) ordination of the weighted UniFrac distance for soil bacterial and fungal communities. PERMANOVA indicates significant effect of organic N treatment and Year on both bacterial and fungal community compositions ($p < 0.001$).

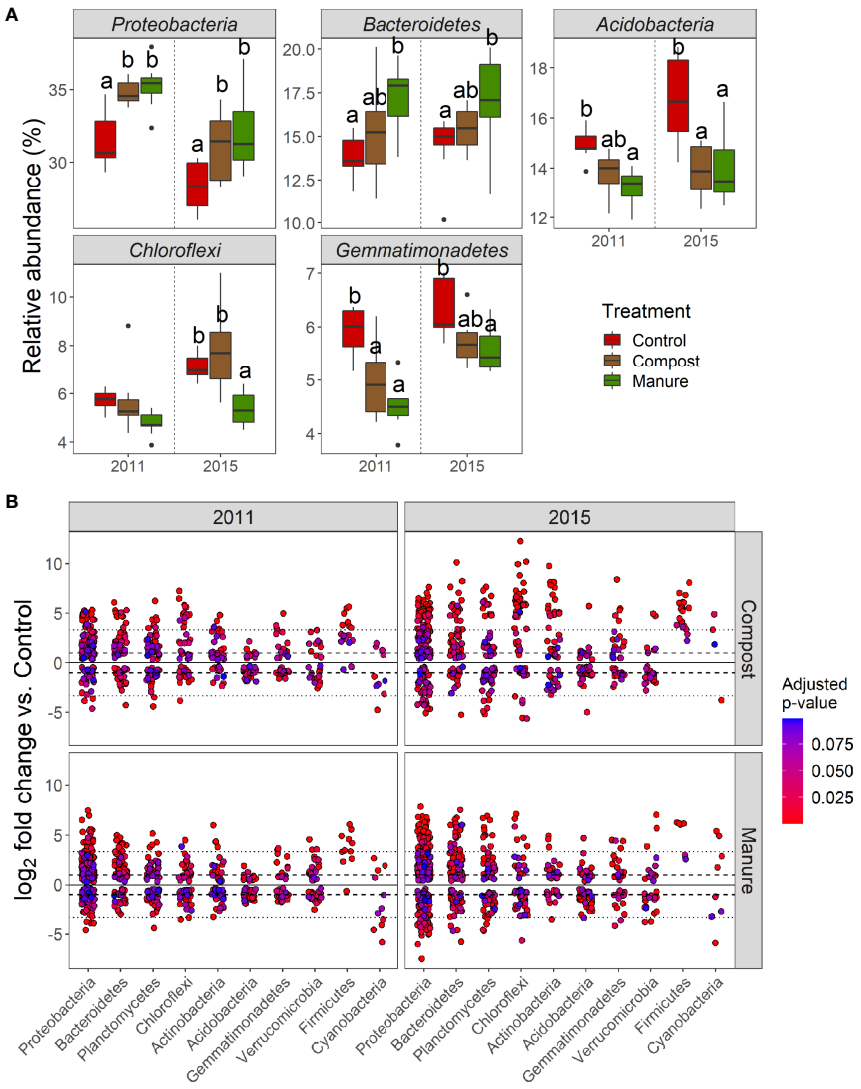


FIGURE 2
(A) Relative abundance of several bacteria phyla (> 1%) are significantly different among organic N treatments ($p < 0.05$, ANOVA). Lowercase letters indicate significant differences among organic N treatment in a specific year. **(B)** Relative abundance changes (log_2-fold change) of bacterial ASVs in soils treated with compost and manure compared with the control soil. Each circle represents a single bacterial ASV with an adjusted p value of < 0.1. Dashed line: 2-fold change. Dotted lines: 10-fold change.

soil microbial communities. Compost treated soils had a higher microbial alpha diversity than manure treated soils, while manure treatment had a stronger effect on the bacterial and fungal community compositions. In our study, fresh cattle manure was selected with a higher total N content than the compost so that manure plots received less overall material for the same total N application rate. Therefore, the compost treatment had higher organic C, while the manure treatment had a higher available N in soils (28). Higher organic C may increase the microbial richness in the compost treatment. In addition, although both compost and manure could introduce exogenous species to soils, microbes from compost may be more adaptive to the soil environment than those from manure, which originate from the cattle's gut microbiome. Thus, microbes from compost have a higher chance to survive in soils. In contrast, the changes in soil bacterial and fungal community composition may be more driven by the N availability, which was higher in manure treatment. Furthermore, we found more ASVs were responsive to manure than compost. The sensitivity of microbes to organic fertilizers may result in a difference in microbial community composition at finer taxonomic levels.

