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Long-term and legacy effects of manure application on soil microbial community composition

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Abstract

We analyzed soil prokaryotic and fungal community composition in soils with varying histories of cattle manure application. The manure treatments were (i) annual application for 43 years (MF), (ii) annual application for 14 years followed by 29 years without application (MF14), and (iii) annual application for 30 years followed by 13 years without application (MF30). An annual application of chemical nitrogen (N) fertilizer (CNF) and a non-amended control (Con) were also included. Soil prokaryotic evenness and diversity significantly decreased in MF relative to other treatments in fall, but were similar to the other fertilizer treatments in spring and summer. Distinct prokaryotic and fungal community composition was observed in MF compared to other treatments across fall, spring, and summer seasons. The MF treatment significantly increased the relative abundance of *Firmicutes*, *Gammaproteobacteria*, and *Gemmatimonadetes*, but significantly decreased the relative abundance of *Acidobacteria*. In fall, the soil prokaryotic and fungal community composition with MF30 was significantly different than the other fertilization treatments. Overall, the study showed that annual manure application (MF) led to a different microbial community composition than the other fertilizer treatments. Soil without manure application for 13 years (MF30) had a significantly different microbial community composition from other fertilizer treatments in fall, while the soil without manure application for 29 years (MF14) resembled a microbial community that had never received manure.

Keywords Cattle manure · Long-term fertilization · Microbial α-diversity · Microbial community composition · Residual effect

Introduction

Soils usually contain inadequate amounts of the nutrients that crops require to grow. The extra nutrients are usually supplied through application of inorganic and/or organic fertilizers to the soil. These fertilizers can have unintended positive or negative effects on soil microorganisms. Inorganic fertilizers can have direct positive effects on soil microorganisms because the nutrients that they supply can be used by the microorganisms (Geisseler and Scow 2014; Leff et al. 2015). Such

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fertilizers can also have indirect positive effects through increased root exudation by crops or increased crop biomass that adds organic C to the soil through crop residues (Geisseler and Scow 2014). Inorganic fertilizers can also have direct negative effects due to osmotic effects induced by the fertilizer or indirect effects through changes in soil pH (Zhang et al. 2017). By contrast, organic fertilizers (including manures) usually have positive effects on soil microbial communities (Chinnadurai et al. 2014; Francioli et al. 2016; Hartmann et al. 2015). For example, an 8-month field trial showed that application of manure increased microbial biomass carbon (C), N mineralization, soil respiration, and enzyme activities (Dinesh et al. 2010), and a 3-month trial showed that the relative abundance of soil Gram-negative bacteria increased more by applying manure than inorganic fertilizer (Lazcano et al. 2013). One reason for the positive manure effects on soil microorganisms is that manures add organic C to the soil, in addition to nutrients such as N, P, and K, and organic C is the main metabolic substrate used by saprophytic soil microorganisms. However, the effects of organic fertilizers are not always apparent over short time periods (Calleja-Cervantes et al. 2015; Ghosh et al.

2010), highlighting the importance of studying responses of the soil microbial communities to manure application over a long time. Although some long-term studies have been conducted (Francioli et al. 2016; Neufeld et al. 2017; Zhong et al. 2010), further characterization of long-term manure effects on multiple aspects of soil microbial communities in different agro-ecological systems is required.

Because organic fertilizers slowly release nutrients during decomposition, they can sustain soil microorganisms for a long time. The continued effects after suspending application of organic fertilizers are called legacy or residual effects (Wyngaard et al. 2016). In a degraded semi-arid Mediterranean soil of southeast Spain, 5, 17, or 25 years after applying different rates of municipal solid wastes (65 to 300 Mg ha^{-1}, respectively) one time, soil microbial biomass and enzyme activity remained significantly greater compared with the control (Bastida et al. 2007, 2008; Torres et al. 2015), and soil microbial community composition was also altered depending on the amendment application rate (Bastida et al. 2008, 2013; Torres et al. 2015). After suspending fertilization for 4 years, soil microbial biomass C was 20 to 40% greater with manure and compost than with chemical N fertilizer (Ginting et al. 2003). However, 8 years after the last fertilization, the abundance of both ammonia-oxidizing bacteria and archaea were similar in the topsoil (0 to 5 cm) and subsurface soil (5 to 20 cm) that received inorganic fertilizer or poultry litter (Wyngaard et al. 2016). Therefore, it is important to determine the duration of the legacy effects of organic fertilizers and understand what factors influence them.

The response of soil microbial communities to organic or inorganic fertilizers depends on many environmental and edaphic factors, including soil moisture. Soil moisture is important because it affects the microbial-mediated decomposition of organic fertilizers and the dissolution of nutrients from inorganic fertilizers. Both direct and indirect effects of soil moisture on soil microbial biomass, community composition, and enzyme activities have been reported (Bastida et al. 2017; Manzoni et al. 2012; Hartmann et al. 2016). In arid and semi-arid areas, soil moisture limitations can be overcome by irrigation. Because southern Alberta is in a semi-arid region, both irrigated and rain-fed agricultural systems exist. Southern Alberta also has the highest concentration of cattle in Canada, producing large quantities of manure. However, the extent to which water management affects long-term and legacy effects of cattle manure in such regions is not clear.

In this investigation of long-term manure and legacy effects on soil microbial composition, we used a manure fertilization field trial that was established in 1973 under irrigation or rain-fed conditions. The trial treatments included continuous annual manure application (1973–2015), suspended manure application for 13 years (2003–2015) and 29 years (1987–2015) after 30 and 14 annual manure applications, respectively, chemical N fertilizer (1990–2016), and non-amended

controls. The objectives of this study were to determine (a) the effects of annual manure application on soil microbial α-diversity and community composition, (b) the legacy effect of manure application by assessing how discontinuing manure application affects soil α-diversity and community composition, and (c) how water management (irrigation vs. rain-fed) influences manure effects. We hypothesized that (a) annual manure application would have more beneficial effects on soil microbial α-diversity and community composition than chemical N, (b) suspending manure application would leave legacy effects on soil α-diversity and community composition, and (c) water management would affect these responses.

Materials and methods

Experimental design and soil sampling

A long-term manure field experiment was established in 1973 at the Agriculture and Agri-Food Canada Research and Development Centre in Lethbridge (49° 42′ N, 112° 48′ W), Alberta, Canada. This region has a semi-arid climate with a mean annual temperature of 14.7 °C and precipitation of 251 mm during the growing season (May–September) (Benke et al. 2013). The soil is a calcareous, dark brown Chernozemic (Typic Haplustoll) with a clay loam texture (IUSS Working Group 2015).

Details of the randomized split-block experimental design were previously reported by Sommerfeldt and Chang (1985), Hao et al. (2004), and Benke et al. (2013). Briefly, at the beginning in 1973, solid beef cattle feedlot manure was applied each fall after harvest, and immediately incorporated by three main tillage methods: plow, rototiller, or cultivator plus disk. Within each main plot of tillage treatment, manure was applied at rates of 0 (control, Con) and 60 (MF) Mg ha^{-1} year^{-1} as subplots (7.5 by 15 m) to both irrigated and rain-fed blocks. The manure application rate of 60 Mg ha^{-1} year^{-1} was the local recommended agronomic rate (N based) for irrigated crop production at the time the experiment was initiated in 1973. Main and subplot treatments were randomly assigned and replicated three times. In 1987, tillage treatments were discontinued due to lack of tillage effect (Chang et al. 1991), and manure in all plots was incorporated with a cultivator, which increased the number of replicated manure treatments from three to nine. From 1987, three replications of 60 Mg ha^{-1} year^{-1} manure treatment received no further manure to investigate its legacy effect. In 2003, manure application was discontinued in another three replications. These discontinued applications created two new manure treatments: discontinued after 14 annual applications (1973–1987) (MF14) and discontinued after 30 annual applications (1973–2003) (MF30), in both the irrigated and rain-fed blocks. In 1990, each control subplot was split, one half

received 100 kg N ha^{-1} (NH$_4$NO$_3$ fertilizer, CNF treatment) each spring and the other half remained unchanged. No other inorganic fertilizer was used in any of the subplots. Overall, there were three different manure fertilization treatments (MF, MF14, and MF30) within the irrigated and rain-fed block, respectively. In addition, within the same blocks, there were control plots that never received manure (Con) and plots that received chemical nitrogen (N) fertilization (1990–2016; CNF). Each treatment had three replicated subplots.