Soil microbial communities often exhibit strong temporal dynamics (47). We collected soils in the corn phase at the end of two cropping cycles in 2011 and 2015. Organic fertilizers were applied twice from 2011 to 2015. We expected that soil microbial communities would be different between 2011 and 2015, and

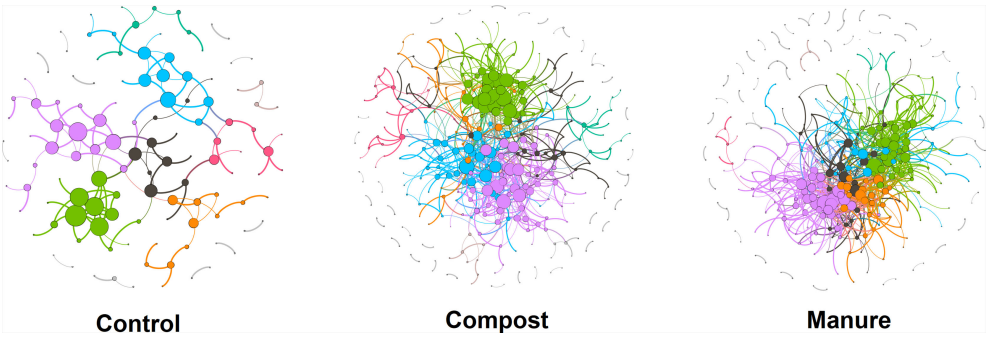


FIGURE 3
Microbial networks under three organic N treatments. Networks are built using the random matrix theory. Nodes represent ASVs and links indicate significant correlation. Modules are randomly colored. Detailed topological features of networks are shown in Table 1.

that there would be a stronger organic fertilization effect in 2015 due to repeated application of organic fertilizers. Bacterial and fungal communities did significantly vary between 2011 and 2015 in our study. More interestingly, both bacterial and fungal communities had a higher number of responsive ASVs and a higher magnitude of their response to compost and manure in 2015 than 2011. These results suggest that both bacterial and fungal communities could be gradually manipulated by agricultural management practices, such as crop rotation and organic fertilization. Organic N fertilizers increased *Proteobacteria* and *Bacteroidetes*, but decreased *Acidobacteria*, *Chloroflexi*, and *Gemmatimonadetes*. Similar changes in the relative abundances of these phyla have been identified through on-farm surveys of manure or compost treated soils (48).

Organic fertilization affected the microbial network

Co-occurrence networks reveal shared ecological niches and potential biological interactions among organisms (38). In this study, several topological properties of the networks (e.g. average path distance, average clustering coefficient, and modularity) suggested that organic fertilizer intensified soil microbial co-occurrence networks. Previous studies also showed that organic amendment supported more interactions within the soil microbial community (22, 23, 25, 26). The probable mechanism is that the degradation of organic amendments, mainly complex and polymeric organic compounds, was mediated by many specialized microbes. Microbes interacted to each other closely to efficiently degrade these polymeric compounds. To support this assumption, we found that organic fertilizers increased the interaction among four N-cycling genes, especially the links between the ureolytic and chitinolytic communities. Furthermore, we also found that manure treated soils had a more intensive microbial co-occurrence network than compost treated soils. This may be associated with higher N transformation rates in manured soils.

Negative interactions may indicate competition and niche separation between organisms (49). We found that compost and manure treated soils had higher negative associations than the control soils, indicative of increased microbial competition in soil treated with organic fertilizers. Although organic fertilizers increase soil organic C and nutrient availability, microbial biomass and its diversity are also increased. The application of organic fertilizers is a pulse event. Fresh labile substrates thus may be consumed quickly. Thereafter the reduced available C and nutrients may lead to an intense competition among microbes. However, it is notable that most network analyses cannot reveal specific ecological interactions based on positive or negative correlations between nodes.

Organic fertilization affected N-cycling functional groups

The ureolytic community composition was significantly changed by organic fertilizers in our soil, which is consistent with other studies in agricultural soils (12, 13). However, our study further demonstrated that manure had a stronger effect than compost on the ureolytic community composition. The shift of the ureolytic community between compost and manure treatments were closely associated with total N and nitrate suggesting that N availability plays a key role in shaping ureolytic communities. Additionally, there were more responsive OTUs to manure than to compost additions. Urea turnover or concentration may be higher with manure than compost treatment, therefore exerting a stronger effect on urea degraders at both the community and species levels. Supporting this observation, we also found manured soils had higher urease activity than compost treated soils (28).

10.3389/fsoil.2022.869136

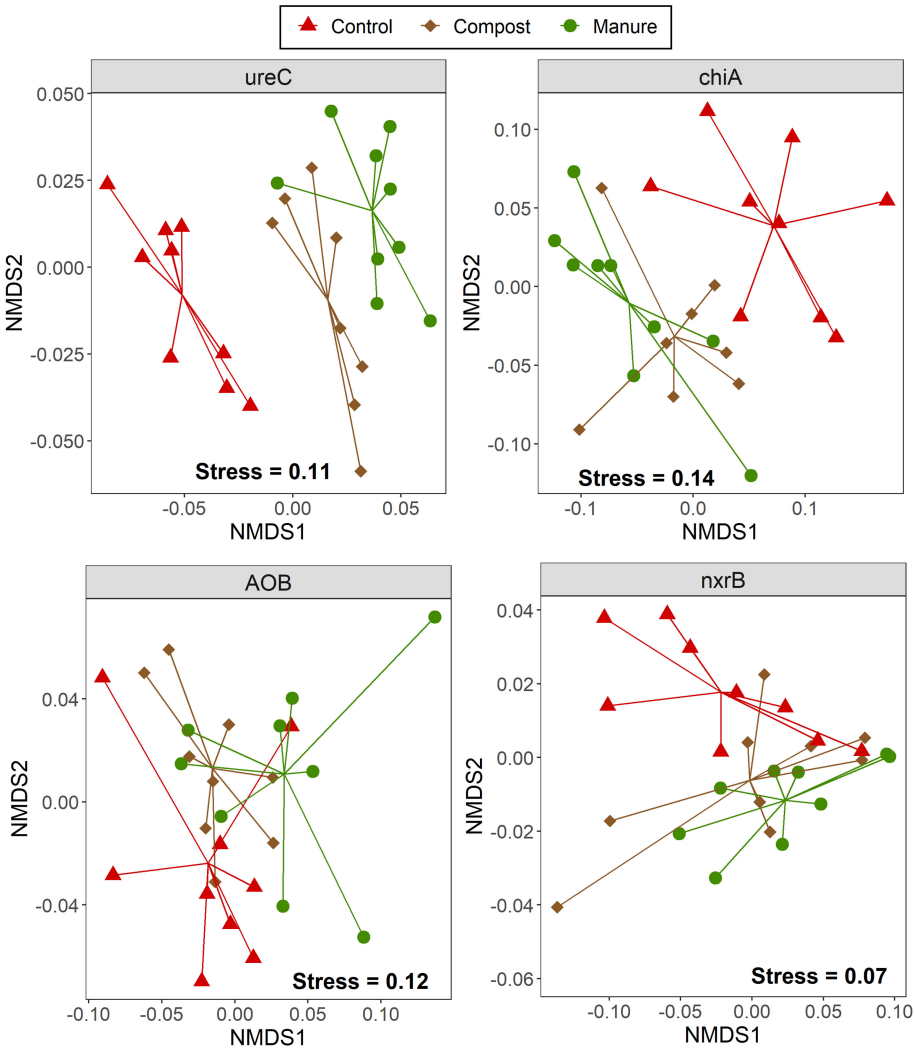


FIGURE 4
NMDS ordination of the weighted UniFrac distance for four N-cycling functional groups. Four N-cycling genes were *ureC* (encoding urease), *chiA* (encoding chitinase), bacterial *amoA* (encoding ammonia monooxygenase) and *Nitrospira nxrB* (encoding nitrite oxidoreductase). Except for *Nitrospira nxrB*, organic N treatment significantly changed the community composition of other N-cycling functional groups ($p < 0.01$).

Numerous studies have focused on the effect of chitin amendment on the chitinolytic communities (14, 50–52). It is largely unknown how organic fertilizers directly mediate the chitinolytic community. In our study, both compost and manure treatment significantly changed the chitinolytic community composition. However, in our previous study on a conventional farm, we found that compost treatment effect was not significant on the chitinolytic community, largely due to the heterogeneity of the microbiome in compost-treated soil (2). The change in the chitinolytic community was associated with pH in this study. Previous studies have also demonstrated that pH was a key factor for shaping the chitinolytic community (14, 53). Furthermore, we reveal that the compost and manure treated soils harbored distinct chitinolytic communities. This difference was correlated with SOC, which was higher in compost treated soils. Compost treatment also had higher proportions of *Actinobacteria* than the manure treated soils. These outcomes suggest that organic C availability may play a more important role than N availability in shaping the chitinolytic community composition.