On average, the manure in this study had total C of 195.6 ± 12.1 g kg^{-1}, total N of 17.0 ± 0.2 g kg^{-1}, and total P of 6.6 ± 0.1 g kg^{-1}. Barley was grown in most years except 1996 when canola (*Brassica rapa* L.) was grown, 1997 to 2000 when corn (*Zea mays* L.) was grown, and 2016 when soybean (*Glycine max* (L.) Merr.) was grown. Crops were generally seeded in May and harvested in October. The amount of irrigation water averaged 160 mm year^{-1}, with a maximum value of 432 mm in 2002 and a minimum value 0 mm in 1995. A solid-set sprinkler system was used for irrigation. For each irrigation event, 25–90 mm of water were applied depending on soil moisture conditions. Soil samples for this study were collected in 2015 and 2016. During the 2015 growing season (May to August), June rainfall was only 23% (19.5 mm) of the long-term average for that month, and August rainfall (16.1 mm) was 40% of normal rainfall. In 2016, May and July rainfall were 150 and 215%, respectively, of normal rainfall for these months. Air temperatures did not deviate much from normal, except in June of both years when the average maximum air temperatures were 117% (25.3 °C) and 113% (24.5 °C), respectively, of normal levels.

Soil samples were collected in November 2015 (fall, 2 ays after manure application to the MF plots), May 2016 (spring, 182 days after manure application), and August 2016 (summer, 276 days after manure application). Six randomly selected soil cores (core size 240–660 cm^3 for the 0–15 cm depths) from each subplot were collected and thoroughly mixed to form one composite sample per subplot. In total, 90 soil samples were collected (5 fertilization treatment × 2 water management × 3 replications × 3 seasons). Soil samples were sieved (< 2 mm) and then either air-dried for chemical analyses or stored at − 20 °C until DNA extraction.

Determination of soil basic properties

Soil pH was measured in 1:2 soil-to-water ratio using an Accumet AB pH meter (Fisher Scientific, Hampton, NH). After filtering (0.45 μm) the soil slurry, the supernatant solution was used to determine electrical conductivity (EC) and non-purgeable water-soluble organic C (C_{water}). The EC of the filtrate was measured with an EC meter (Model 125A, Orion, Boston, MA). The C_{water} contents of the filtrate were measured with a Shimadzu TOC-Vcsh. Soil available P (AP) was determined by extracting soil

with 0.5 M NaHCO$_3$ solution at 1:10 soil-to-NaHCO$_3$ ratio, and measured with an automated colorimeter (EasyChem Pro, System Analytical Technology, Anagni, Italy). Soil available N (AN) was determined by extracting with a 2 M KCl solution at 1:5 soil-to-KCl ratio, and measured with an automated colorimeter (Auto-Analyzer III, Bran + Luebbe, Norderstedt, Germany).

DNA extraction, Amplicon library preparation, and MiSeq sequencing

Soil DNA was extracted from 0.25 g frozen soil using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., USA) according to the manufacturer's instructions. The DNA was quantified using a NanoDrop 2000 (Thermo Fisher Scientific, USA) and visualized using a 1% agarose gel after electrophoresis.

Two PCR reactions were performed to prepare the amplicon library. Briefly, for the first PCR reaction, the V4 hypervariable region of the prokaryotic (bacterial and archaeal) 16S rRNA gene was amplified using the primer set 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (Caporaso et al. 2011), and the internal transcribed spacer region 1 (ITS1) of the fungal ribosomal operon was amplified using ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) (Bellemain et al. 2010). This PCR reaction was performed in a 25-μl reaction mixture, containing 1 μl of template DNA, 1 × PCR-buffer (Qiagen, Germany), 1.8 mM MgCl$_2$, 1.25 μl of 5% dimethylsulfoxide (DMSO), 0.2 mM dNTP, 0.5 U *Taq* DNA polymerase (Roche, Germany), and 0.6 μM of each primer (for 16S rRNA) or 0.2 μM of each primer (for ITS1). The 5′ ends of the forward and reverse primers were tagged with CS1 (ACACTGAC GACATGGTTCTACA) and CS2 (TACGGTAGCAGAGA CTTGGTCT), respectively, which were used as anchors for the second PCR reaction. The conditions to amplify the prokaryotic 16S rRNA fragments consisted of an initial denaturation at 94 °C for 2 min, 33 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and elongation at 72 °C for 30 s followed by a final elongation at 72 °C for 7 min, and the conditions to amplify the ITS1 fragments consisted of an initial denaturation at 96 °C for 15 min, 33 cycles of denaturation at 96 °C for 30 s, annealing at 52 °C for 30 s and elongation at 72 °C for 1 min followed by a final elongation at 72 °C for 10 min.

The second PCR reaction was used to add barcodes to each sample and the Illumina sequencing adapters. This PCR reaction was performed in a 20-μl reaction mixture, containing 1 × PCR-buffer (Qiagen, Germany), 1.8 mM MgCl$_2$, 1 μl of 5% DMSO, 0.2 mM dNTP, 0.5 U *Taq* DNA polymerase (Roche), 2 μM of Illumina barcode, and 1 μl of 1/150 dilution

of the first PCR product. The PCR conditions were as follows: initial denaturation at 95 °C for 10 min (for 16S rRNA) or 15 min (for ITS1), 15 cycles of denaturation at 95 °C for 15 s (for 16S rRNA) or 30 s (for ITS1), annealing at 60 °C (for 16S rRNA) or 58 °C (for ITS1) for 30 s, and elongation at 72 °C for 1 min followed by a final elongation at 72 °C for 3 min.

After the second amplification, PCR products were quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, Canada) and the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (D-Mark, Canada). Multiple samples were pooled together to form a 16S rRNA and an ITS1 amplicon library in equal proportions based on their molecular weight and DNA concentrations, respectively. The two amplicon libraries were purified using calibrated Ampure XP beads (Agencourt, USA), and the average size and quantity of each library were assessed on the LabChip GX (Perkin Elmer, USA) instrument. Each library was then separately sequenced on Illumina MiSeq using the paired-end 250 kit at Génome Québec Innovation Centre at McGill University (Montréal, Canada). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA390038).

Bioinformatics analysis

The raw data were quality-filtered by QIIME (Version 1.9.1) (Caporaso et al. 2010). Briefly, sequences were joined and depleted of barcodes and primers. The reads were then scanned with a 4-base wide sliding window and cut when the average quality per base dropped below 20. Sequences shorter than 150 bp and homo-polymers and ambiguous base calls longer than six nucleotides were removed. The operational taxonomic units (OTUs) were clustered with 97% similarity using UPARSE (Edgar 2013), and the chimeric sequences were then identified and removed using UCHIME (Edgar et al. 2011). The OTUs with three or fewer reads were removed as they potentially originated from sequencing artifacts (Kunin et al. 2010). For soil prokaryotes, the representative sequence for each OTU was picked and its taxonomic affiliation was assigned by the Ribosomal Database Project classifier against the Silva (SSU123) 16S rRNA database (Quast et al. 2013; Wang et al. 2007). For soil fungi, a representative sequence for each OTU was picked and classified against the UNITE database (alpha version 12_11) (Nilsson et al. 2015). A total of 4,038,065 and 3,310,704 16S rRNA and ITS paired-end high-quality sequences were obtained from 90 soil samples, with average lengths 285 ± 19 bp and 279 ± 27 bp, respectively. Each sample had a different number of reads, which would introduce bias when estimating diversity indices (e.g., observed OTUs richness index, Heip_e evenness index, and Shannon diversity index) (Schöler et al. 2017; Vestergaard

et al. 2017). Therefore, to even the varying read numbers, all the samples were randomly re-sampled to the lowest read number in the fall, spring, and summer season by QIIME (Weiss et al. 2015). Therefore, 40,910 (Fall), 9640 (spring), and 26,780 (summer) randomly selected 16S rRNA sequences were used to calculate prokaryotic α-diversity, and 26,230 (fall), 26,920 (spring), and 21,880 (summer) randomly selected ITS sequences were used to calculate fungal α-diversity. The prokaryotic weighted UniFrac distance was calculated based on the phylogenetic information of samples.