Organic fertilizers significantly changed the community of AOB, but not the *Nitrospira* community in this study. There was no distinction between manure and compost treatments on the community of AOB, though we found manured soils had higher nitrate, octyne-sensitive nitrification potential, and AOB abundance than compost treated soils (28). Ammonium was slowly released by N mineralization in both compost and

10.3389/fsoil.2022.869136

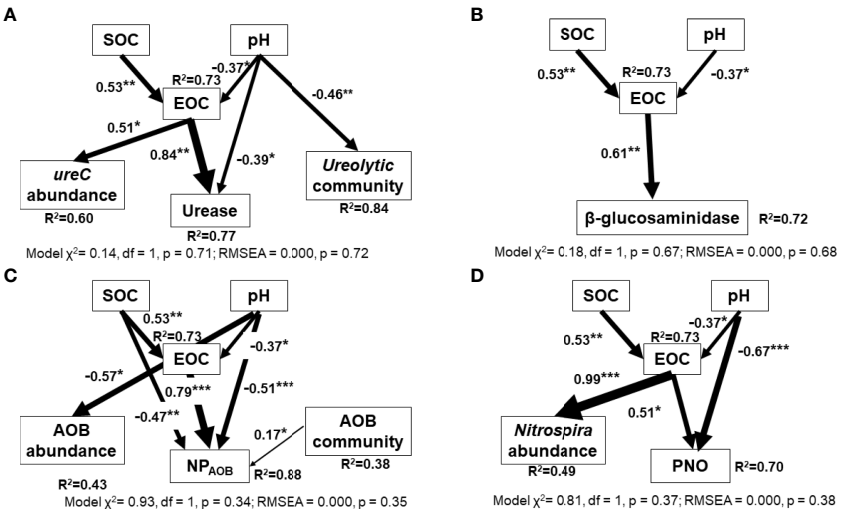


FIGURE 5
Structural equation models (SEM) showing the effect of soil chemical properties (SOC, EOC, and pH) and the abundance and community of N-cycling functional groups on their corresponding N process rates. Four selected N-cycling processes are **(A)** urease, **(B)** β-glucosaminidase (NAGase), **(C)** octyne-sensitive nitrification potential (nitrification potential contributed by AOB, NP_{AOB}), and **(D)** potential nitrite oxidation (PNO). NMDS1 is used to represent the community composition of N-cycling functional groups. The arrow width is proportional to the strength of the relationship. R^2 represents the proportion of variance explained for every dependent variable. SOC, soil organic carbon; EOC, extractable organic C. Asterisks indicate significant p values (***p < 0.001, **p < 0.01, *p < 0.05)..

manure treated soils, which may result in similar selective forces on the community of AOB. At the nearby site under conventional management, the same compost was applied at the same rate and we found that compost had no effect on the community of AOB with three consecutive years of application in a continuous corn cropping system (15). This contrasting result suggests that the duration of compost application or agricultural management may have an important role in shaping the AOB community, since the organic site reported in this current study received six applications of compost with organic management in a diverse rotation. In contrast, the community composition of *Nitrospira* was not changed by the quality and duration of organic fertilizers (17). The response of the *Nitrospira* community to organic fertilizer may be masked by their physiologically diverse life-strategies (54). Collectively, our results suggest the quality of organic fertilizers has relatively limited effect on the composition of AOB and *Nitrospira* communities particularly when compared to the effect of conventional ammonia based fertilizers.

Relative importance of soil chemical properties and functional genes on soil processes

In a previous study, we demonstrated that soil edaphic properties rather than functional gene abundance dominantly impacted N transformation processes (28). In this study, we complemented these findings by including the community composition of four selected N-cycling groups. We expected that the community composition would be strongly linked to N-cycling processes. We did find that community composition was often significantly correlated with the corresponding process rates based on Mantel test. However, our SEM results showed that the direct effects of community composition on soil N-cycling process rates were not maintained after considering multiple biotic and abiotic factors together. Soil pH and EOC often directly affected selected N-cycling activities and rates. This result was contradicted by Trivedi et al. (55), who found that several C-cycling functional genes directly drove the corresponding soil enzyme activities. This discrepancy may be partly explained by the low coverage of some N-cycling groups by amplicon sequencing. We only targeted bacterial *ureC* and *chiA*, but did not include fungi, which are also involved in urea and chitin degradation (56, 57). We only targeted *Nitrospira* in our study, but both *Nitrospira* and *Nitrobacter* may be important nitrite oxidizers in soils (58, 59). However, we found the AOB community with a high coverage of AOB *amoA* by amplicon sequencing directly controlled the octyne-sensitive nitrification potential (contributed by AOB). Collectively, our study suggests that improved primer coverage of N-cycling groups may be necessary to link functional genes and their corresponding process rates especially when the level of functional redundancy in communities is unknown.

Conclusion

We investigated the community compositions of soil microbiome and N-cycling bacteria under fresh and composted manure treatments in a long-term organic farming system. We found that fresh manure treatment had a stronger impact than compost treatment on the composition of bacterial and fungal communities, as well as on the ureolytic and chitinolytic community. Both taxonomic and functional microbial groups were more responsive to fresh manure than compost. Fresh manure treated soils also had more complex microbial interactions than compost treated soils. Collectively, our study suggests that fresh manure selects for a microbial community with an increased functional capacity of supplying available N including nitrate. Further investigation should include crop yield, soil C storage, disease suppression, N loss and greenhouse gas emissions to assess holistically the advantages and disadvantages of using fresh manure versus compost for organic farming systems.

Data availability statement

The data presented in this study are deposited in the NCBI Sequence Read Archive, accession PRJNA804976 (https://www.ncbi.nlm.nih.gov//bioproject/PRJNA804976).

Author contribution

JN and JR designed the study and revised the manuscript. YO conducted the experiment, analyzed the data, and wrote the draft of the manuscript. All authors have read and approved the final manuscript.

Funding

The USDA National Institute of Food and Agriculture funded this work under awards 2011- 67019-30178 and 2016-35100-25091. The research was supported by the Utah Agricultural Experiment Station, Utah State University and approved as journal paper number 9392. ITS and 16S Illumina sequencing was conducted by the U.S. Department of Energy Joint Genome Institute, an Office of Science User Facility, and was supported by the Office of Science of the U.S. Department of Energy under contract DE-AC02-05CH11231.

Acknowledgments

We would like to thank Craig Marlen Rice, Jeremiah Moore and Henry Linford for soil sampling and laboratory assistance. Davie Olsen and Kareem Adeleke performed organic farm plot maintenance.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsoil.2022.869136/full#supplementary-material

References

1. Bernard E, Larkin RP, Tavantzis S, Erich MS, Alyokhin A, Sewell G, et al. Compost, rapeseed rotation, and biocontrol agents significantly impact soil microbial communities in organic and conventional potato production systems. *Appl Soil Ecol* (2012) 52:29–41. doi: 10.1016/j.apsoil.2011.10.002

2. Ouyang Y, Norton JM. Short-term nitrogen fertilization affects microbial community composition and nitrogen mineralization functions in an agricultural soil. *Appl Environ Microbiol* (2020) 86:e03378-19. doi: 10.1128/AEM.02278-19

3. Wang J, Song Y, Ma T, Raza W, Li J, Howland JG, et al. Impacts of inorganic and organic fertilization treatments on bacterial and fungal communities in a paddy soil. *Appl Soil Ecol* (2017) 112:42–50. doi: 10.1016/j.apsoil.2017.01.005

4. Sun R, Zhang X-X, Guo X, Wang D, Chu H. Bacterial diversity in soils subjected to long-term chemical fertilization can be more stably maintained with the addition of livestock manure than wheat straw. *Soil Biol Biochem* (2015) 88:9–18. doi: 10.1016/j.soilbio.2015.05.007

5. Zhang Y, Hao X, Alexander TW, Thomas BW, Shi X, Lupwayi NZ. Long-term and legacy effects of manure application on soil microbial community composition. *Biol Fertil Soils* (2018) 54:269–83. doi: 10.1007/s00374-017-1257-2

6. Carrera LM, Buyer JS, Vinyard B, Abdul-Baki AA, Sikora LJ, Teasdale JR. Effects of cover crops, compost, and manure amendments on soil microbial community structure in tomato production systems. *Appl Soil Ecol* (2007) 37:247–55. doi: 10.1016/J.APSOIL.2007.08.003

7. Marschner P, Kandeler E, Marschner B. Structure and function of the soil microbial community in a long-term fertilizer experiment. *Soil Biol Biochem* (2003) 35:453–61. doi: 10.1016/S0038-0717(02)00297-3

8. Pérez-Piqueres A, Edel-Hermann V, Alabouvette C, Steinberg C. Response of soil microbial communities to compost amendments. *Soil Biol Biochem* (2006) 38:460–70. doi: 10.1016/j.soilbio.2005.05.025

9. Schimel JP, Bennett J. Nitrogen mineralization: challenges of a changing paradigm. *Ecology* (2004) 85:591–602. doi: 10.1890/03-8002

10. Norton J, Ouyang Y. Controls and adaptive management of nitrification in agricultural soils. *Front Microbiol* (2019) 10:1931. doi: 10.3389/fmicb.2019.01931

11. Norton JM, Schimel JP. "Nitrogen mineralization immobilization turnover,". In: PM Huang, Y Li and ME Summers, editors. *Handbook of soil science, 2nd ed*. Boca Raton, FL USA: CRC Press (2011).