Statistical analysis

All the data analyses were computed by the Vegan package in R (R Core Team 2013). There were three factors for the statistical analyses of data: fertilization (MF, MF14, MF30, CNF, and Con), water management (irrigated and rain-fed), and season (fall, spring, and summer). Univariate permutational analysis of variances test (PERMANOVA; (Anderson 2001)) with 999 permutations was performed to analyze the effect of fertilization and water management on soil microbial α-diversity and soil chemical properties. Differences in the microbial α-diversity index, soil chemical properties, and relative abundances of major microbial taxa among the five fertilization treatments or two water management were compared using analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test. The p values in the Tukey's HSD test were adjusted by the Benjamini-Hochberg false discovery rate correction. Multivariate PERMANOVA test with 999 permutations was performed to determine how fertilization and water management factors affected soil microbial community composition based on weighted Unifrac distance and Bray-Curtis distance for prokaryotes and fungi, respectively. Dissimilarities in the microbial community composition between pairwise fertilization treatments were investigated by multivariate PERMANOVA, in which the "strata" option was performed to constrain permutation of the fertilization treatments within each water management group (Anderson 2001). Principal coordinate analyses (PCoA) based on the weighted Unifrac distance (prokaryotes) and Bray-Curtis distance (fungi) were used to visualize the distribution patterns of microbial community composition in relation to fertilization treatments in each season. Distance-based redundancy analysis (dbRDA) based on the weighted Unifrac distance (prokaryotes) and Bray-Curtis distance (fungi) were performed to estimate the proportion of variability in the microbial community composition that was explained by each soil chemical property, and marginal tests were performed to test the significance of each explanation.

Results

Long-term fertilization and water management effect on soil microbial α-diversity

Fertilization significantly affected prokaryotic Heip_e and Shannon indices in fall, which were both significantly lower in MF than the other fertilization treatments (Table 1). In spring, soil prokaryotic OTUs, Heip_e, and Shannon indices were significantly greater with rain-fed than irrigated management, but fertilization treatments had no significant effect (Table 1). The opposite result was observed for the prokaryotic OTUs in summer, where significantly more OTUs were detected in irrigated than rain-fed treatments (Table 1). The fertilization × water management interaction on soil prokaryotic α-diversity was not significant at any time (Table 1).

Fertilization had no effect on fungal OTUs and Shannon indices across the three seasons, but significantly affected fungal the Heip_e index in fall, with this index being significantly lower in MF30 than MF14 soils (Table 2). Water management significantly affected the fungal Heip_e index in all three seasons with significantly greater values in rain-fed than irrigated plots in spring and summer (Table 2). By contrast, there were more OTUs in irrigated than rain-fed plots in spring (Table 2).

Long-term fertilization and water management effect on soil microbial community composition

Fertilization and water management significantly affected soil prokaryotic and fungal community composition in all three seasons. There was also a significant fertilization × water management interaction on soil prokaryotic community composition in fall and summer, and on soil fungal community composition in summer (Table 3). For both prokaryotes and fungi, MF14 and CNF community compositions were similar to the Con.

For soil prokaryotic community composition, the PCoA ordinations mainly separated irrigated and rain-fed management (PERMANOVA: $F > 5.1$, $p = 0.001$) (Table 3) on the second axis in fall and summer (Fig. 1a, c), and on the first axis in spring (Fig. 1b), explaining 23.31, 23.04, and 53.76% of the total variation, respectively. The influence of fertilization on prokaryotic community composition became evident on the first axis in fall and summer (Fig. 1a, c), and on the second axis in spring (Fig. 1b), which explained 38.38, 17.32, and 35.71% of the total variation, respectively. The MF treatment resulted in distinct clusters from other fertilizer treatments in the ordination patterns in all three seasons regardless of the water management (PERMANOVA: $F > 2.8$, $p < 0.01$)

Table 1 Effects of fertilization and water management on soil prokaryotic α-diversity[a]

Main test[b]	Fall			Spring			Summer		
	OTUs F (P)	Heip_e	Shannon	OTUs F (P)	Heip_e	Shannon	OTUs F (P)	Heip_e	Shannon
Fertilization ($F_{4,20}$)	1.2 (0.33)	**5.1 (0.005)**	**4.6 (0.009)**	1.5 (0.25)	1.3 (0.31)	1.5 (0.25)	0.6 (0.65)	1.2 (0.34)	1.1 (0.38)
Water management ($F_{1,20}$)	0.1 (0.71)	0.1 (0.79)	0.0 (0.91)	**21.0 (< 0.001)**	**177.2 (< 0.001)**	**79.6 (< 0.001)**	**6.5 (0.02)**	0.1 (0.72)	3.6 (0.07)
Fertilization × water management ($F_{4,20}$)	0.8 (0.53)	1.3 (0.31)	1.5 (0.25)	0.9 (0.47)	0.7 (0.63)	0.7 (0.61)	0.3 (0.88)	1.2 (0.34)	0.49 (0.74)
Multiple comparison[c]	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
Con ($n = 6$)	6143 a	**0.173 a**	**10.06 a**	3874 a	0.461 a	10.84 a	7349 a	0.303 a	11.11 a
CNF ($n = 6$)	6128 a	**0.174 a**	**10.06 a**	3878 a	0.464 a	10.81 a	7515 a	0.303 a	11.15 a
MF ($n = 6$)	6115 a	**0.157 b**	**9.90 b**	4059 a	0.475 a	10.90 a	7632 a	0.317 a	11.23 a
MF30 ($n = 6$)	6159 a	**0.171 a**	**10.04 a**	4014 a	0.460 a	10.84 a	7220 a	0.302 a	11.09 a
MF14 ($n = 6$)	6254 a	**0.177 a**	**10.11 a**	3810 a	0.459 a	10.76 a	7181 a	0.302 a	11.08 a
Multiple comparison[c]	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
Irrigated ($n = 15$)	6152 a	0.171 a	10.04 a	**3775 b**	**0.431 b**	**10.66 b**	**7655 a**	0.304 a	11.18 a
Rain-fed ($n = 15$)	6168 a	0.170 a	10.03 a	**4119 a**	**0.497 a**	**11.00 a**	**7102 b**	0.306 a	11.08 a

[a] Prokaryotic included bacterial and archaeal 16S r RNA sequences

[b] Effect of fertilization, water management, and their interactions were analyzed by univariate permutational analysis of variance (PERMANOVA; degrees of freedom for each factor and residuals are given in brackets). Values represent the pseudo-F ratio (F) and the level of significance (P). Fertilization factor included Con, CNF, MF, MF30, and MF14, and water management included irrigated and rain-fed plots

[c] Mean value (mean) under each fertilization treatment or each water management. *CNF* chemical fertilizers, *Con* no fertilizer control, *MF* manure, *MF30* manure application discontinued in 2003, *MF14* manure application discontinued in 1997. Means with different letters are significantly different with $p < 0.05$ assessed by Tukey's HSD test. Values of $P < 0.05$ are shown in bold