12. Luo G, Li L, Friman VP, Guo J, Guo S, Shen Q, et al. Organic amendments increase crop yields by improving microbe-mediated soil functioning of agroecosystems: A meta-analysis. *Soil Biol Biochem* (2018) 124:105–15. doi: 10.1016/j.soilbio.2018.06.002

13. Wang L, Luo X, Liao H, Chen W, Wei D, Cai P, et al. Ureolytic microbial community is modulated by fertilization regimes and particle-size fractions in a black soil of northeastern China. *Soil Biol Biochem* (2018) 116:171–8. doi: 10.1016/j.soilbio.2017.10.012

14. Kielak AM, Cretoiu MS, Semenov AV, Sørensen SJ, van Elsas JD. Bacterial chitinolytic communities respond to chitin and pH alteration in soil. *Appl Environ Microbiol* (2013) 79:263–72. doi: 10.1128/AEM.02546-12

15. Ouyang Y, Norton JM, Stark JM, Reeve JR, Habteselassie MY. Ammonia-oxidizing bacteria are more responsive than archaea to nitrogen source in an agricultural soil. *Soil Biol Biochem* (2016) 96:4–15. doi: 10.1016/j.soilbio.2016.01.012

16. Ouyang Y, Norton JM. Nitrite oxidizer activity and community are more responsive than their abundance to ammonium-based fertilizer in an agricultural soil. *Front Microbiol* (2020) 11:1736. doi: 10.3389/fmicb.2020.01736

17. Han S, Zeng L, Luo X, Xiong X, Wen S, Wang B, et al. Shifts in nitrobacter- and nitrospira-like nitrite-oxidizing bacterial communities under long-term fertilization practices. *Soil Biol Biochem* (2018) 124:118–25. doi: 10.1016/j.soilbio.2018.05.033

18. He J, Shen J, Zhang L, Zhu Y, Zheng Y, Xu M, et al. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. *Environ Microbiol* (2007) 9:2364–74. doi: 10.1111/j.1462-2920.2007.01358.x

19. Zhou J, Deng Y, Luo F, He Z, Tu Q, Zhi X. Functional molecular ecological networks. *MBio* (2010) 1. doi: 10.1128/mBio.00169-10

20. de Vries FT, Griffiths RI, Bailey M, Craig H, Girlanda M, Gweon HS, et al. Soil bacterial networks are less stable under drought than fungal networks. *Nat Commun* (2018) 9:1–12. doi: 10.1038/s41467-018-05516-7

21. Banerjee S, Schlaeppi K, van der Heijden MGA. Keystone taxa as drivers of microbiome structure and functioning. *Nat Rev Microbiol* (2018) 16:567–76. doi: 10.1038/s41579-018-0024-1

22. Hartmann M, Frey B, Mayer J, Mäder P, Widmer F. Distinct soil microbial diversity under long-term organic and conventional farming. *ISME J* (2015) 9:1177–94. doi: 10.1038/ismej.2014.210

23. Banerjee S, Walder F, Büchi L, Meyer M, Held AY, Gattinger A, et al. Agricultural intensification reduces microbial network complexity and the abundance of keystone taxa in roots. *ISME J* (2019) 13:1722–36. doi: 10.1038/s41396-019-0383-2

24. Hartman K, van der Heijden MGA, Wittwer RA, Banerjee S, Walser J-C, Schlaeppi K. Cropping practices manipulate abundance patterns of root and soil microbiome members paving the way to smart farming. *Microbiome* (2018) 6:14. doi: 10.1186/s40168-017-0389-9

25. Schmid CAO, Schröder P, Armbruster M, Schloter M. Organic amendments in a long-term field trial–consequences for the bulk soil bacterial community as revealed by network analysis. *Microb Ecol* (2018) 76:226–39. doi: 10.1007/s00248-017-1110-z

26. Ling N, Zhu C, Xue C, Chen H, Duan Y, Peng C, et al. Insight into how organic amendments can shape the soil microbiome in long-term field experiments as revealed by network analysis. *Soil Biol Biochem* (2016) 99:137–49. doi: 10.1016/j.soilbio.2016.05.005

27. Ramos TM, Jay-Russell MT, Millner PD, Shade J, Misiewicz T, Sorge US, et al. Assessment of biological soil amendments of animal origin use, research needs, and extension opportunities in organic production. *Front Sustain Food Syst* (2019) 3:73. doi: 10.3389/fsufs.2019.00073

28. Ouyang Y, Reeve JR, Norton JM. Soil enzyme activities and abundance of microbial functional genes involved in nitrogen transformations in an organic farming system. *Biol Fertil Soils* (2018) 54:437–50. doi: 10.1007/s00374-018-1272-y

29. Parada AE, Needham DM, Fuhrman JA. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol* (2016) 18:1403–14. doi: 10.1111/1462-2920.13023

30. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* (2013) 10:996–8. doi: 10.1038/nmeth.2604

31. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* (2007) 73:5261–7. doi: 10.1128/AEM.00062-07

32. Price MN, Dehal PS, Arkin AP. FastTree 2 – approximately maximum-likelihood trees for Large alignments. *PloS One* (2010) 5:e9490. doi: 10.1371/journal.pone.0009490

33. McMurdie PJ, Holmes S. Phyloseq: An r package for reproducible interactive analysis and graphics of microbiome census data. *PloS One* (2013) 8: e61217. doi: 10.1371/journal.pone.0061217

34. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, et al. Ribosomal database project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res* (2014) 42:D633–42. doi: 10.1093/nar/gkt1244

35. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* (2011) 27:2194–200. doi: 10.1093/bioinformatics/btr381

36. Wang Q, Quensen JF, Fish JA, Lee TK, Sun Y, Tiedje JM, et al. Ecological patterns of nifH genes in four terrestrial climatic zones explored with targeted metagenomics using FrameBot, a new informatics tool. *MBio* (2013) 4:e00592–13. doi: 10.1128/mBio.00592-13

37. Fish JA, Chai B, Wang Q, Sun Y, Brown CT, Tiedje JM, et al. FunGene: the functional gene pipeline and repository. *Front Microbiol* (2013) 4:291. doi: 10.3389/fmicb.2013.00291

38. Deng Y, Jiang YH, Yang Y, He Z, Luo F, Zhou J. Molecular ecological network analyses. *BMC Bioinf* (2012) 13:1–20. doi: 10.1186/1471-2105-13-113

39. Wu L, Yang Y, Chen S, Zhao M, Zhu Z, Yang S, et al. Long-term successional dynamics of microbial association networks in anaerobic digestion processes. *Water Res* (2016) 104:1–10. doi: 10.1016/j.watres.2016.07.072

40. Shi S, Nuccio EE, Shi ZJ, He Z, Zhou J, Firestone MK. The interconnected rhizosphere: High network complexity dominates rhizosphere assemblages. *Ecol Lett* (2016) 19:926–36. doi: 10.1111/ele.12630

41. Bastian M, Heymann S, Jacomy M. Gephi : An open source software for exploring and manipulating networks. *Proceedings of the International AAAI Conference on Web and Social Media* (2009) 3(1):361–2. Retrieved from https://ojs.aaai.org/index.php/ICWSM/article/view/13937.

42. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res* (2003) 13:2498–504. doi: 10.1101/gr.1239303

43. Oksanen J, Blanchet FG, Kindt R, Legendre P, McGlinn D, Wagner H, et al. Vegan: Community ecology package. (version 2.5-6) *The Comprehensive R Archive Network (CRAN)* (2019). https://cran.r-project.org/web/packages/vegan/vegan.pdf

44. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* (2014) 15:550. doi: 10.1186/s13059-014-0550-8

45. Lori M, Symnaczik S, Mäder P, De Deyn G, Gattinger A. Organic farming enhances soil microbial abundance and activity–a meta-analysis and meta-regression. *PloS One* (2017) 12:e0180442. doi: 10.1371/journal.pone.0180442

46. Reeve JR, Hoagland LA, Villalba JJ, Carr PM, Atucha A, Cambardella C, et al. Organic farming, soil health, and food quality: Considering possible links. *Adv Agron* (2016) 137:319–67. doi: 10.1016/bs.agron.2015.12.003

47. Shade A, Gregory Caporaso J, Handelsman J, Knight R, Fierer N. A meta-analysis of changes in bacterial and archaeal communities with time. *ISME J* (2013) 7:1493–506. doi: 10.1038/ismej.2013.54

48. Peng M, Tabashsum Z, Millner P, Parveen S, Biswas D. Influence of manure application on the soil bacterial microbiome in integrated crop-livestock farms in Maryland. *Microorganisms* (2021) 9:2586. doi: 10.3390/microorganisms9122586

49. Ghoul M, Mitri S. The ecology and evolution of microbial competition. *Trends Microbiol* (2016) 24:833–45. doi: 10.1016/j.tim.2016.06.011