Table 2 Effects of fertilization and water management on soil fungal α-diversity

Main test[a]	Fall			Spring			Summer		
	OTUs F (P)	Heip_e	Shannon	OTUs F (P)	Heip_e	Shannon	OTUs F (P)	Heip_e	Shannon
Fertilization ($F_{4,20}$)	0.6 (0.64)	**6.0 (0.002)**	2.3 (0.10)	1.0 (0.42)	1.0 (0.45)	1.8 (0.17)	0.2 (0.93)	1.4 (0.27)	1.1 (0.37)
Water management ($F_{1,20}$)	0.9 (0.35)	**5.4 (0.03)**	1.3 (0.27)	2.0 (0.17)	**6.2 (0.02)**	2.2 (0.15)	**14.4 (0.001)**	**8.5 (0.009)**	0.5 (0.48)
Fertilization × water management ($F_{4,20}$)	0.8 (0.54)	2.3 (0.09)	2.4 (0.09)	1.2 (0.34)	0.3 (0.85)	0.5 (0.74)	0.4 (0.79)	**5.0 (0.006)**	2.8 (0.06)
Multiple comparison[b]	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
Con ($n = 6$)	355 a	**0.126 ab**	5.50 a	470 a	0.115 a	5.74 a	398 a	0.110 a	5.41 a
CNF ($n = 6$)	373 a	**0.121 ab**	5.48 a	473 a	0.109 a	5.68 a	402 a	0.111 a	5.47 a
MF ($n = 6$)	368 a	**0.120 ab**	5.47 a	428 a	0.092 a	5.25 a	377 a	0.106 a	5.33 a
MF30 ($n = 6$)	365 a	**0.097 b**	5.16 a	450 a	0.105 a	5.56 a	392 a	0.093 a	5.16 a
MF14 ($n = 6$)	339 a	**0.142 a**	5.60 a	476 a	0.107 a	5.67 a	338 a	0.103 a	5.28 a
Multiple comparison[b]	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
Irrigation ($n = 15$)	367 a	**0.114 b**	5.39 a	472 a	**0.096 b**	5.48 a	**431 a**	**0.097 b**	5.37 a
Rain-fed ($n = 15$)	353 a	**0.128 a**	5.50 a	445 a	**0.115 a**	5.68 a	**348 b**	**0.113 a**	5.30 a

[a] Effect of fertilization, water management, and their interactions were analyzed by univariate permutational analysis of variance (PERMANOVA; degrees of freedom for each factor and residuals are given in brackets). Values represent the pseudo-F ratio (F) and the level of significance (P). Fertilization factor included Con, CNF, MF, MF30, and MF14, and water management included irrigated and rain-fed plots

[b] Mean value (mean) under each fertilization treatment or each water management. *CNF* chemical fertilizers, *Con* no fertilizer control, *MF* manure, *MF30* manure application discontinued in 2003, *MF14* manure application discontinued in 1997. Means with different letters are significantly different with $p < 0.05$ assessed by Tukey's HSD test. Values of $P < 0.05$ are shown in bold

(Fig. 1a, b, c and Table 3), as well as the MF30 treatment in rain-fed management in fall (PERMANOVA: $F > 2.0$, $p < 0.01$) (Fig. 1a and Table 3).

For soil fungal community composition, a weak separation of the two water management treatments was observed along the first axis in fall (Fig. 1d) and the second axis in spring and summer (Fig. 1e, f). The MF treatment produced distinct clusters from other fertilization treatments in fall and summer (PERMANOVA: $F > 2.8$, $p = 0.003$) (Fig. 1d, f and Table 3).

The effect of fertilization on relative abundance of major microbial taxa

Overall, prokaryotic sequences were assigned to 23 bacterial phyla and 2 archaeal phyla (Fig. S1). The dominant phyla were Actinobacteria (mean relative abundance ± standard deviation, 20.61 ± 3.91%), Proteobacteria (20.02 ± 7.15%), Acidobacteria (15.23 ± 4.19%), Chloroflexi (12.92 ± 5.24%), Planctomycete (6.26 ± 1.56%), Bacteroidetes (6.23 ± 4.97%), Crenarchaeota (5.93 ± 2.03%), Gemmatimonadetes (3.30 ± 0.94%), Firmicutes (2.47 ± 1.28%), and Verrucomicrobia (2.19 ± 1.34%), accounting for more than 95% of the total prokaryotic relative abundance (Fig. S1 and Fig. 2).

The relative abundance of *Firmicutes* in fall (irrigated plot; Fig. 2a), *Chloroflexi* in spring and summer (rain-fed plot; Fig. 2d, f), and *Gemmatimonadetes* in summer (rain-fed plot; Fig. 2f) was greater with MF than the other four fertilizer treatments. Compared to the Con, MF treatment significantly decreased the relative abundance of *Bacteroidetes* in fall (irrigated plot; Fig. 2a), *Actinobacteria* in fall (rain-fed plot; Fig. 2b) and summer (irrigated and rain-fed plots; Fig. 2e, f), and *Acidobacteria* in fall and spring (rain-fed plot; Fig. 2b, d). Similar effects of MF on prokaryotes at the class level were also observed. The MF treatment had greater abundance of *Gammaproteobacteria* (Fig. S2b, d, f) and *Gemmatimonadetes* (Fig. S2f), and lower abundance of *Thermoleophilia* (Fig. S2a, b, c, d, e, f) and *Acidobacteria-6* (Fig. S2b, c, d) compared to the Con. The effect of MF30 was more pronounced at the class level, with greater abundance of *Gitt-GS-136* (Fig. S2a), *Ellin6529* (Fig. S2a, b), *Acidimicrobiia* (Fig. S2a), *Chloracidobacteria* (Fig. S2b), and *Thaumarchaeota* (Fig. S2b) than the Con.

The fungal sequences were assigned to six phyla. *Ascomycota* (32.32 ± 8.82%) and *Basidiomycota* (7.30 ± 8.84%) were the major phyla (Fig. S3). The fungal data were presented at the class level since there were only six phyla. The dominant classes were *Sordariomycetes* (19.24 ± 6.38%), *Dothideomycetes* (2.91 ± 2.38%), *Eurotiomycetes* (1.81 ± 2.08%), *Agaricomycetes* (1.47 ± 3.33%), *Tremellomycetes* (1.25 ± 2.14%), and *Incertae_sedis* (1.00 ± 0.99%), accounting for more than 27% of the total fungal relative abundance (Fig. S3 and Fig. 3).

The relative abundances of most fungal class taxa showed no effects of manure application history, except

Table 3   Effects of fertilization and water management on soil microbial community composition

Main test[b]	Prokaryotic[a]						Fungi					
	Fall		Spring		Summer		Fall		Spring		Summer	
	F	P	F	P	F	P	F	P	F	P	F	P
Fertilization ($F_{4,20}$)	**5.42**	**0.001**	**1.94**	**0.009**	**3.69**	**0.001**	**2.66**	**0.001**	**2.38**	**0.002**	**2.40**	**0.001**
Water management ($F_{1,20}$)	**10.52**	**0.001**	**5.17**	**0.001**	**8.97**	**0.001**	**4.61**	**0.001**	**3.40**	**0.009**	**4.58**	**0.001**
Fertilization × water management ($F_{4,20}$)	**1.60**	**0.04**	0.61	0.96	**1.51**	**0.02**	0.97	0.53	0.66	0.93	**1.37**	**0.03**
Pairwise comparison[c]												
CNF vs. Con	0.53	0.80	0.42	0.82	0.65	0.59	0.99	0.27	0.42	0.94	0.72	0.66
MF vs. Con	**5.65**	**0.004**	**3.64**	**0.01**	**4.53**	**0.005**	**3.36**	**0.004**	**3.46**	**0.04**	**2.55**	**0.004**
MF vs. CNF	**6.65**	**0.007**	**2.80**	**0.02**	**4.70**	**0.006**	**4.93**	**0.003**	**3.79**	**0.02**	**3.90**	**0.006**
MF vs. MF30	**6.43**	**0.005**	**2.83**	**0.01**	**3.64**	**0.004**	**3.69**	**0.004**	**4.39**	**0.005**	**2.83**	**0.006**
MF vs. MF14	**6.99**	**0.006**	**3.60**	**0.006**	**5.12**	**0.008**	**3.62**	**0.008**	**5.85**	**0.003**	**4.45**	**0.006**
MF30 vs. MF14	**2.03**	**0.01**	0.51	0.68	1.04	0.18	**1.65**	**0.006**	1.15	0.23	1.33	0.09
MF30 vs. Con	**3.85**	**0.006**	1.17	0.18	**1.66**	**0.004**	**2.36**	**0.003**	1.66	0.08	1.11	0.18
MF14 vs. Con	0.95	0.17	0.71	0.62	0.90	0.24	0.99	0.27	1.37	0.14	0.96	0.25

[a] Prokaryotic included bacterial and archaeal 16S r RNA sequences

[b] Effect of fertilization, water management, and their interactions were analyzed by multivariate permutational analysis of variance (PERMANOVA; degrees of freedom for each factor and residuals are given in brackets). Fertilization factor included Con, CNF, MF, MF30, and MF14, and water management included irrigated and rain-fed plots

[c] Pairwise comparisons were analyzed by multivariate permutational analysis of variance (PERMANOVA) and constrained the permutation of samples within each water management group. *CNF* chemical fertilizers, *Con* no fertilizer control, *MF* manure, *MF30* manure application discontinued in 2003, *MF14* manure application discontinued in 1997. Values represent the pseudo-F ratio (*F*) and the level of significance (*P*). Values of $p < 0.05$ are shown in bold
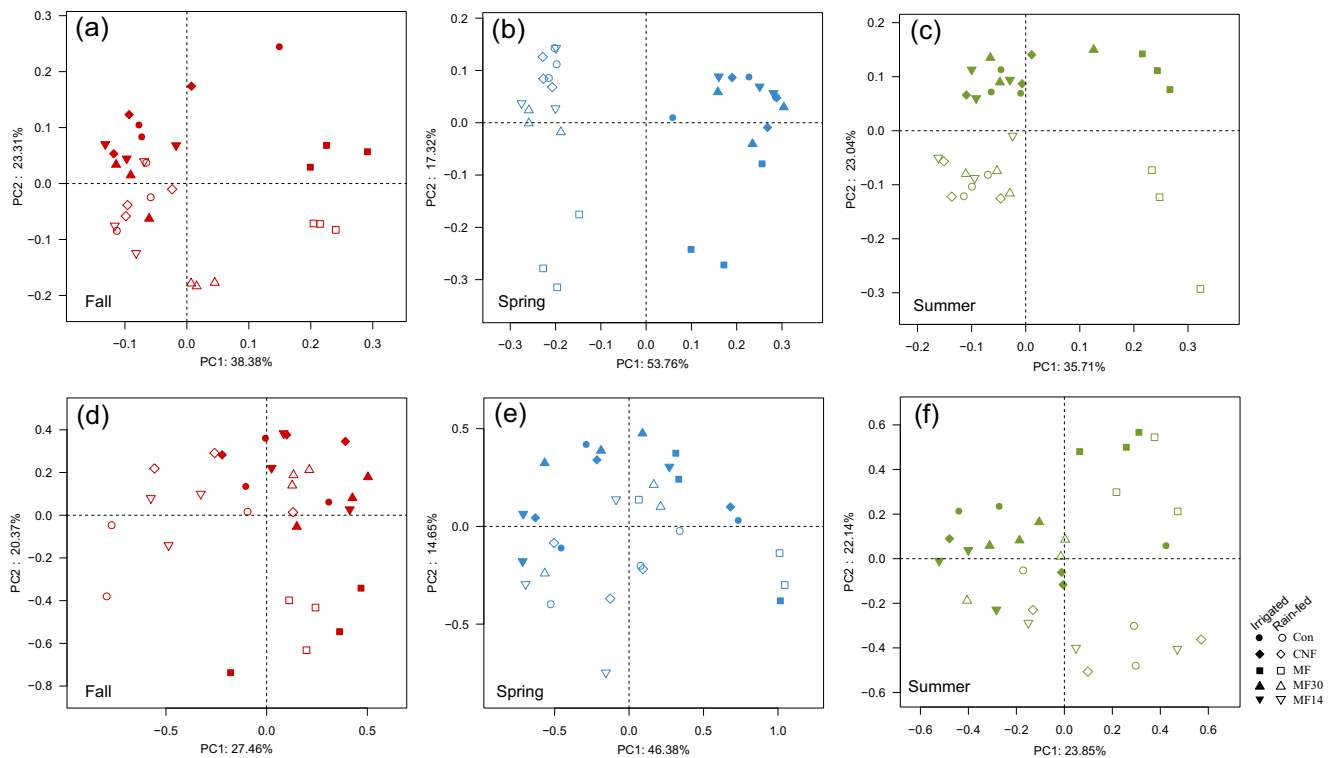

Fig. 1 Principal coordinate analysis (PCoA) of prokaryotic (**a**, **b**, and **c**) and fungi (**d**, **e**, and **f**) community composition. *CNF* chemical fertilizers, *Con* no fertilizer control, *MF* manure, *MF30* manure application discontinued in 2003, *MF14* manure application discontinued in 1997

Fig. 2 Relative abundances of the dominant prokaryotic phyla after long-term continuous and discontinued fertilization. Prokaryotic included bacterial and archaeal 16 S r RNA sequences. The horizontal and vertical panels show different seasons (fall, spring and summer) and water management (irrigated and rain-fed), respectively. *CNF* chemical fertilizers, *Con* no fertilizer control, *MF* manure, *MF30* manure application discontinued in 2003, *MF14* manure application discontinued in 1997. Different letters indicate significant differences based on Tukey's HSD test *p* < 0.05. Only the phyla with significantly different relative abundances among fertilizations were labeled with letters. Error bars represent standard deviation (*n* = 3)
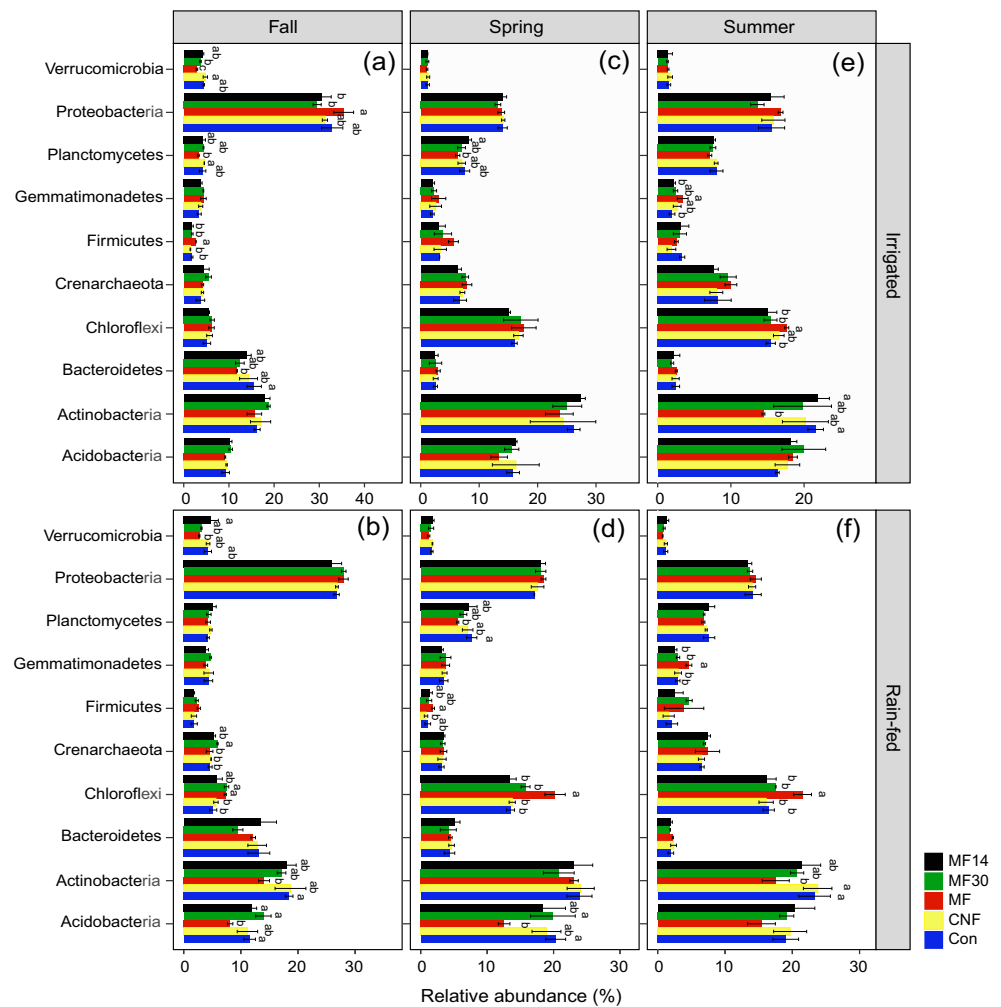
Sordariomycetes (Fig. 3b, d, f). The major family-level taxon of *Sordariomycetes*, *Microascaceae*, showed greater relative abundance in irrigated and rain-fed MF30 plots than Con and MF14 soils in fall and spring (Fig. S4 a, b, c, d) and also in summer for the rain-fed plots (Fig. S4f). A greater relative abundance of *Microascaceae* was also observed in the MF than the Con and MF14 soils in rain-fed plots in all three seasons (Fig. S4, b, d, f).

Relationships between soil chemical properties and microbial community composition

Fertilization, water management, and season significantly affected soil moisture, pH, and EC, while fertilization and season significantly affected soil C_{water} and AN. Soil AP was only significantly affected by fertilization (Table 4). Overall, soil chemical properties showed the most pronounced differences between MF and other fertilization treatments; the greatest were soil moisture, EC, C_{water},

AN, and AP, and the lowest was pH (Table 4). The MF30 also had greater C_{water} and AP and lower pH than Con, CNF, and MF14 (Table 4).

Distance-based RDA model analysis showed the individual soil chemical properties that explained the changes in soil microbial community composition (Table 5). In fall, soil moisture had the strongest influence on the soil prokaryotic and fungal community composition, explaining 11.64 and 7.25% of the total variance, respectively (Table 5). Soil AP also explained 8.91 and 5.09% of the total prokaryotic and fungal variance, respectively (Table 5). The effect of AP was most pronounced in spring when AP explained 12.48% of prokaryotic and 5.05% of fungal variance (Table 5). In summer, the most influential soil property was soil moisture, which explained 8.00 and 7.41% of the prokaryotic and fungal variance, respectively (Table 5). Soil AN explained an additional 5.79% of the prokaryotic variance, and soil AP, pH, and C_{water} explained 4.78, 4.52, and 4.46% of the fungal variance, respectively (Table 5).
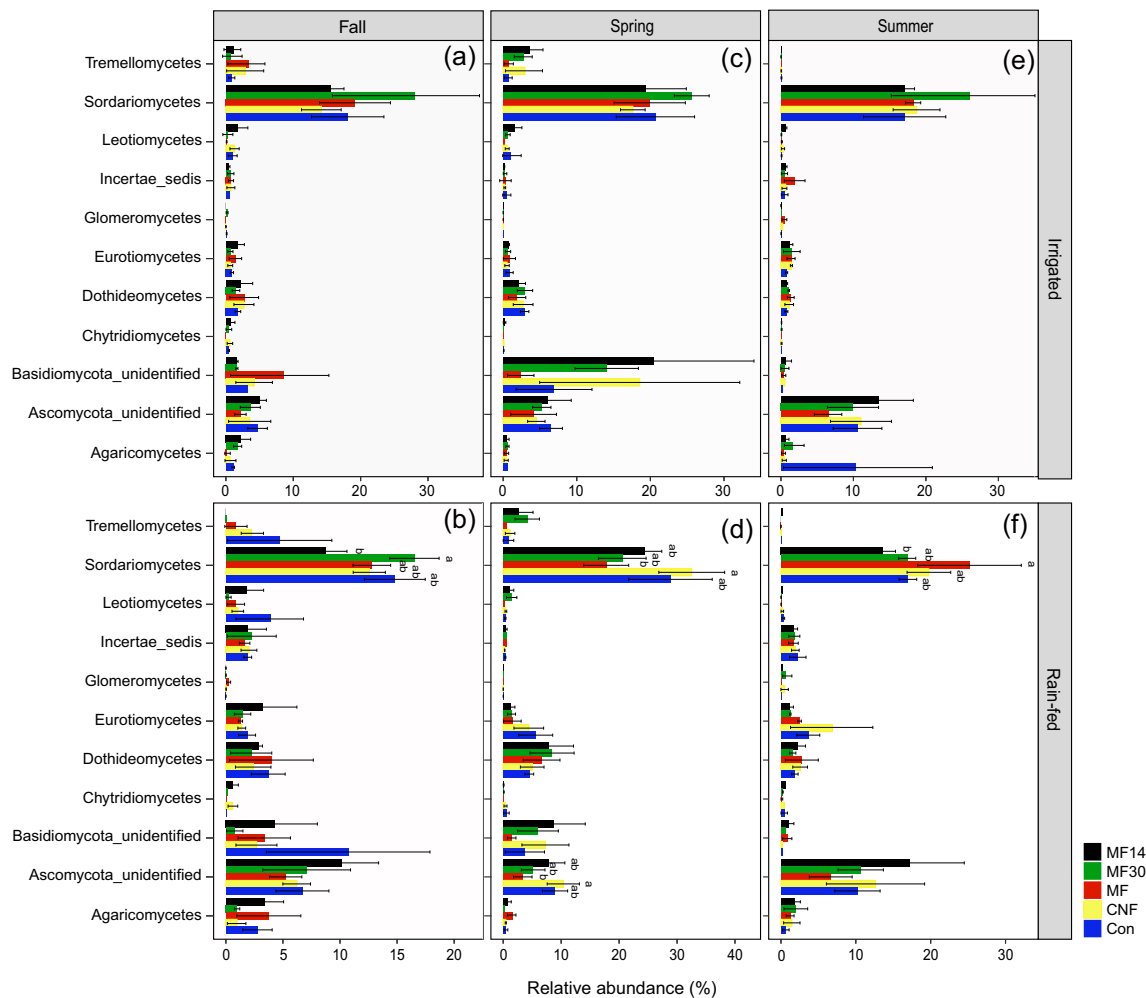
Fig. 3 Relative abundances of the dominant fungal classes after long-term continuous and discontinued fertilization. The horizontal and vertical panels show different seasons (fall, spring and summer) and water management (irrgation and rain-fed), respectively. *CNF* chemical fertilizers, *Con* no fertilizer control, *MF* manure, *MF30* manure application discontinued in 2003, *MF14* manure application discontinued in 1997. Different letters indicate significant differences based on Tukey's HSD test $p < 0.05$. Only the phyla with significantly different relative abundances among fertilizations were labeled with letters. Error bars represent standard deviation ($n = 3$)

Discussion

Effects of water management on the microbial community diversity and composition

Soil moisture appeared to be the strongest determinant of the soil microbial community composition in fall and summer. Our study was located in a semi-arid region of Canada, and soil moisture content was mainly regulated by water management (irrigated vs. rain-fed) (Table 4), which showed a strong effect on soil prokaryotic α-diversity in spring, fungal evenness, and prokaryotic and fungal community composition. Soil water availability has also been shown to directly (Manzoni et al. 2012) or indirectly (Hartmann et al. 2016) impact soil microbial community composition in other studies. Bastida et al. (2017) reported that restricted irrigation

could reduce microbial biomass and enzyme activities. Although water management had significant impacts on soil moisture, pH, and EC in our study, it did not affect soil C, N, and P concentrations (Table 4). In addition, the long-term and legacy effects of manure on soil microbial community composition were similar under irrigated and rain-fed soil conditions, i.e., the soil microbial community composition responded similarly to manure regardless of water management (see below).

Effect of annual manure fertilization on the microbial composition

Continuous annual fertilization with manure (MF) for 43 years (1973–2016) provided a unique opportunity to evaluate the effect of long-term manure application on soil microbial

Table 4 Effects of fertilization and water management on soil chemical properties

Main test[a]	Moisture $F(P)$	pH $F(P)$	EC $F(P)$	C_{water} $F(P)$	AN $F(P)$	AP $F(P)$
Fertilization ($F_{4,60}$)	35.8 (< 0.001)	38.9 (< 0.001)	75.9 (< 0.001)	59.3 (< 0.001)	54.1 (< 0.001)	88.1 (< 0.001)
Water management ($F_{1,60}$)	185.1 (< 0.001)	12.2 (< 0.001)	7.4 (0.008)	2.6 (0.11)	3.5 (0.06)	3.9 (0.05)
Season ($F_{2,60}$)	401.3 (< 0.001)	20.9 (< 0.001)	10.3 (< 0.001)	5.4 (0.007)	45.9 (< 0.001)	1.8 (0.17)
Fertilization × water management ($F_{4,60}$)	1.5 (0.22)	3.7 (0.009)	8.4 (< 0.001)	1.2 (0.34)	12.1 (< 0.001)	2.7 (0.04)
Fertilization × season ($F_{8,60}$)	10.3 (< 0.001)	0.4 (0.93)	0.5 (0.89)	0.4 (0.90)	1.8 (0.11)	1.7 (0.12)
Management × season ($F_{2,60}$)	21.9 (< 0.001)	2.2 (0.12)	8.8 (< 0.001)	2.5 (0.09)	18.4 (<0.001)	2.1 (0.13)
Fertilization × water management × season ($F_{8,60}$)	0.6 (0.77)	0.4 (0.94)	3.8 (0.001)	0.9 (0.58)	2.0 (0.06)	0.7 (0.70)
Multiple comparison[b]	Mean	Mean	Mean	Mean	Mean	Mean
	%		μs/cm	mg/kg	mg/kg	mg/kg
Con ($n = 18$)	14.72 b	7.87 a	362.7 b	86.3 c	40.21 b	152.0 d
CNF ($n = 18$)	15.26 ab	7.87 a	383.5 b	81.0 c	43.94 b	145.1 d
MF ($n = 18$)	20.70 a	7.35 c	1198.7 a	202.1 a	97.66 a	438.3 a
MF30 ($n = 18$)	18.00 ab	7.61 b	368.6 b	118.2 b	41.55 b	302.3 b
MF14 ($n = 18$)	16.25 ab	7.72 ab	292.5 b	93.9 bc	30.71 b	213.9 c
Multiple comparison[b]	Mean	Mean	Mean	Mean	Mean	Mean
	%		μs/cm	mg/kg	mg/kg	mg/kg
Irrigated ($n = 45$)	19.45 a	7.63 a	468.4 a	121.0 a	47.76 a	261.9 a
Rain-fed ($n = 45$)	14.52 b	7.74 a	484.5 a	111.6 a	53.86 a	238.8 a

[a] Effect of fertilization, water management, season, and their interactions were analyzed by univariate permutational analysis of variance (PERMANOVA; degrees of freedom for each factor and residuals are given in brackets). Values represent the pseudo-F ratio (F) and the level of significance (P). Fertilization factor included Con, CNF, MF, MF30, and MF14. Water management included irrigated and rain-fed plots. Season factor included fall, spring, and summer

[b] Mean value (mean) under each fertilization treatment or each water management. *AN* available nitrogen, *AP* available phosphorus, *CNF* chemical fertilizers, *Con* no fertilizer control, C_{water} non-purgeable water-soluble organic carbon, *EC* electrical conductivity, *MF* manure, *MF30* manure application discontinued in 2003, *MF14* manure application discontinued in 1997. Means with different letters are significantly different with $p < 0.05$ assessed by Tukey's HSD test. Values of $p < 0.05$ are shown in bold

diversity and community composition compared to soil that had stopped receiving manure (MF14, MF30), chemical N fertilizer (CNF) (1990–2016), and a non-amended control (Con) soil. Soil prokaryotic evenness and diversity immediately decreased after manure application in the MF treatment in fall, but it rebounded to slightly greater values than other fertilization treatments by the following spring and summer. Our previous work showed that when fertilization reduced soil pH to below 5.0, soil bacterial evenness and diversity also significantly declined, but these indices did not change when soil pH remained above 7.0 (Zhang et al. 2017). In the present study, although a slight decline of soil pH was observed in the manured plots, the pH remained above 7.3, which probably explains why microbial evenness and diversity did not change. In other studies, prokaryotic evenness (Hartmann et al. 2015) and diversity (Bonanomi et al. 2016; Tian et al. 2015) also declined after organic fertilization. However, Calleja-Cervantes et al. (2015) did not observe any changes in bacterial Shannon diversity 15 days after organic fertilization, and some studies reported an increase of soil prokaryotic diversity after long-term manure application (Francioli et al.

2016; Hartmann et al. 2015). These inconsistent findings may be partly explained by the sampling time because the soil microbial community will be disturbed for several days after the fertilization process, before recovering by their resilience (Allison and Martiny 2008). In the present study, the decreased prokaryotic evenness and diversity with MF was probably related to manure increasing the relative abundances of *Firmicutes* and *Proteobacteria* (Fig. 2a), which are major microbial groups in manure (Ryckeboer et al. 2003; St-Pierre and Wright 2014). During spring and summer, the weakening of the manure effect and increased microbial activity likely supported the recovery of prokaryotic evenness and diversity.

Compared to CNF and Con, the MF treatment had significantly distinct effects on the soil prokaryotic and fungal community composition regardless of water management during fall, spring, and summer, as shown on the PCoA ordination plots (Fig. 1) as well as PERMANOVA analyses (Table 3). Such manure effects on the soil microbial community composition are consistent with a number of other studies in different agroecosystems (Francioli et al. 2016; Hartmann et al. 2015; Tian et al. 2015). Unlike those previous studies, our study did

Table 5 Relationships between soil chemical properties and microbial community composition

	Prokaryotic[a]						Fungi					
	Moisture VC (P)	pH VC (P)	EC VC (P)	C_{water} VC (P)	AN VC (P)	AP VC (P)	Moisture VC (P)	pH VC (P)	EC VC (P)	C_{water} VC (P)	AN VC (P)	AP VC (P)
Fall	**11.64%**	2.03%	1.71%	2.10%	3.40%	**8.91%**	7.25%	3.33%	1.89%	3.03%	1.94%	**5.09%**
	(0.001)	(0.40)	(0.62)	(0.38)	(0.04)	**(0.001)**	**(0.003)**	(0.22)	(0.83)	(0.36)	(0.81)	**(0.03)**
Spring	2.73%	3.12%	4.60%	4.37%	4.83%	**12.48%**	2.96%	3.62%	4.72%	4.11%	3.34%	**5.05%**
	(0.35)	(0.23)	(0.07)	(0.10)	(0.07)	**(0.002)**	(0.33)	(0.16)	(0.08)	(0.14)	(0.22)	**(0.05)**
Summer	**8.00%**	2.25%	3.12%	2.67%	**5.79%**	1.95%	**7.41%**	**4.52%**	3.74%	**4.46%**	3.91%	**4.78%**
	(0.001)	(0.31)	(0.08)	(0.16)	**(0.004)**	(0.49)	**(0.001)**	**(0.03)**	(0.10)	**(0.04)**	(0.06)	**(0.03)**

Values represent the estimation variance component (VC) that explained the distribution of microbial community composition, and the level of significance (P). Values at $P < 0.05$ are shown in bold

AN available nitrogen, *AP* available phosphorus, C_{water} non-purgeable water-soluble organic carbon, *EC* electrical conductivity

[a] Prokaryotic included bacterial and archaeal 16S r RNA sequences

not reveal any differences in the prokaryotic and fungal community composition between CNF and Con. This was probably related to the similar soil chemical properties between the two treatments, as the shift in soil microbial community composition generally corresponded with changes in soil nutrient availability. These differences reinforce the observation that the effects of inorganic fertilizers on soil microorganisms can be negative, neutral, or positive.

Similar to manure effects on soil microbial diversity, soil pH has been suggested to be the major factor that drives soil bacterial community composition (Pietri and Brookes 2008; Rousk et al. 2009, 2010; Zhang et al. 2017). Our previous study showed that soil bacterial community composition would not be affected by soil pH if the soil was buffered against pH changes in an alkaline soil (Zhang et al. 2017). Therefore, soil pH was unlikely to be the major driver of soil bacterial community composition in fall, spring, and summer seasons in the current study because the pH remained above 7.3. However, the MF treatment elevated soil C, N, and P concentrations relative to other fertilization regimes (Table 4), and it was probably these elements that were important drivers of bacterial community composition. An oligotroph-copiotroph strategy shift of soil bacteria with changes of soil nutrient availability has been observed, i.e., high nutrient availability promotes the growth of copiotrophic organisms while low nutrient levels lead to an increase of slow-growing oligotrophic microorganisms (Fierer et al. 2007, 2012). *Proteobacteria* are considered as copiotrophic microorganisms (Fierer et al. 2007, 2012) and were one of the predominant taxa in the soils of the present study. Although the relative abundance of the *Proteobacteria* phylum was not different among MF and other fertilizer treatments (Fig. 2), *Gammaproteobacteria* showed the greatest relative abundance with MF (Fig. S2b, d, f). *Firmicutes* is considered a major copiotrophic microbial group and some *Firmicutes* genera play important roles in the manure

degradation process (Ryckeboer et al. 2003). Hartmann et al. (2015) and Bonanomi et al. (2016) reported that *Firmicutes* were significantly more abundant in manured farming systems. However, in our study, the increased relative abundance of *Firmicutes* associated with manure was only observed in fall. Consistent with previous studies (Li et al. 2017; Whitman et al. 2016), *Gemmatimonadetes* also showed copiotrophic features in this study, and had greater relative abundance with MF than the Con soil. *Bacteroidetes* is another typically copiotrophic group (Fierer et al. 2007) and has been positively correlated with soil total P (Tian et al. 2015) and soluble P (Yashiro et al. 2016). However, our study found a lower relative abundance of *Bacteroidetes* with MF. Most members of *Acidobacteria* have been suggested to be slow-growing oligotrophic microorganisms (Fierer et al. 2007), and we observed the lower relative abundance of the *Acidobacteria* phylum in MF than the Con soil. This is probably a reflection of the *Acidobacteria* being more adapted to nutrient-limited soil environments (Fierer et al. 2012).

Legacy effect of manure on microbial composition

Discontinued manure application after 14 and 30 years represented suspending manure application for 29 (MF14) and 13 years (MF30), respectively, which provided unique conditions to evaluate the legacy effect of manure on the soil microbial community composition in irrigated and rain-fed farming systems under semi-arid climatic conditions. The legacy effect of organic amendments on soil microorganisms has been attributed to the decomposition of organic matter, plant growth, and changing edaphic factors (Bastida et al. 2008, 2013; Torres et al. 2015). In agroecosystems, soil microorganisms are directly affected by soil nutrients (Geisseler and Scow 2014; Leff et al. 2015), and the slow release of nutrients and dissolved C during manure decomposition can sustain soil microorganisms for long periods of time (Diacono and

Montemurro 2010). Previous studies have reported that changes in soil C, N (Hartmann et al. 2015; Li et al. 2017; Sun et al. 2015), or pH (Sun et al. 2015; Zeng et al. 2016; Zhang et al. 2017) caused by fertilization have the most pronounced effects on the soil microbial community composition. In the present study, distance-based RDA analysis showed that soil AP was the second strongest predictor of the soil microbial community composition, which explained 1.9–12.5% of the total variance (Table 5). This was probably due to soil P accumulating after manure application. When the application rate of manure is determined on the basis of N, the P added by manure often exceeds plant requirements, resulting in P accumulation in soil (Diacono and Montemurro 2010). Previous work at the study site has shown that P levels remained elevated even after suspending manure application for 16 years (Hao et al. 2008). The present study showed a greater concentration of AP in MF30 than MF14 and Con soils (Table 4).

The soil fungal community composition was less affected by the P accumulation resulting from manure application. Negative correlations between soil P concentration and the relative abundance of soil Glomeromycota have been reported (Lin et al. 2012; Tedersoo et al. 2014). However, in the present study, the relative abundance of *Glomeromycetes* did not differ among fertilization treatments (Fig. 3). Generally, most of the soil fungal taxa did not significantly respond to fertilizer treatments, except for *Sordariomycetes* (*Ascomycota*) and its major family member *Microascaceae*. This may be due to the limited coverage by the primers used. In the rain-fed plots of this study, *Microascaceae* had a greater relative abundance in MF and MF30 than Con and MF14 soils, indicating a strong residual effect of manure on soil *Microascaceae*, but the effect was inconsistent when manure application was suspended for 29 years. The ecological role of *Microascaceae* is not clear; only some members of this fungal taxon are recognized as plant pathogens (Zhang et al. 2006), while Lueders et al. (2006) suggested that *Microascaceae* may play an important role in degrading labile C.

After suspending manure application for 13 years (MF30), the relative abundances of soil *Acidimicrobia* (*Actinobacteria*), *Ellin6529* (*Chloroflexi*), *Gitt-GS-136* (*Chloroflexi*), and *Thaumarchaeota* were greater in MF30 than Con soil. Although Zhang et al. (2013) reported that the relative abundances of *Acidimicrobia* and *Gitt*-GS-136 were positively related to the manure application rate, there were no differences in these microbial taxa between MF30 and MF14 in our study (Fig. S2). The members of *Ellin6529* and *Acidimicrobia* were described as free-living N$_2$-fixing microbial groups (Dos Santos et al. 2012; Lopes et al. 2015), and *Thaumarchaeota* contains members that play important roles in ammonia oxidation (Stieglmeier et al. 2014). Free-living N$_2$-fixing bacteria are likely to be more abundant in N-limited environments (Deluca et al. 1996). As MF30 and

Con soils had similar AN concentrations, the higher relative abundance of these microbial groups in MF30 was probably related to the higher water-soluble C concentration than Con soil because when free-living bacteria fix atmospheric N$_2$, they require energy supplied by soil organic C. The soil with MF30 provided more C than the Con soil. But the legacy effect of manure was only observed in fall, and it was weaker or disappeared in the spring and summer, which was probably related to increased C and N mineralization rates in response to increasing soil temperatures and microbial activity. It is noteworthy that the microbial community composition was significantly different between MF and MF30, and the predominant microbial taxa in MF may not have persisted when manure application was discontinued, leading to the predominance of other microbial taxa that regulate soil C (*Microascaceae*) and N (*Acidimicrobiia*, *Ellin6529*, and *Thaumarchaeota*) cycling.

Conclusions

There were differences in soil microbial community compositions between irrigated and rain-fed plots, but fertilization treatments had similar effects on the prokaryotic and fungal community composition in both irrigated and rain-fed plots. Soil prokaryotic community composition was more sensitive than soil fungi to manure application in both the irrigated and rain-fed plots. Soil prokaryotic evenness and diversity significantly decreased 2 days after manure application (in fall), but had a slightly higher evenness and diversity than other fertilizer treatments in the spring and summer. Long-term fertilization with manure significantly increased the relative abundance of *Chloroflexi*, *Firmicutes*, *Gammaproteobacteria*, and *Gemmatimonadetes*, while significantly decreasing the relative abundance of *Acidobacteria*, *Bacteroidetes*, *Thermoleophilia*, and *Verrucomicrobia*. The changes in relative abundances of major microbial taxa induced a significantly distinct community composition in soils with 43 annual manure applications. Suspending manure application for 13 years led to a significantly different microbial community composition in fall, with a greater relative abundance of *Acidimicrobiia*, *Ellin6529*, *Gitt-GS-136*, *Microascaceae*, and *Thaumarchaeota* than in soil that did not receive manure. The increased relative abundance of *Acidimicrobiia*, *Ellin6529*, and *Thaumarchaeota* indicated the important legacy effect that manure may have in regulating soil N$_2$-fixing and ammonia-oxidizing microorganisms. Discontinuing manure applications for 29 years led to similar microbial diversity and community composition as that in the soil that did not receive manure. This study showed that manure application still had a legacy effect on soil microbial community composition after suspending manure application for 13 years, while the legacy effect of manure application was not evident after

suspending manure application for 29 years. In future studies, soil microbiome technology, such as metagenomics, transcriptomics, and proteomics, etc., should be complemented with enzyme assays to improve our understanding of the mechanisms by which manure affects the soil microbiome.

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