50. Hui C, Jiang H, Liu B, Wei R, Zhang Y, Zhang Q, et al. Chitin degradation and the temporary response of bacterial chitinolytic communities to chitin amendment in soil under different fertilization regimes. *Sci Total Environ* (2020) 705:136003. doi: 10.1016/j.scitotenv.2019.136003

51. Jacquiod S, Franqueville L, Cécillon S, M. Vogel T, Simonet P. Soil bacterial community shifts after chitin enrichment: An integrative metagenomic approach. *PloS One* (2013) 8:e79699. doi: 10.1371/journal.pone.0079699

52. Cretoiu MS, Kielak AM, Schluter A, Van Elsas JD. Bacterial communities in chitin-amended soil as revealed by 16S rRNA gene based pyrosequencing. *Soil Biol Biochem* (2014) 76:5–11. doi: 10.1016/j.soilbio.2014.04.027

53. Terahara T, Ikeda S, Noritake C, Minamisawa K, Ando K, Tsuneda S, et al. Molecular diversity of bacterial chitinases in arable soils and the effects of environmental factors on the chitinolytic bacterial community. *Soil Biol Biochem* (2009) 41:473–80. doi: 10.1016/J.SOILBIO.2008.11.024

54. Daims H, Lücker S, Wagner M. A new perspective on microbes formerly known as nitrite-oxidizing bacteria. *Trends Microbiol* (2016) 24:699–712. doi: 10.1016/j.tim.2016.05.004

55. Trivedi P, Delgado-Baquerizo M, Trivedi C, Hu H, Anderson IC, Jeffries TC, et al. Microbial regulation of the soil carbon cycle: evidence from gene–enzyme relationships. *ISME J* (2016) 10:2593–604. doi: 10.1038/ismej.2016.65

56. Mobley HLT, Island MD, Hausinger RP. Molecular biology of microbial ureases. *Microbiol Rev* (1995) 59:451–80. doi: 10.1128/mr.59.3.451-480.1995

57. Treseder KK, Lennon JT. Fungal traits that drive ecosystem dynamics on land. *Microbiol Mol Biol Rev* (2015) 79:243–62. doi: 10.1128/mmbr.00001-15

58. Poly F, Wertz S, Brothier E, Degrange V. First exploration of nitrobacter diversity in soils by a PCR cloning-sequencing approach targeting functional gene nxrA. *FEMS Microbiol Ecol* (2008) 63:132–40. doi: 10.1111/j.1574-6941.2007.00404.x

59. Pester M, Maixner F, Berry D, Rattei T, Koch H, Lücker S, et al. NxrB encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic marker for nitrite-oxidizing n itrospira. *Environ Microbiol* (2014) 16:3055–71. doi: 10.1111/1462-2920.12300

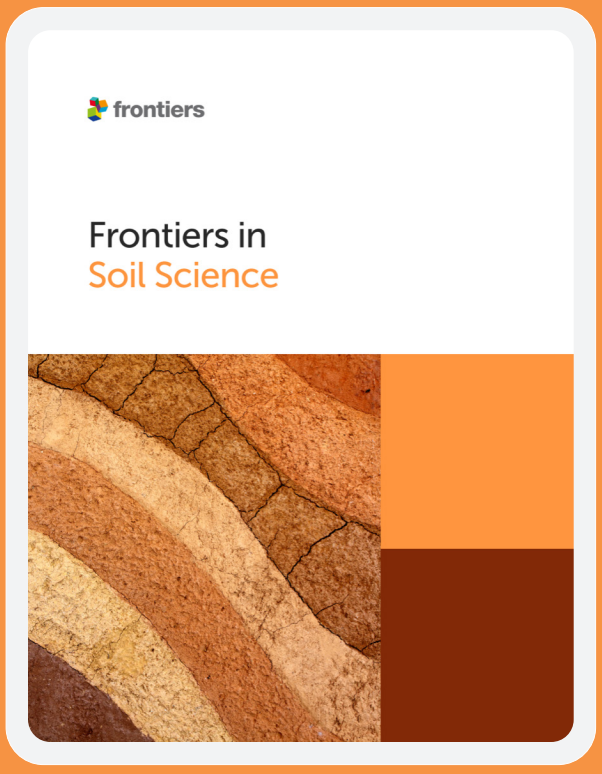
Frontiers in
Soil Science

Digs into the science of soil functioning, classification, and management

A forum for excellence in soil science - from plant-soil interactions to mathematical modelling on soil processes - deepening our knowledge of the subject.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact