ROLE OF MICROBES IN CLIMATE SMART AGRICULTURE

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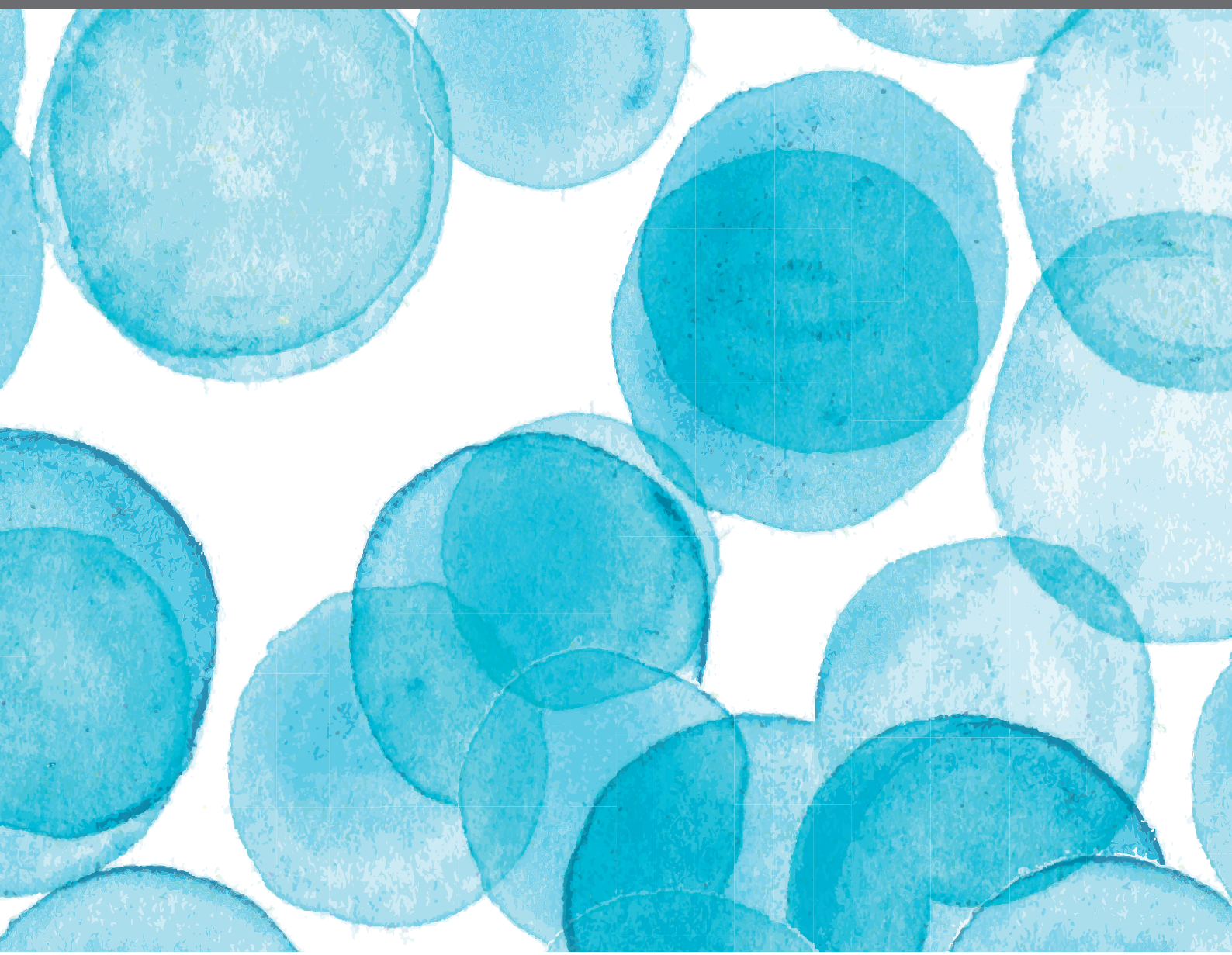

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ROLE OF MICROBES IN CLIMATE SMART AGRICULTURE

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EDITORIAL
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Editorial: Role of Microbes in Climate Smart Agriculture

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Keywords: sustainable agriculture, greenhouse gas emissions and mitigation, C transformation and stability, extreme weather events, elevated CO_2 and O_3

Editorial on the Research Topic

Role of Microbes in Climate Smart Agriculture

Soil microbes play an essential role in virtually all ecosystem processes, such that microbial abundance and activity determines the sustainable productivity of agricultural lands, ecosystem resilience against nutrient mining, degradation of soil and water resources, and GHG emissions (Wagg et al., 2014). Their activity is directly affected by changes in the environment. In this context, climate change is a relevant factor, with the potential to affect the role of microbes in the soil, which is vital to support agriculture worldwide. Climate-smart agriculture (CSA) is an approach that can help to reduce these impacts. CSA is an integrative approach to develop agricultural strategies for sustainably increasing agricultural productivity, adapting, and building resilience of agricultural and food security systems, and reducing agricultural greenhouse gas emissions under climate change scenarios (Lipper et al., 2014; Paustian et al., 2016). In this Research Topic, we aimed to provide the reader with a selection of studies to highlight novel experimental concepts such as process-oriented omics approaches with state-of-the-art technological advances in agricultural science to better understand how consequences of climate change such as elevated atmospheric CO_2 concentration (eCO_2), temperature, and drought affect soil microbes and associated ecosystem processes. In addition, the role of microbes in agricultural management that contribute to climate change adaptation, GHG mitigation, and soil carbon storage has been discussed.

As two core issues of global climate change, the constant rise in atmospheric CO_2 concentration and temperature have significant influences on ecosystem functioning (Mueller et al., 2016). In a study in semiarid grassland ecosystems, Yu et al. revealed the potential feedback response mechanism of soil microbiome to multiple climate change factors by the decrease in N cycling processes under warming, and increase in C and N cycling processes under either eCO_2 alone or in interaction with warming. In the context of increasing global atmospheric CO_2 concentration, grasslands behave as a potential C sink (Roy et al., 2016). Clipping (removal of aboveground plant biomass) is a common practice in grassland ecosystems, and this practice may reduce nutrient inputs into soils (Garibaldi et al., 2007), which in turn may affect microbial functionality and by extension, other ecosystem services. Accordingly, Guo et al. concluded that annual clipping shifted functional communities and enhanced the relative abundance of genes related to labile and recalcitrant C degradation with potential links to a clipping-induced acceleration of decomposition of C stored in grassland ecosystems.

The impact of climate warming on soil C and N dynamics has recently received considerable interest. Waghmode et al. revealed that climate warming and dried soil conditions remarkably increased the abundance of ammonia-oxidizing bacterial (AOB), concomitant to a reduction in the abundance of ammonia-oxidizing archaea and denitrifying bacteria, potentially affecting

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nitrogen turnover in the agro-ecosystem. The authors further suggested that, compared to regular irrigation (60 mm), the high irrigation (90 mm) overrode the warming effects on soil microbial community structure. The effects of extreme weather events on pathogen-antagonist interactions were evaluated in a perspective article by Meisner and de Boer. Extreme weather events like droughts or heavy precipitation are becoming more frequent and affect agricultural ecosystems (e.g., plant health and productivity). Soil-borne plant pathogens might become a bigger problem if microbial antagonists in soils are more strongly affected by the extreme weather conditions than the pathogens and can thus not suppress pathogens in soils. Different strategies of microorganisms to cope with water stress were discussed, and the potential for controlling soil-borne plant pathogens through enhancing growth of beneficial microorganisms under extreme weather conditions was highlighted in the perspective.

CSA emphasizes developing agricultural strategies not only to protect food security under climate change but also to mitigate GHG emissions and to improve soil C sequestration (Lipper et al., 2014). Biochar (the C-rich solid formed by pyrolysis of biomass) amendment in agricultural soil has been proposed as a way to abate climate change by sequestering C and mitigating GHG (particularly N_2O), while simultaneously increasing the crop yield (Woolf et al., 2010; Jiang et al., 2019). In an innovative research, Wang et al. revealed that the biochar predominately reduces CH_4 and N_2O emissions with high straw load, but not with low straw load, and this could be because biochar competes for electrons against methanogens and promotes methanotrophs, nitrifiers and denitrifiers. Agricultural intensification results in the enhanced re-investment of bio-based residues in agricultural soils, with consequences for GHG emissions (Ho et al., 2017). In this contest, Brenzinger et al. suggested that the combination of compost with one of the more nutrient-rich organic amendments such as sewage sludge digestate provides a trade-off between sustaining crop yield and reducing GHG emissions. Duan et al. documented that the application of catch crop residues leads to higher N_2O emissions, which could be due to net N mineralization and O_2 depletion coupled with the residue degradation in organic hotspots. The catch crop residue amendment can influence the N_2O production, but not the genetic potential of the community to produce and reduce N_2O. Further, Mohanty et al. advocated that biogenic nitrate and microbial volatile organic compounds (mVOCs) could have positive feedback effects on the nitrification rate in arable soils. To this end, Norton and Ouyang reviewed the status quo of the controlling factors and management practices of soil nitrification. Management strategies to reduce N losses, improve N use efficiency, and mitigate global climate change were recommended based on the latest understanding of the nitrification process.

The Research Topic further focused on the potential use of slag (byproducts generated during iron and steel manufacturing) fertilizer for sustainable agricultural production. Iron and steel production rose dramatically with the advent of the industrial revolution, and the volume of slag produced outpaced its consumption. Slags are rich in fertilizer components and their use in agriculture holds great promise for sustainable and eco-friendly agriculture (Gwon et al., 2018; Das et al., 2019). In a mini-review, Das et al. discussed the potential mechanisms of slag-microbe interactions in soil and how the interactions influence crop yield, GHG emissions, soil carbon sequestration, and heavy metal stabilization in contaminated soils.

CSA also emphasizes the sustainable development of livestock manure production for mitigating CH_4 emissions, since livestock production is a significant source of methane, mainly from enteric fermentation, dairy farming operations, and manure management (Laubach et al., 2015). In a study, Habtewold et al. concluded that the acidified dairy slurry suppressed CH_4 emissions, which could be due to the inhibition of *Methanosarcina* species.

The need for increased food production under CSA interventions increasingly shifted the focus to the role of soil biodiversity in general and arbuscular mycorrhizal (AM) fungi in particular. In a review, Sosa-Hernández et al. presented an overview on the current knowledge of subsoil ecology with the focus on arbuscular mycorrhizal fungi (AMF) and their potential significance for a sustainable agriculture. Practices of no-tillage, crop rotations, and cover cropping with deep rooting mycorrhizal plants may promote subsoil AM communities.

A deep understanding of microbial ecology and soil–plant–microbe interactions in a changing climate scenario is essential to use microbial technology for climate change adaptation and mitigation. This Research Topic contributes to the understanding of how climate changes affect soil microbes and ecosystem processes, and how agricultural practices under CSA interventions shifted microbiome for climate change adaptation, GHG mitigation, and soil C storage.

AUTHOR CONTRIBUTIONS

SD wrote the first draft of the editorial. All authors edited the editorial.

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Elevated CO_2 and Warming Altered Grassland Microbial Communities in Soil Top-Layers

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As two central issues of global climate change, the continuous increase of both atmospheric CO_2 concentrations and global temperature has profound effects on various terrestrial ecosystems. Microbial communities play pivotal roles in these ecosystems by responding to environmental changes through regulation of soil biogeochemical processes. However, little is known about the effect of elevated CO_2 (eCO_2) and global warming on soil microbial communities, especially in semiarid zones. We used a functional gene array (GeoChip 3.0) to measure the functional gene composition, structure, and metabolic potential of soil microbial communities under warming, eCO_2, and eCO_2 + warming conditions in a semiarid grassland. The results showed that the composition and structure of microbial communities was dramatically altered by multiple climate factors, including elevated CO_2 and increased temperature. Key functional genes, those involved in carbon (C) degradation and fixation, methane metabolism, nitrogen (N) fixation, denitrification and N mineralization, were all stimulated under eCO_2, while those genes involved in denitrification and ammonification were inhibited under warming alone. The interaction effects of eCO_2 and warming on soil functional processes were similar to eCO_2 alone, whereas some genes involved in recalcitrant C degradation showed no significant changes. In addition, canonical correspondence analysis and Mantel test results suggested that NO_3-N and moisture significantly correlated with variations in microbial functional genes. Overall, this study revealed the possible feedback of soil microbial communities to multiple climate change factors by the suppression of N cycling under warming, and enhancement of C and N cycling processes under either eCO_2 alone or in interaction with warming. These findings may enhance our understanding of semiarid grassland ecosystem responses to integrated factors of global climate change.

Keywords: elevated carbon dioxide, warming, soil microbial community, Prairie Heating and CO_2 Enrichment (PHACE) experiment, functional genes, grassland ecosystem

INTRODUCTION

With the rapid and continuous increase in fossil fuel emissions since the beginning of the Industrial Revolution, the concentration of atmospheric CO_2 has risen sharply from 280 to 406.53 ppm in 2017 (Ruddiman, 2013; Pieter Tans, 2017). The high levels of CO_2 and other greenhouse gasses have led to an increased global temperature and reduced precipitation (IPCC, 2014). Soil microbial communities (i.e., bacteria, archaea and fungi) are regarded as sensitive indicators of soil quality and are responsible for belowground carbon (C) and nutrient cycling in various ecosystems. The community structure and functional processes can be influenced by temperature and elevated CO_2 (eCO_2) both directly and indirectly via biotic and abiotic factors, such as soil C inputs, moisture and temperature (Castro et al., 2010). Therefore, it is crucial to understand the combined effect of eCO_2 and warming on the functional diversity, composition, structure and dynamics of soil microbial communities and their correlations with ecosystem processes.

Grass-dominated terrestrial ecosystems contain more than 10% of the global carbon (C) stock and account for over 30% of the global aboveground net primary production (NPP) (Jones and Donnelly, 2004; Grosso et al., 2008), and also provides the majority of forage for feeding livestock. The priming effects of CO_2 in grasslands are well known and include increased above- and belowground plant biomass, photosynthetic C allocation to roots, belowground C inputs and rhizodeposition (Pendall et al., 2004; Carol Adair et al., 2009; Adair et al., 2011; Carrillo et al., 2011; Lee et al., 2011). The combination of eCO_2 with warming and warming alone showed uncertain effects on above- and belowground production, C allocation, and the soil nitrogen (N) status, which may be highly correlated with soil water availability (Dijkstra et al., 2010, 2013a; Carrillo et al., 2011; Morgan et al., 2011). However, how eCO_2 and warming, particularly when combined, impact the functional diversity, composition, structure and functional processes of soil microbial communities are still unclear in water-constrained grasslands. For example, warming may increase biomass and microbial activity in a prairie ecosystem (Belay-Tedla et al., 2009), but the pattern may be altered under water limited conditions or reduced soil C inputs (Castro et al., 2010). Rising CO_2 may increase soil water availability, improving plant water-use efficiency (Wan et al., 2007; Leakey, 2009), but this effect may be offset by warming-induce desiccation in water-constrained ecosystems (Morgan et al., 2011). The effect of CO_2 and temperature on soil C may be mediated by the impact of these variables on soil water availability via regulation of decomposition and plant inputs in semiarid grassland ecosystems (Carrillo et al., 2011), which in turn alters the composition, structure and functional processes of microbial communities. However, the interactive effects of multiple global change factors (e.g., eCO_2, warming, elevated O_3 and precipitation) on soil microbial communities had been less well studied (Castro et al., 2010). Therefore, a comprehensive evaluation of the effect of warming and eCO_2 on soil microbial communities, especially in water limited ecosystems, is necessary.

To model the effects of eCO_2 and warming, a Prairie Heating and CO_2 Enrichment (PHACE) experiment was conducted on semiarid temperate mixed grass prairies in Wyoming, United States (Parton et al., 2007). The gross primary production, root biomass, ecosystem respiration, soil organic carbon, net soil nitrogen (N) release and mineralization associated with soil moisture were altered under multiple factor conditions (Dijkstra et al., 2010; Carrillo et al., 2011, 2012; Ryan et al., 2015, 2017; Mueller et al., 2016). For example, a previous study showed that eCO_2 significantly decreased soil inorganic N due to the increase of microbial N immobilization, and warming significantly increased soil inorganic N and plant N pool sizes, while the combined effects of eCO_2 and warming on N pool sizes were not significant (Dijkstra et al., 2010). These changes may directly or indirectly affect the structure and functional processes (e.g., C and N cycling) of the soil microbial community.

A high-throughput functional gene array (GeoChip 3.0) (He et al., 2010a) was employed to analyze the soil microbial communities in the above mentioned semiarid grassland experimental site. GeoChip 3.0 contains approximately 28,000 oligonucleotide probes involved in many biogeochemical functional processes [such as C, N, sulfur (S) and phosphorus (P) cycling], and has been used to examine the microbial communities from various environments (Yu et al., 2014a; Cai et al., 2015; Xiong et al., 2015; Xue et al., 2016a; Yu et al., 2018). In this study, we attempted to address whether (i) the functional composition and structure of soil microbial communities would be dramatically altered as soil C inputs and soil properties change in response to multiple climate factors; (ii) soil microbial functional processes (e.g., C and N cycling) would have different responses to warming, eCO_2 and the interaction between these two factors. This study has important implications for soil microbial communities in response to global climate changes in grassland ecosystems.

MATERIALS AND METHODS

Site Description and Sampling

The PHACE experiment was conducted at the United States Department of Agriculture's Agricultural Research Service (USDA-ARS) High Plains Grasslands Research Station in Cheyenne, WY, United States (latitude 41°11′N, longitude 104° 54′W). The ecosystem is dominated by two C3 grasses, *Hesperostipa comata* Trin and Rupr. and *Pascopyrum smithii* (Rydb.) and a C4 grass, *Bouteloua gracilis* (H.B.K.) Lag. The average annual precipitation is 388 mm (Zelikova et al., 2014), and the mean air temperature is −2.5°C in winter and 17.5°C in summer. The soil at the experimental site is a fine-loamy, mixed, mesic Aridic Argiustoll (Morgan et al., 2011).

Twenty 3.4 m diameter circular plots were constructed with a 60 cm deep impermeable barrier. The PHACE experiment was conducted in a full factorial design to evaluate the combined effect of CO_2 and temperature with five replicates per treatment. Plots were randomly assigned to four treatments including two concentrations of CO_2 treatment (ambient vs. 600 μmol mol^{-1}) since 2006, and two levels of warming treatment [ambient vs. warming of the canopy above ambient (+1.5°C,

day; +3.0°C, night)] since 2007: (i) ambient, ambient CO_2 and ambient temperature; (ii) warming, ambient CO_2 and elevated temperature; (iii) eCO_2, elevated CO_2 and ambient temperature; (iv) eCO_2 + warming, elevated CO_2 and elevated temperature. Warming and Free Air CO_2 Enrichment (FACE) technology was used as previously reported (Dijkstra et al., 2010; Morgan et al., 2011).

Five replicate samples were collected form each treatment plot (ambient, warming, eCO_2, eCO_2 + warming) at a soil depth of 0–5 cm in 2008. After the removal of plant residual roots and rocks, all PHACE soil samples were immediately stored at −80°C or 4°C for DNA extraction and soil property analysis, respectively.

Soil Property Analysis

Soil total carbon (TC) and nitrogen (TN) were measured by dry combustion using a Leco TruSpec carbon and nitrogen analyzer. The NO_3-N and NH_4-N were extracted from soil samples by the use of 1 M KCl solution and quantified by a Lachat Quickchem 8500 series 2 instrument (Lachat, Loveland, CO, United States). Soil pH was measured using a glass electrode in a 1:2.5 (soil:water) solution (w/v).

DNA Extraction and GeoChip Analysis

Soil DNA was extracted from 5 g soil samples using a freeze-grinding method (Zhou et al., 1996) and was purified using a Promega Wizard DNA clean-up system (Madison, WI, United States). DNA quality was measured using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, NC, United States) to determine 260/280 nm and 260/230 nm ratios, and DNA concentration was quantified with Quant-It PicoGreen (Invitrogen, Carlsbad, CA, United States). Approximately 3 μg purified DNA per sample was labeled with the fluorescent dye Cy-5 (GE Healthcare) using a random priming method (He et al., 2014; Yu et al., 2014b, 2018).

Hybridizations were performed with the GeoChip 3.0 on a MAUI hybridization system (Biomicro Systems, Salt Lake City, UT, United States) at 42°C and 40% formamide for 12 h. After washing and drying, GeoChip slides were scanned by a ProScan array microarray scanner (PerkinElmer, Boston, MA) (Xue et al., 2016b) at a laser power of 95% and a photomultiplier tube (PMT) gain of 75%, and the images were quantified using ImaGene 6.0 (Biodiscovery, El Segundo, CA, United States) to determine the intensity of each spot.

Poor-quality spots with a signal-to-noise ratio (SNR) (SNR = [signal mean - background mean]/background standard deviation) of >2.0 were removed as previously described (He and Zhou, 2008). After removal of poor-quality spots, the signal intensities of the probes were normalized within and across all samples on our microarray processing pipeline[1] (He et al., 2010a; Liang et al., 2010). Those gene probes that were detected in at least two of the 5 replicate samples were considered positive, and data can be found on our website[2].

Statistical Analysis

Significant changes in soil properties between ambient and warming or eCO_2 and eCO_2 + warming were determined by unpaired *t*-tests and analysis of variance (ANOVA). The overall changes in microbial functional and phylogenetic structure were determined by detrended correspondence analysis (DCA) and permutational multivariate analysis of variance (Adonis). The significant differences in individual genes between ambient and the three treatments were calculated by unpaired *t*-tests. The correlation between the microbial functional structure and soil properties was analyzed by canonical correspondence analysis (CCA) and Mantel test. All statistical analyses were performed by R project v.3.2.1[3] using the Vegan and Agricolae package.

RESULTS

Effects of Warming, eCO_2, eCO_2 + Warming on Soil Properties

Soil parameters showed different trends under warming, eCO_2, and eCO_2 + warming treatments (**Table 1**). First, NO_3-N was significantly lower ($P < 0.05$, *t*-test) under eCO_2 and eCO_2 + warming conditions compared with control, while there were no significant differences between ambient and warming. Second, NH_4-N was significantly lower ($P < 0.05$, *t*-test) under eCO_2 than ambient but the difference was not significant between ambient and warming or eCO_2 + warming. Third, soil moisture was significantly lower ($P < 0.05$, *t*-test) under warming than ambient, but was higher at significant ($P < 0.05$, *t*-test) and marginal ($P < 0.1$, *t*-test) levels under eCO_2 and eCO_2 + warming than ambient, respectively. Fourth, no significant differences were observed in TN, TC, C/N ratio and pH between ambient and warming, or eCO_2, and eCO_2 + warming. These results indicated that eCO_2 significantly

[1]http://ieg.ou.edu/microarray/

[2]http://mem.rcees.ac.cn/download.html

[3]www.r-project.org

TABLE 1 | Effects of warming, eCO_2 and eCO_2 + Warming on soil properties.

	NO_3-N (mg/kg)	NH_4-N (mg/kg)	TN (%)	TC (%)	C/N	Moisture (%)	pH
Warming effect[a]	0.860	0.536	0.001	−0.033	−0.183	**−1.255***	−0.145
eCO_2 effect	**−2.136**[b]	**−0.759***	−0.019	−0.198	0.003	**1.980***	−0.134
eCO_2 + Warming effect	**−1.476***	−0.034	−0.024	−0.194	0.357	1.072•	−0.033

*TN, total nitrogen; TC, total carbon; C/N, TC/TN ratio; ANOVA, analysis of variance; [a]Soil property values were analyzed and represented with differences of mean (treatment – ambient). [b]The significance of treatment effects were analyzed by t-tests. Significant differences (P < 0.05) indicated by bold type. Asterisks denote the P-value for the difference: **P ≤ 0.01, *P ≤ 0.05, •P < 0.1.*

affected soil NO_3-N, NH_4-N and moisture, while warming and eCO_2 + warming significantly affected only soil moisture and NO_3-N, respectively.

Effects of Warming, eCO_2, eCO_2 + Warming on Functional and Phylogenetic Structure of Soil Microbial Communities

A total of 3,624 microbial function genes were detected under four treatments across 20 samples. A significantly ($P < 0.05$) greater number of genes were detected under eCO_2 (2,217 ± 269) than ambient (1,269 ± 78) (**Supplementary Table S1**), but the difference was not significant between ambient and either warming or eCO_2 + warming. Analysis of alpha-diversity indexes showed similar patterns. eCO_2 significantly ($P < 0.05$) increased the Shannon index (H′) and the Simpson's reciprocal index (1/D) compared to ambient, but no significant differences were found between ambient and other treatments. The overall taxonomic composition of soil microbial community under different treatments was further analyzed at phylum level based on GeoChip data (**Supplementary Figure S1**). The detected functional genes were taxonomically derived from 2 archaeal phyla, 17 bacterial phyla, and 3 eukaryotic phyla. Proteobacteria (69.45% – 66.13%), Actinobacteria (17.78% – 13.68%), Firmicutes (4.41% – 3%), Ascomycota (4.35% – 2.69%) and Chloroflexi (2.02% – 1.11%) were detected as the five dominant phyla. eCO_2 and eCO_2 + warming significantly impacted the abundance of key genes derived from these five dominant phyla (**Supplementary Figure S2**).

The Adonis test of all detected genes showed that eCO_2, warming, and their combined effect significantly ($P < 0.05$) impacted soil microbial communities (**Table 2**). About 41.1% of the total variation can be explained by this model with eCO_2 (26.4%) as the main factor, followed by warming (7.6%) and eCO_2 + warming (7.1%). Moreover, the soil microbial phylogenetic structure based on the analysis of *gyrB*, a phylogenetic marker gene, was significantly ($P < 0.05$) influenced by all treatments (eCO_2, 24.5%; warming, 6.9%; eCO_2 + warming, 7.9%) (**Table 2**). Detrended correspondence analysis of all detected functional genes and of *gyrB* genes indicated that samples from the four treatment plots were distinct from each other (**Figure 1** and **Supplementary Figure S3**).

TABLE 2 | Adonis analysis of the effect of eCO_2, Warming and eCO_2 + Warming on the functional and phylogenetic structure of microbial communities based on all detected genes and *gyrB* genes, respectively.

	eCO_2		Warming		eCO_2 + Warming	
	R2	*P*	R2	*P*	R2	*P*
Functional structure	0.264	**0.001*** **	0.076	**0.030***	0.071	**0.047***
Phylogenetic structure (*gyrB*)	0.245	**0.001*** **	0.069	0.056•	0.079	**0.042***

*Asterisks denote the P-value for the difference: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, • $P \leq 0.1$.*

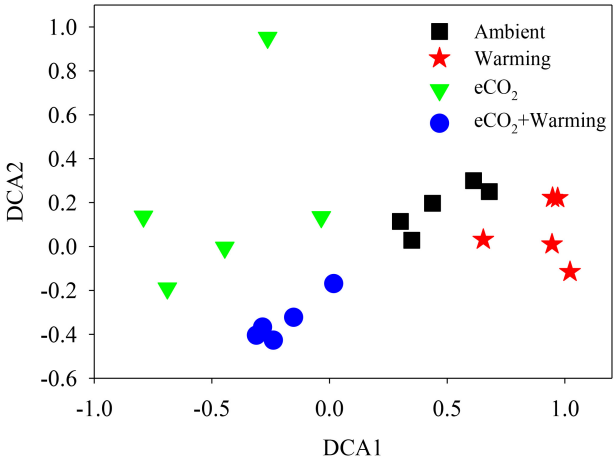


FIGURE 1 | Detrended correspondence analysis (DCA) of all detected functional genes across three treatments and the ambient samples.

These results indicated that the diversity, composition, and phylogenetic and functional gene structures of the soil microbial communities was changed under eCO_2, warming and eCO_2 + warming treatments in semiarid grassland.

Effects of Warming, eCO_2, eCO_2 + Warming on Key Functional Genes Involved in Major Biochemical Process

A total of 138 ± 9, 94 ± 12, 245 ± 26, and 200 ± 9 genes involved in C cycling (including C fixation, degradation and methane metabolism) showed positive signals under ambient, eCO_2, warming and eCO_2 + warming treatments, respectively. Compared with ambient, detected gene numbers were significantly ($P < 0.05$) higher in the samples from eCO_2 treatments.

Two key carbon fixation genes were detected, including Pcc (propionyl-CoA carboxylase) and Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) (**Supplementary Figure S4**). Elevated CO_2 and warming had opposite effects on these genes. The signal intensities of Pcc and Rubisco genes were significantly higher ($P < 0.05$) under eCO_2, but relatively lower under warming compared to ambient. However, the combination of eCO_2 and warming also showed a significantly ($P < 0.01$) positive effect on these two genes. These results suggested that eCO_2 and eCO_2 + warming potentially increased carbon fixation.

The signal intensities of genes involved in methane production and oxidation showed different patterns in response to three treatments. Elevated CO_2 alone significantly ($P < 0.05$) increased the signal intensities of *mcrA* for CH_4 production and *pmoA* for CH_4 oxidation, while the signal intensities of these two genes decreased under warming at marginally significant ($P = 0.085$) or significant ($P = 0.033$) levels, respectively (**Supplementary Figure S5**). The combination of eCO_2 and warming significantly ($P < 0.05$) increased the signal intensities of *mcrA*, but had no

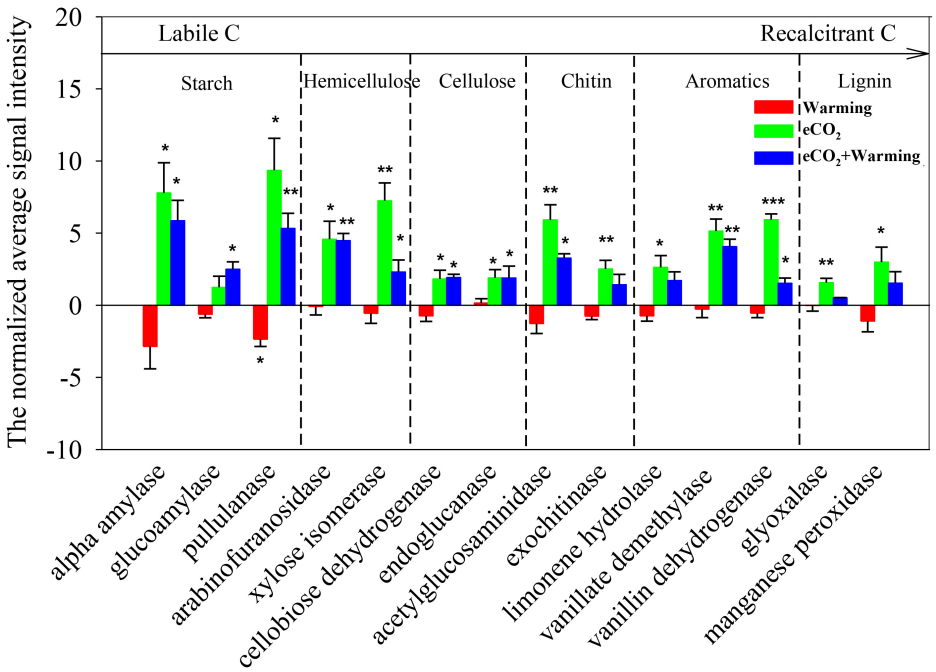


FIGURE 2 | Significant differences of detected genes involved in C degradation in response to treatments. All data are presented as differences of mean (treatment-ambient) ± standard errors (SEs). Significant differences were calculated by *t*-tests and marked by asterisks. ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$.

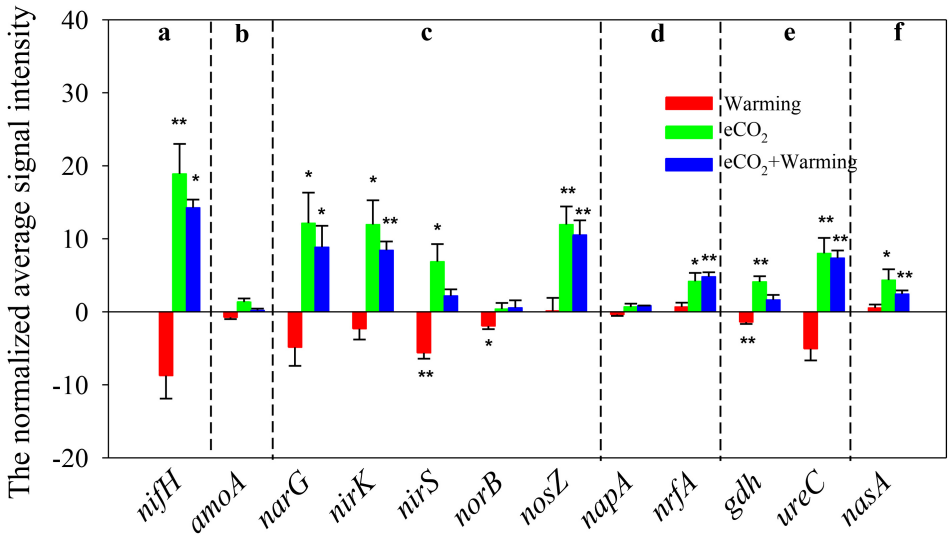


FIGURE 3 | Significant differences of detected genes involved in the N cycle under Warming, eCO_2, eCO_2 + Warming treatments. **(a)** N_2 fixation; **(b)** Nitrification; **(c)** Denitrification; **(d)** Dissimilatory N reduction to ammonium; **(e)** Ammonification; **(f)** Assimilatory N reduction. All data are presented as differences of mean (treatment-ambient) ± standard errors (SEs). Significant differences were calculated by *t*-tests and marked by asterisks. ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$.

effect on *pmoA*. These results indicate that warming may have negative effects on soil methane metabolism, while eCO_2 had significant positive effects. When combined, warming may, to some extent, counteract the positive effects of eCO_2.

Notably, genes involved in C degradation were also dramatically affected by all three treatments (**Figure 2**). Among these, only the signal intensities of genes encoding pullulanase for starch degradation decreased by a significant ($P < 0.05$) level under warming alone. However, eCO_2 alone significantly ($P < 0.05$) increased the signal intensities of functional genes for degradation of both labile C (starch, hemicellulose, cellulose and chitin) and recalcitrant C (aromatic and lignin) (Zhou et al., 2011; Xue et al., 2016a), including those encoding alpha amylase and pullulanase for starch decomposition,

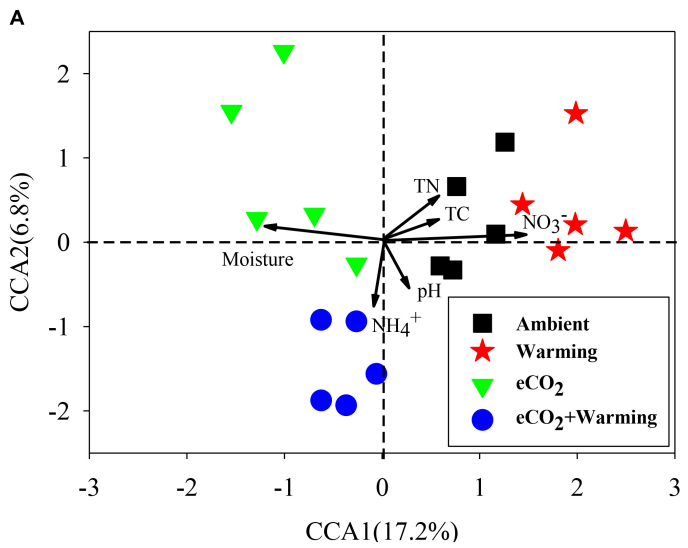
arabinofuranosidase and xylose isomerase for hemicellulose decomposition, cellobiose dehydrogenase and endoglucanase for cellulose decomposition, acetylglucosaminidase and exochitinase for chitin decomposition, limonene hydrolase, vanillate demethylase, and vanillin dehydrogenase for aromatic component degradation, glyoxalase and manganese peroxidase for lignin decomposition. The combination of eCO_2 and warming significantly increased ($P < 0.05$) the signal intensities of most of the functional genes involved in the degradation of labile C. These results revealed that eCO_2 had a dramatically positive effect on labile and recalcitrant C degradation, while warming likely had a relatively strong offset effect on the genes involved in degradation of recalcitrant C, especially for lignin-degradation genes, whereas the signal intensities of these genes had no significant change under warming alone.

There were 126 ± 7, 96 ± 12, 211 ± 22, and 188 ± 10 genes involved in N cycling detected under ambient, warming, eCO_2 and eCO_2 + warming treatments, respectively (**Supplementary Table S1**). Elevated CO_2 significantly ($P < 0.05$) increased the signal intensity of genes involved in N_2 fixation (*nifH*), denitrification (*narG*, *nirS/K* and *nosZ*), dissimilatory N reduction to ammonium (*nrfA*), ammonification (*gdh* and *ureC*) and assimilatory N reduction (*nasA*), while warming significantly ($P < 0.05$) decreased the signal intensity of *nirS*, *nosB* (denitrification) and *gdh* (**Figure 3**). In addition, among the 13 functional genes detected in N cycling, 7 were stimulated significantly under the eCO_2 + warming treatment, including *nifH*, *narG*, *nirK*, *nosZ*, *nrfA*, *ureC* and *nasA*. The signal intensities of *nirS* and *gdh* were significantly enhanced ($P < 0.05$) under eCO_2 and suppressed ($P < 0.05$) under warming, while they remained unchanged under eCO_2 + warming. These results suggest that eCO_2, either alone or in combination with warming, may have a positive effect on soil N cycling by increasing the abundance of functional genes, though for some genes the effect was counteracted by warming.

Two functional genes involved in P cycling were detected by GeoChip, exopolyphosphatase (Ppx) for inorganic polyphosphate degradation and polyphosphate kinase (Ppk) for polyphosphate biosynthesis in prokaryotes (**Supplementary Figure S6**). The signal intensity of Ppk was significantly increased ($P < 0.05$) under eCO_2, and the signal intensity of Ppx was significantly decreased ($P < 0.05$) under warming. The combination of warming and eCO_2 had no apparent effect on these two genes.

Linkages Between Microbial Community Structure and Soil Properties

To investigate the relationship between microbial community structure and soil properties (NO_3-N, NH_4-N, TN, TC, pH and moisture), a canonical correspondence analysis (CCA) was performed (**Figure 4A**). The communities from ambient, eCO_2 + warming treatments separated clearly along the first canonical axis. Among these soil properties only NO_3-N and moisture significantly ($P < 0.01$) correlated with all detected genes (**Figure 4B**), while other soil properties showed significant correlations with individual functional genes. The correlation between individual functional genes involved in C, N and P cycling and soil properties were further analyzed by the Mantel test. In total, 9, 2, 2, 25, and 3 genes involved in C and N cycling significantly ($P < 0.05$) correlated with soil NO_3-N, TN, TC, moisture, and all soil properties, respectively (**Supplementary Table S2**). For example, genes involved in C degradation (*amyA*, isopullulanase, *pulA*, *ara*, *xylA*, *CDH*, acetylglucosaminidase, exochitinase, pectinase, *vanA*, *vdh*, *mnp*), C fixation (Pcc and Rubisco), methane metabolism (*mcrA and pmoA*), N fixation (*nifH*), ammonification (*gdh* and *ureC*), denitrification (*narG*, *nirK*/S, *nosB*, *nosZ*) and P



B

	F	P
NO_3-N	2.661	**0.003****
NH_4-N	0.765	0.827
TN	1.209	0.248
TC	1.016	0.447
pH	0.739	0.847
Moisture	2.871	**0.001*****
Whole	1.229	0.107

FIGURE 4 | Canonical correspondence analysis (CCA) of GeoChip data and soil properties **(A)**. Model significances **(B)**. Asterisks denote the *P*-value for the difference: ***$P \leq 0.001$, **$P \leq 0.01$.

cycling (Ppk and Ppx) were significantly ($P < 0.05$) correlated with soil moisture. In addition, the genes involved in C degradation (*ara*, *CDH*, acetylglucosaminidase), C fixation (Pcc), methane metabolism (*mcrA* and *pmoA*), N fixation (*nifH*), ammonification (*ureC*) and P cycling (Ppx) were significantly ($P < 0.05$) correlated with NO_3-N. These results indicated that NO_3-N and moisture may be the main environmental factors influencing the microbial functional structure in this grassland.

DISCUSSION

Soil microbial communities regulate many biogeochemical processes (e.g., C, N cycling) in response to global climate change, which in turn shape ecosystem functions (Castro et al., 2010). Here, we conducted a multi-factor experiment for climate change in a warmed semi-arid grassland to evaluate how these factors (warming, eCO_2 and their combined effect) impact soil microbial communities. By using GeoChip, our results demonstrated that the composition and functional structure of the communities shifted substantially under warming, eCO_2, and eCO_2 + warming treatments. In addition, key functional genes involved in C, N, and P cycling produced distinct changes under the different treatments and were significantly correlated with soil properties. This study gives new insights into microbial responses and feedbacks to global climate change in grasslands.

Treatment Effects on Microbial Communities Structures

At this experimental site the composition and phylogenetic and functional structures of soil microbial communities were dramatically altered under warming, eCO_2, and eCO_2 + warming. Previous studies have shown that microbial community structure shifted under eCO_2 (He et al., 2010b, 2014; Yu et al., 2018) and warming (Sheik et al., 2011; Xue et al., 2016a,b). Our results agreed with these reports, which were supported by both the Adonis and DCA analysis of all detected genes. Moreover, the relative abundance of functional genes derived from five dominant phyla was also significantly altered, suggesting that the abundances of these microorganisms may increase under both eCO_2 and eCO_2 + warming. In this water-constrained grassland, eCO_2 increased soil water availability by inducing the leaf transpiration of plant and increasing plant water-use efficiency (Morgan et al., 2004, 2011), while warming had the opposite effect (Reyes-Fox et al., 2014; Zelikova et al., 2014). In compared with ambient, soil moisture significantly ($P = 0.039$, *t*-test) decreased under warming alone, but greatly increased under both eCO_2 ($P = 0.024$, *t*-test) and eCO_2 + warming ($P = 0.066$, *t*-test) treatments, suggesting that the eCO_2-induced water conserving effects may be greater than the desiccating effects of the warming-induced in this semiarid grassland (**Table 1**). These results agree with the previous report from this site (Carrillo et al., 2014). Previous studies of the BioCON site demonstrated that eCO_2 significantly increased soil pH and moisture as well as shifted the functional and phylogenetic composition and structure of microbial communities in a grassland ecosystem (He et al., 2010b; Deng et al., 2012). In addition, a multifactor warming experiment showed that warming and added precipitation altered the soil microbial community composition in a grass prairie (Castro et al., 2010). Most importantly, the combined effects of eCO_2 and warming were also significant for both total functional genes and *gyrB* genes by Adonis analysis, implying significant impacts by eCO_2 and warming on the soil microbial community.

Warming Effect on Functional Genes

How soil microbial functional processes (e.g., C, N, and P dynamics) will respond to climate change is critical issue for PHACE studies. In our results, the abundance and diversity of functional genes involved in functional processes were modified under warming. Several previous studies showed inconclusive responses by soil microbial communities under warming. For example, some key metabolic pathways, such as labile C degradation and nitrogen cycling, were enriched under warming (Zhou et al., 2011; Luo et al., 2014), or altered (increased or decreased) depending on the individual gene (Xue et al., 2016a). Moreover, some experimental sites found declines in microbial biomass respiration and carbon degradation processes within microbial communities in response to warming (Allison and Treseder, 2008; Allison et al., 2010; Romero-Olivares et al., 2017). A previous study also showed that the abundance of genes associated with C and N cycling decreased with warming in a Tibetan grassland (Yue et al., 2015). Those findings are generally consistent with the results presented here, in which the signal intensities of 13 genes involved in carbon degradation decreased under warming, though the differences were significant for only one gene (pullulanase), suggesting a relatively weak effect of warming on soil C dynamics. In addition, the signal intensities of 18 genes involved in C fixation, methane metabolism, N cycling, and P cycling were also decreased under warming, especially for 5 genes (*pmoA*, *nirS*, *norB*, *gdh*, and Ppx) which showed a significant ($P < 0.05$) decrease. These phenomena could be attributed to the fact that warming decreases soil water availability in this semiarid grassland (**Table 1**), which may suppress soil microbial activity and microbial functional processes (Allison and Treseder, 2008). Moreover, the microorganisms may harbor one gene which could also harbor the other genes catalyzing the processes involved in denitrification. Experimental warming often increases soil microbial functional processes in water unconstrained ecosystems (Zhou et al., 2011), however, soil water availability is a limiting factor for biological activity in this semiarid grassland (Dijkstra et al., 2010). This inference is also supported by our Mantel test, showing that many of the functional genes involved C, N, and P cycling have significant ($P < 0.05$) correlation with soil moisture (**Supplementary Table S2**). In addition, the significant decrease in abundance of *nirS* and *norB* may lead to an inhibition of microbial denitrification processes, and accordingly we also found a relatively higher concentration of soil nitrate under warming than under ambient (**Table 1** and **Figure 3**). Moreover, the abundance of *pmoA* genes significantly decreased, suggesting

that CH_4 uptake may reduce under warming. Although the CH_4 flux was not measured in this study, the inference was confirmed by a previous study of this PHACE site (Dijkstra et al., 2013b). Results of the current study revealed a possible weak negative microbial feedback to warming in this semiarid grassland.

Elevated CO_2 Effect on Functional Genes

Elevated CO_2 stimulated microbial functional processes and relevant soil functions. A study of this PHACE experimental site showed a positive feedback of microbial communities under eCO_2 (Nie et al., 2013), while other reports showed that eCO_2 has no significant response (Sinsabaugh et al., 2003; Austin et al., 2009) at the FACE site. Additionally, several previous studies showed that key genes involved in C degradation, C fixation, and methane metabolism cycling were stimulated under eCO_2 in grassland, agricultural, and forest ecosystems (He et al., 2010b; Xiong et al., 2015; Yu et al., 2018). These results appear consistent with the present study, using the same GeoChip technology, revealing that the abundances of most of the functional gene involved in C cycling were significantly enhanced under eCO_2. The effect of eCO_2 on soil microbial communities possibly occurs via altered soil properties (e.g., pH and moisture) and increased C allocation to fine roots (He et al., 2010b; Morgan et al., 2011). However, in this water constrained ecosystem, the decomposition and plant inputs to soil may be regulated by soil water availability (Carrillo et al., 2011). In the current study, the signal intensities of 13 genes involved in both labile and recalcitrant C degradation were significantly increased, suggesting that microbial C decomposition may be stimulated under eCO_2. The C fixation process was also enhanced by the significant increase of Pcc and Rubisco gene abundances, which is probably involved in the microbial community mediation response strategy to the gradual decrease in soil organic C due to faster decomposition (Carrillo et al., 2011). The decrease of soil total carbon has been observed not only in this PHACE site, but also in an agricultural FACE site (Xiong et al., 2015). The total signal intensities of *mcrA* and *pmoA* genes were significantly enhanced under eCO_2, which is in agreement with previous studies of forest and agricultural FACE sites (Xiong et al., 2015; Yu et al., 2018). The methane production may be stimulated under eCO_2, which could enhance methane uptake by increasing substrate availability for the methanotrophs. Moreover, this was also supported by a study at this PHACE site, showing that CH_4 uptake was enhanced by increased soil moisture under eCO_2 (Dijkstra et al., 2013b). eCO_2 not only impacted soil C cycling driven by belowground microorganisms, but also altered the soil microbial N cycling process. The current study showed that the signal intensities of most N cycling genes (e.g., *nifH*, *nrfA*, *gdh*, *ureC*, *nasA*, *narG*, *nirK/S*, *nosZ*) were significantly increased under eCO_2. This is most likely due to the fact that the greater soil water availability and C inputs from eCO_2 may enhance the soil microbial activity and N demand (Carrillo et al., 2012; He et al., 2014). In addition, this conclusion was supported by soil properties data which showed a significant decrease of soil NO_3-N, NH_4-N under eCO_2 (**Table 1**). Consequently, our results showed a potentially positive microbial response to eCO_2.

Elevated CO_2 + Warming Effect on Functional Genes

The combined effects of eCO_2 and warming altered microbial functional processes in a manner similar to eCO_2 alone. It has been previously shown that warming can offset the positive effects of eCO_2 on soil water availability in this PHACE site (Carrillo et al., 2014; Reyes-Fox et al., 2014). Consistent with these studies, the soil moisture under eCO_2 + warming was lower than under eCO_2 alone, but was marginally ($P = 0.066$) higher than ambient conditions (**Table 1**). However, whether the combination of eCO_2 and warming had similar effects on soil microbial functional processes remains unknown. In the present study, the signal intensities of genes involved in labile C degradation were significantly increased under eCO_2 + warming treatment, but for some recalcitrant C degradation genes (limonene hydrolase, glyoxalase, and manganese peroxidase) the changes were not significant (**Figure 2**). In comparison with the effect of eCO_2 alone, the offset of warming was relatively weak for soil labile C, but comparatively strong for soil recalcitrant C dynamics. These phenomena could be explained by a previous study of this site, showing that the labile C pool size was greatly altered under eCO_2 + warming in 2008 due to the increase in C input mediated by soil water availability (Carrillo et al., 2011). The signal intensities of two genes involved in C fixation were significantly enhanced under both eCO_2 and eCO_2 + warming treatments, indicating that eCO_2 may have a robust effect on C fixation processes (**Supplementary Figure S4**). In addition, a significantly higher signal intensity of *mcrA* for methane production was observed with the eCO_2 + warming treatment (**Supplementary Figure S5**). We speculate that methanogenic activity was promoted by the large input of labile carbon (Wachinger et al., 2000; Knorr et al., 2008). For N cycling, 7 and 9 genes abundances were significantly increased under eCO_2 + warming or eCO_2 alone treatments, respectively. These results potentially suggest that eCO_2 + warming has a relatively positive effect on soil microbial functional process, although warming, to some extent, offset the priming effect of eCO_2. Our results provide support to previous studies that suggested the response of soil processes to eCO_2 + warming are more similar to those of eCO_2 alone than of warming alone (Dieleman et al., 2012; Nie et al., 2013).

This study demonstrated that microbial community structure and functional processes were altered in response to climate change in this semiarid grassland ecosystem. Our results highlight three major mechanisms by which microbial communities could regulate soil microbial functional processes in response to global climate change. eCO_2 had strong positive effects on microbial communities by increasing the microbial functional diversity and soil microbial C and N cycling, while warming had a weak negative effect on microbial communities. The combination of eCO_2 and warming induced a relatively positive feedback from microbial communities although warming offset part of the priming effect caused by eCO_2.

However, this study only examined microbial communities in single season of a year that might not reflect the changes of all microorganisms. Our future study may focus on the temporal dynamics of soil microbial communities in response to multiple climate change factors with the substantiation of actual process measurements.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01790/full#supplementary-material

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Taxonomic and Functional Responses of Soil Microbial Communities to Annual Removal of Aboveground Plant Biomass

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Clipping, removal of aboveground plant biomass, is an important issue in grassland ecology. However, few studies have focused on the effect of clipping on belowground microbial communities. Using integrated metagenomic technologies, we examined the taxonomic and functional responses of soil microbial communities to annual clipping (2010–2014) in a grassland ecosystem of the Great Plains of North America. Our results indicated that clipping significantly ($P < 0.05$) increased root and microbial respiration rates. Annual temporal variation within the microbial communities was much greater than the significant changes introduced by clipping, but cumulative effects of clipping were still observed in the long-term scale. The abundances of some bacterial and fungal lineages including *Actinobacteria* and *Bacteroidetes* were significantly ($P < 0.05$) changed by clipping. Clipping significantly ($P < 0.05$) increased the abundances of labile carbon (C) degrading genes. More importantly, the abundances of recalcitrant C degrading genes were consistently and significantly ($P < 0.05$) increased by clipping in the last 2 years, which could accelerate recalcitrant C degradation and weaken long-term soil carbon stability. Furthermore, genes involved in nutrient-cycling processes including nitrogen cycling and phosphorus utilization were also significantly increased by clipping. The shifts of microbial communities were significantly correlated with soil respiration and plant productivity. Intriguingly, clipping effects on microbial function may be highly regulated by precipitation at the interannual scale. Altogether, our results illustrated the potential of soil microbial communities for increased soil organic matter decomposition under clipping land-use practices.

Keywords: clipping land-use, taxonomic and functional response, microbial community, metagenomics, GeoChip

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INTRODUCTION

The grassland ecosystem is an important terrestrial C pool containing almost 12% of Earth's organic matter (Schlesinger, 1977), more than 90% of which is stored belowground in the form of roots and soil organic matter (SOM) (Shahzad et al., 2012). Thus, grasslands are potential C sinks in the context of increasing global atmospheric CO_2 concentration provided that they are properly managed (Lal et al., 2007; Li et al., 2008). Plant tissue removal via grazing, mowing and clipping plant matter, is a central issue in land-use practices and has been reported to change plant-litter decomposition (Semmartin et al., 2008; Klumpp et al., 2009), biodiversity of plants (Ward et al., 2007; Wu et al., 2009), and nutrient cycling (Garibaldi et al., 2007; de Faccio Carvalho et al., 2010). Reduced plant coverage can also change the litter layer on the soil surface, increase soil energy absorbed and emitted, and amplify the diurnal soil-temperature range (Wan et al., 2002). In addition, clipping can increase evaporation from soil and decrease transpiration of vegetation, resulting in an unpredictable net effect on soil moisture (Zhang et al., 2005).

As microbial communities play important roles in biogeochemical cycles of C, nitrogen (N), phosphorus (P), and sulfur (S), a mechanistic understanding of annual clipping effects on microbial community structure and function is crucial for a robust prediction of soil C stocks and fluxes under the context of land-use practices (Zhang et al., 2005; Belay-Tedla et al., 2009). However, until now, how microbial structure and function respond to clipping is poorly understood and remains controversial in many cases. For example, some previous studies reported that plant tissue removal can significantly reshape microbial community structure and function by increasing the ratio of oligotrophic to copiotrophic taxa (Fierer et al., 2007; Carey et al., 2015), which was mainly associated with decreased plant photosynthesis, reducing C supply to roots and belowground microbial communities (Craine et al., 1999; Bahn et al., 2006; Ingram et al., 2008). However, another study reported that little variations in microbial composition and diversity were observed under clipping treatment, although removal of aboveground plant biomass can increase soil temperature while decreasing C and nutrient pools in an experimental semi-arid grassland (Carey et al., 2015). Also, it is uncertain how plant tissue removal affects N mineralization processes and consequently alters N availability for decomposition processes (Cheng et al., 2010). In addition, interacting environmental variations such as climate (Castro et al., 2010), soil physical and chemical properties (Bell et al., 2009), vegetation (Mitchell et al., 2010), and substrate quantity and quality (Hernández and Hobbie, 2010) can significantly affect soil microbial communities, which convolute the direct impacts of plant tissue removal. Therefore, long-term monitoring of taxonomic and functional shifts of soil microbial communities in response to annual clipping is necessary for a comprehensive understanding of the effects of plant tissue removal on soil microbial communities.

The advances and applications of metagenomic technologies such as next generation sequencing and functional gene arrays (e.g., GeoChip) have revolutionized our analysis of soil microbial communities (Caporaso et al., 2012; Shokralla et al., 2012; Tu et al., 2014; Yue et al., 2015; Xue et al., 2016b). High-throughput amplicon sequencing has been successfully used to analyze the diversity of soil microbial communities in forests (Nacke et al., 2011; Brown et al., 2013; Cong et al., 2015), grasslands (Sheik et al., 2011), farmland (Su et al., 2015), and permafrost (Penton et al., 2013; Deng et al., 2015). Meanwhile, the functional gene structure and functional potentials of soil microbial communities have been rapidly analyzed using functional gene arrays, which are still quicker and less consumable for now than metagenomic shotgun sequencing especially for complex microbial communities (Liang et al., 2015; Yue et al., 2015). Therefore, the complementarity in terms of experimental data and analysis between high-throughput sequencing and functional gene arrays allows us to comprehensively estimate the composition and functional structure of soil microbial communities.

In this study, we examined taxonomic and functional responses of grassland microbial communities to annual clipping in a native, tall-grass prairie ecosystem of the US Great Plains in Central Oklahoma (latitude 34°59′ N, longitude 97°31′ W). This multifactor climate change experiment was established in 2009, with warming (+3°C), half precipitation (−50%), double precipitation (+100%), clipping (annual biomass removal) and their combined treatments (Xu et al., 2013). In this study, we primarily focus on the clipping treatment and 40 soil surface samples were collected in the clipped and control plots from 2010 to 2014 to test three central hypotheses. First, taxonomic and functional structures of soil microbial communities would be progressively altered, as the cumulative clipping effect may reduce nutrient (e.g., C, N, P) inputs from litter and change soil properties (e.g., temperature, moisture) in the long-term (5 years) scale (Hamilton and Frank, 2001; Bahn et al., 2006; Xue et al., 2016a). Also, different taxonomic and functional groups would show different sensitivities to clipping in the interannual scale due to the regulation of some temporal background variations (e.g., precipitation) on clipping effects. Lastly, clipping would significantly affect soil C and nutrient cycles by stimulating genes involved in C and N fixation and labile/recalcitrant C degradation. In this study, microbial communities were analyzed using GeoChip 5.0 as well as sequencing of bacterial/archaeal 16S rRNA gene and fungal ITS amplicons with Illumina MiSeq technology. This study provides novel insights into the taxonomic and functional responses of soil microbial communities to annual clipping and implies the potential for increased SOM decomposition under clipping land-use practices.

MATERIALS AND METHODS

Site and Sampling

The annual clipping experiment was conducted in the Kessler Atmospheric and Ecological Field Station (KAEFS) in McClain County, OK, United States (latitude 34°59′ N, longitude 97°31′ W). KAEFS is located in the tall-grass prairie of central red-bed plains of Oklahoma, dominated by C_3 forbs (*Ambrosia trifida*, *Solanum carolinense,* and *Euphorbia dentate*) and C_4 grasses

(*Tridens flavus*, *Sporobolus compositus,* and *Sorghum halepense*) (Xu et al., 2013). The site is on an old field prairie that had been abandoned from field cropping 40 years ago. The herbivores were excluded at this site in 2008 to prevent light grazing, which occurred before. Based on Oklahoma Climatological Survey data from 1948 to 1999, the temperature ranges from 3.3°C in January to 28.1°C in July (mean annual temperature, 16.3°C) and the precipitation ranges from 82 mm in January and February to 240 mm in May and June (mean annual precipitation, 914 mm) (Zhou et al., 2012). The soil is part of the Nash-Lucien complex with a high available water holding capacity (37%), neutral pH, and a deep (ca. 70 cm), moderately penetrable root zone (Xu et al., 2013).

This experiment was established in July of 2009 with a blocked split-plot design, in which warming (+3°C), half precipitation (−50%) and double precipitation (+100%) are primary factors nested by clipping (annual removal of above-ground biomass). The site was divided into four experimental blocks, each containing six 2.5 m × 3.5 m plots, which were further divided into two 2.5 m × 1.75 m subplots with a half for clipping. Treatments were randomly distributed across the plots within each block. Plants in the southern subplots were clipped at a height of 10 cm above the ground once to mimic the land-use practice of hay harvest at approximately the date of peak plant biomass on: 25 September, 2009; 28 September, 2010; 5 October, 2011; 17 October, 2012; 22 September, 2013; 9 October, 2014. Whereas the northern subplots were unclipped control subplots (Xu et al., 2013). The clipped plant materials were removed completely from the plots. This study focused on eight subplots with control (ambient) temperature and normal precipitation treatments, four of which were from clipped subplots and four from control (unclipped) subplots. Annual samples from the topsoil (0–15 cm) were collected one day before annual clipping from 2010 to 2014 (no samples were available in 2009). Three soil cores (2.5 cm diameter × 15 cm deep, ∼40 g) were collected in each subplot by using a soil sampler tube and composited to have enough samples for soil chemistry and molecular biology analyses. Holes were immediately refilled with root-free soils collected just adjacent to the plots. Soil samples were immediately transported to the laboratory and stored at −80°C until molecular analysis. A total of 40 annual soil samples (four clipped samples and four control samples in each year) were further analyzed in this study.

Ambient Temperature, Precipitation, and Soil and Vegetation Property Measurements

A series of measurements were routinely performed in the experimental field. Aboveground plant biomass investigations were conducted as described previously (Sherry et al., 2008). In brief, plant biomass, separated into C_3 and C_4 species, was directly measured by annual clipping in the clipped subplots and indirectly estimated by the pin-contact method in the control subplots (Frank and McNaughton, 1990). Total and heterotrophic soil respirations were measured once or twice a month between 10:00 and 15:00 (local time) using a LI-8100 portable soil CO_2 flux measurement system (LI-COR Inc., Lincoln, NE, United States), and autotrophic respiration (AR) was evaluated by the difference of total respiration and heterotrophic respiration (HR). Also, volumetric soil water content (θv) from the soil surface to a 15 cm depth was measured once or twice a month using manual Time Domain Reflectometry equipment (Soil Moisture Equipment Crop., Santa Barbara, CA, United States). Three measurements of soil water content were performed in every subplot each time and the average values were used in analysis. Soil temperature was measured every 15 min at the depth of 7.5 cm in the center of every subplot, using Constantan-copper thermocouples wired to a Campbell Scientific CR10x datalogger (T-type; Campbell Science Inst., Logan, UT, United States). Air temperature and precipitation data were obtained online from an Oklahoma Mesonet Station (Washington Station) located approximately 200 m away from our experiment site. All soil samples were analyzed for soil total organic carbon (TOC) and total nitrogen (TN), soil nitrate (NO_3^-) and ammonia (NH_4^+) by the Soil, Water and Forage Analytical Laboratory at the Oklahoma State University (Stillwater, OK, United States). Soil TOC and TN concentrations were determined using a dry combustion C and N analyzer (LECO, St. Joseph, MI, United States). For NO_3^- and NH_4^+, 6 g of soil was shaken thoroughly with 12 mL of 1 M KCl for 30 min, then filtered through a Fisher P4 qualitative filter (Fisher Scientific, Pittsburgh, PA, United States) and analyzed using a Lachat 8000 flow-injection analyzer (Lachat, Milwaukee, WI, United States). Soil pH was measured at a water-to-soil mass ratio of 2.5:1 using a pH meter with a calibrated combined glass electrode (McLean, 1982).

DNA Extraction and GeoChip Analysis

Soil DNA was extracted from all soil samples within the same batch in 2014 by freeze-grinding and SDS-based lysis as described previously (Zhou et al., 1996), and purified by the MoBio Power Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, United States) according to the manufacturer's protocol. DNA quality was assessed on the basis of the ratios of 260/280 nm and 260/230 nm absorbance using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, United States). The final DNA concentrations were quantified by PicoGreen using a FLUOstar Optima (BMG Labtech, Jena, Germany). The DNA samples were stored in −80°C before analyzed by the Illumina MiSeq technology (San Diego, CA, United States) and GeoChip 5.0.

The latest generation of functional gene array, GeoChip 5.0M (180K), was used to analyze the functional structure of soil microbial communities. GeoChip 5.0M contains 167,044 probes targeting 395,894 coding sequences from 1,593 functional gene families involved in C cycling, N metabolism, sulfur cycling, phosphorus cycling, electron transfer, metal homeostasis, organic remediation, stress response, secondary metabolism, and virus and virulence activity. GeoChip 5.0M was manufactured by Agilent (Agilent Technologies Inc., Santa Clara, CA, United States) in the 4 × 180K format. In our study, 800 ng of purified soil DNA of each sample was labeled with the fluorescent

dye Cy-3 (GE Healthcare, Anaheim, CA, United States) using a random priming method as described previously (He et al., 2007), purified using a QIAquick Purification kit (Qiagen, Mountain View, CA, United States) according to the manufacturer's instructions, and then dried in a SpeedVac (Thermo Savant, Holbrook, NY, United States) into powder. Subsequently, labeled DNA was resuspended into 27.5 μL of DNase/RNase-free distilled water, and then mixed completely with 99.4 μL of hybridization solution containing 63.5 μL of 2 × HI-RPM hybridization buffer, 12.7 μL of 10 × aCGH blocking agent, 10% formamide (final concentration), 0.05 μg/μL Cot-1 DNA, and 10 pM universal standard. The solution was denatured at 95°C for 3 min, and then incubated at 37°C for 30 min. Finally, the DNA solution was hybridized with GeoChip 5.0M arrays (180K) at 67°C for 24 h at 20 rpm in a hybridization oven. After hybridization, the slides were washed using Agilent hybridization buffer at room temperature and then scanned with a NimbleGen MS200 Microarray Scanner (Roche NimbleGen, Inc., Madison, WI, United States). The scanned images of the hybridized arrays were converted and extracted using Agilent Feature Extraction 11.5 software.

GeoChip Data Processing

The microarray data were preprocessed using the microarray analysis pipeline on the Institute for Environmental Genomics (IEG) website[1] as described previously (He et al., 2010; Tu et al., 2014). The major steps were as following: (i) Raw signal intensities (Cy3 channel) on each array were multiplied by a normalization weight I, which is the ratio of the maximum average universal standard intensity (Cy5 channel) among all the samples divided by the average universal standard intensity of each array; (ii) The signal intensities on each array were further multiplied by a normalization weight II, which is the ratio of the maximum total raw intensity (Cy3 channel) among all the samples divided by the total raw intensity of each array; (iii) Spots with SNR (signal to noise ratio) $\geq$ 2 were considered as positive. Otherwise they were treated as negative spots with 0 value; (iv) Spots with signal intensity lower than 250 were not considered as positive and were removed in subsequent analysis; (v) If a probe appeared in less than half or fewer of the samples in one treatment group (two out of four samples), it was removed from that group before any further analyses; (vi) The mean ratio in each sample was calculated by dividing the transformed signal intensity of each probe by the average transformed signal intensity for all detected probes in each sample. (vii) Relative change in normalized signal intensities was calculated as the clipping-induced change of gene abundance [(clipped − control)/control] in each year and/or across years.

MiSeq Sequencing of ITS and 16S rRNA Gene Amplicons

The compositions of bacterial and fungal communities were analyzed using Illumina MiSeq sequencing of ITS and 16S rRNA gene amplicons. The V4 region of 16S rRNA genes was amplified in triplicate for each sample with the primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTA CHVGGGTWTCTAAT-3′), and ITS region was amplified in triplicate for each sample with the primers gITS7F (5′-GTG ARTCATCGARTCTTTG-3′) and ITS4R (5′-TCCTCCG CTTATTGATATGC-3′). A two-step PCR was performed for ITS and 16S amplicon sequencing to avoid extra PCR bias that could be introduced by the components added in the long primers (Wu et al., 2015). The first round PCR was performed in a 25 μL reaction containing 2.5 μL 10 × PCR buffer II (including dNTPs), 0.25 U DNA polymerase, 0.4 μM of both forward and reverse target only primers and 4 μL 2 ng/μL soil DNA. Twelve cycles of PCR amplifications were performed in triplicate in the first round PCR. PCR products were purified using Agencourt® Ampure® XP (Beckman Coulter, Inc., Brea, CA, United States) and used as templates for the second PCR amplification of 20 cycles using the same primers, the reverse primer of which, however, contained Illumina adapter sequence and different barcodes to distinguish samples (Wu et al., 2015). The second round PCR was carried out in triplicate in a 25 μL reaction containing 2.5 μL 10 × PCR buffer II (including dNTPs), 0.25 U DNA polymerase, 0.4 μM of both forward and reverse phasing primers and 15 μL aliquot of the first round purified PCR product. PCR conditions for both first and second amplifications were as follows: 94°C for 3 min, then 94°C for 25 s, 53°C (16S rRNA gene) or 51°C (ITS) for 20 s, and 68°C for 45 s, followed by a final extension at 68°C for 10 min. PCR amplification were carried out in triplicate in order to reduce amplification bias. Subsequently, PCR products were quantified by PicoGreen using a FLUOstar Optima, combined equally and then visualized by electrophoresis on 1% agarose gels, and PCR products were purified using the QIAquick gel extraction kit (Qiangen, Valencia, CA, United States). Finally, 2 × 250 bp paired-ends DNA sequencing was performed on Illumina MiSeq platform according to the manufacturer's instructions.

The process of sequence quality control and analysis was conducted on Galaxy pipeline[2]. Raw sequences were split to different sample libraries based on barcodes. Before the combination of forward and reverse reads, primer sequences at the end of reads were trimmed and low-quality reads were removed by the Btrim program (Kong, 2011) with threshold of QC > 20 over 5-bp window size. Forward and reverse reads of same sequence with at least 20 bp overlap and <5% mismatches were combined using FLASH program (Magoč and Salzberg, 2011). The joined sequences without ambiguous bases in length of 245–258 bp for 16S rRNA gene and 210–450 bp for ITS were subjected to chimera removal. OTUs were classified by UPARSE at 97% similarity level (Edgar, 2013), and singletons were removed. Taxonomic assignment was performed by RDP Classifier with 50% confidence estimates (Wang et al., 2007). All samples were resampled at 30,000 sequences for 16S rRNA gene and 10,000 sequences for ITS.

Statistical Analysis

To test the significance of the differences between clipping and control treatment for various environmental variables, paired

[1] http://ieg.ou.edu/microarray

[2] http://zhoulab5.rccc.ou.edu:8080

t-tests were employed in this study. Microbial α-diversity indexes including Shannon index, Simpson index, evenness and richness were calculated based on the three pre-processed datasets (e.g., 16S rRNA gene sequencing, ITS sequencing, GeoChip analysis). Difference of taxonomic lineages and functional genes between clipping and control was compared by the analysis of variance (ANOVA). Temporal patterns of microbial community structures in the clipped and control plots were determined by detrended correspondence analysis (DCA) based on the Bray-Curtis dissimilarity. A dissimilarity test of the microbial community structures between clipping and control was performed using non-parametric multivariate analysis of variance (Adonis) based on the Bray–Curtis dissimilarity. Mantel tests were used to calculate the correlations between environmental factors and the soil microbial communities. Canonical correspondence analysis (CCA) was performed to identify the effect of soil, plant and climate variables, and time on the microbial community structures. Based on CCA results, variation partitioning analysis (VPA) was performed to determine the contributions of each individual variable or groups of variables to total variations in the soil microbial communities. Linear and non-linear (Quadratic) models were used to reveal the correlations between environmental variables and the relative change of functional genes by clipping. All the above statistical analyses were carried out in R (v.3.1.1, The R Foundation for Statistical Computing, Vienna, Austria) using the package vegan (Dixon and Palmer, 2003).

RESULTS

Ambient Temperature, Precipitation, Plant, and Soil Over Time

During the 5 years of the experiment, the average air temperatures over autumn (September to November) were warmest in 2012 (17.2°C) and coolest in 2013 (16.4°C) (**Supplementary Figure S1a**). Autumnal cumulative precipitation ranged from 17.9 to 28.5 cm in all years except 2012 (**Supplementary Figure S1b**), which was extremely low (10.9 cm) possibly due to the most severe drought across the United States in 2012 since the Dust Bowl era of the 1930s (Wolf et al., 2016). The experimental plots were subjected to clipping once a year to mimic the land-use practice of hay harvest since 2009. The total plant biomass across 5 years (2010–2014) was marginally significantly ($P = 0.06$) increased under clipping (**Figure 1A**), based on the one-tailed paired *t*-test. Specifically, clipping did not significantly change total plant biomass in the first 3 years (2010–2012), but significantly increased total plant biomass in 2013 ($P = 0.03$) and 2014 ($P = 0.05$) (**Supplementary Table S1**). The C_4 plant biomass was significantly ($P = 0.02$) higher under clipping than control, but the C_3 plant biomass remained unchanged in 2014 (**Supplementary Table S1**), resulting in a plant community shift toward more C_4 plant species. In addition, plant richness was marginally significantly ($P = 0.06$) increased by 5 years of clipping (**Figure 1A**).

Soil process measurements revealed that overall soil TOC, TN, and soil ammonium-N (NH_4^+-N) remained unchanged under annual clipping (**Figure 1B**). However, the concentrations of NO_3^--N were marginally significantly ($P = 0.06$) decreased by 5 years of clipping. Specifically, NO_3^- became significantly ($P < 0.05$) lower under clipping than control from 2012 to 2014 (**Supplementary Table S1**). Furthermore, soil pH was also significantly ($P = 0.03$) increased by annual clipping (**Figure 1C**), while annual clipping marginally significantly decreased soil water content based on a one-tailed paired *t*-test. In addition, the average temperature in the surface soil (top 15 cm) significantly ($P < 0.01$) increased under clipping in each year, resulting in an increase of 0.4°C across 5 years (**Figure 1D**). Also, plant belowground activity and microbial activity, measured as total soil respiration (TR), HR, and AR, were significantly ($P < 0.05$) higher in clipped plots than control plots. Annual clipping significantly ($P < 0.01$) increased the rates of AR and HR by 17% and 9%, respectively, suggesting that annual clipping stimulated the activities of plant roots and microbial SOM decomposition simultaneously (**Figure 1E**).

Overall Responses of Soil Microbial Communities to Annual Clipping

Soil microbial communities were analyzed by sequencing 16S rRNA gene and ITS amplicons with Illumina Miseq and functional gene arrays (GeoChip 5.0M). The non-parametric multivariate analysis of variance revealed that taxonomic and functional structures of microbial communities were much more strongly influenced by annual temporal variation (explaining 16.9–48.1%) than annual clipping (explaining 2.5–2.8%) (**Table 1**). No significant differences were observed in the overall bacterial and fungal diversities and structures between clipped and control samples in all years (**Table 1**, **Supplementary Figures S2a,b**, and **Supplementary Table S2**). One exception to this was bacterial community in 2012, which had significantly fewer OTUs under clipping than control. Further comparison of the microbial taxonomic composition showed that some key bacterial and fungal phyla were significantly ($P < 0.05$) shifted by annual clipping (**Supplementary Figures S3**, **S4**). Specifically, *Actinobacteria*, *Bacteroidetes*, *Crenarchaeota*, and *Gammaproteobacteria* were significantly ($P < 0.05$) decreased by 5 years of clipping, and *Chloroflexi* and *Planctomycetes* in bacterial community were significantly ($P < 0.05$) increased by 5 years of clipping (**Supplementary Figure S3**). In fungal community, the phyla *Zygomycota* and *Ascomycota* were significantly ($P < 0.05$) decreased under annual clipping across 5 years (**Supplementary Figure S4**). However, different phyla and genera in bacterial and fungal communities showed greatly different sensitivities to clipping in different years, as indicated that different phyla and genera were significantly ($P < 0.05$) or marginally significantly ($P < 0.10$) shifted by clipping in different years (**Supplementary Table S3**). Among these years, significantly and marginally significantly changed bacterial and fungal genera were the most in 2012, most of which belonged to *Actinobacteria* (16 genera), *Alphaproteobacteria* (15 genera), *Bacteroidetes* (9 genera), and *Ascomycota* (6 genera). Intriguingly, the relative abundances of unidentified fungi were greatly increased in 2012, and the relative abundance of unidentified

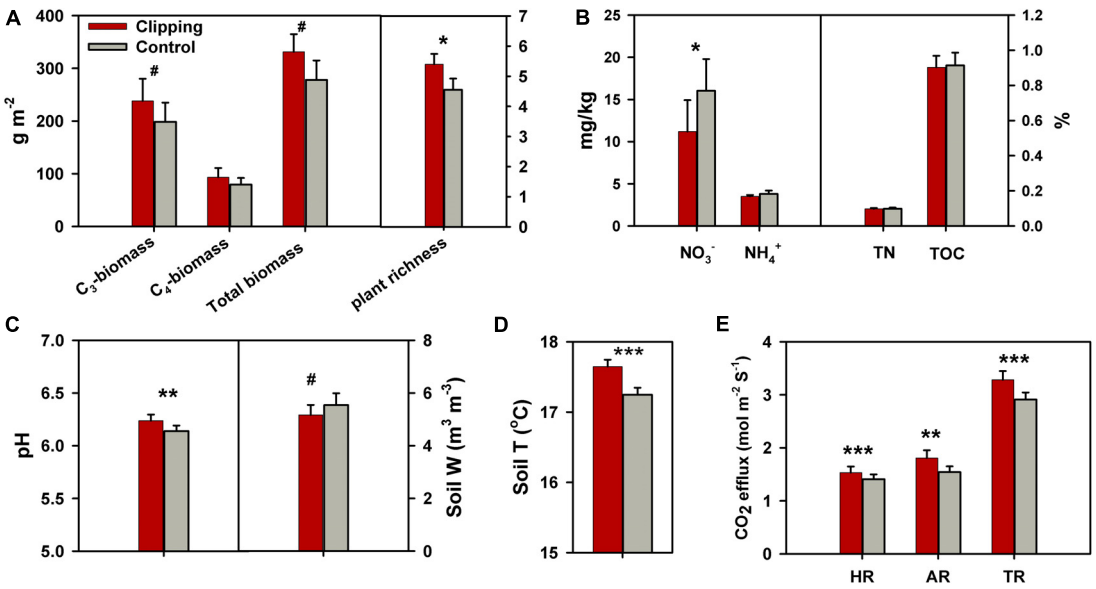


FIGURE 1 | Effects of annual clipping on plant and soil variables across 5 years. **(A–E)** Effects of clipping on C_3 plant biomass, C_4 plant biomass, total plant biomass and plant richness **(A)**; soil nitrate (NO_3^-), ammonia (NH_4^+), total nitrogen (TN) and total organic carbon (TOC) **(B)**; soil pH and soil water content (Soil W) **(C)**; soil temperature (Soil T) **(D)**; and heterotrophic respiration (HR), autotrophic respiration (AR) and total soil respiration (TR) **(E)**. Error bars indicate standard error of the mean. The differences between clippings and controls were tested by two-tailed paired *t*-tests, indicated by $^{***}P < 0.01$, $^{**}P < 0.05$, $^{*}P < 0.10$. The differences for some variables were also tested with one-tailed paired *t*-tests as indicated by $^{\#}P < 0.10$.

fungi was significantly ($P < 0.05$) higher under clipping (49.5%) than control (13.7%) (**Supplementary Figure S4** and **Supplementary Table S3**).

Annual clipping significantly shifted the functional gene richness and diversity, measured as the number of functional genes, Shannon diversity, Simpson diversity and evenness (**Supplementary Table S2**). There were marginally ($P < 0.10$) or significantly ($P < 0.05$) more functional genes detected in clipped samples than control samples in 2011, 2013 and 2014. However, the numbers of functional genes detected and Shannon diversity in 2010 and 2012 were significantly ($P < 0.05$) lower in clipped plots than those in control plots. More importantly, annual clipping also significantly ($P < 0.05$) changed the functional structure of microbial community (**Table 1**). The non-parametric multivariate analysis of variance in each year revealed that no significant clipping effect was observed in the first year (2010), but clipping effects became significant ($P < 0.05$) in the following 4 years (2011–2014) (**Table 1**). These results indicated that the shifts in microbial community functional structure under annual clipping progressively deepened along time and that annual clipping had cumulative effects on microbial community over time. DCA showed that clipped and control samples were clustered together in the first year (2010), while clipped samples gradually separated by the first DCA axis from control samples in the following 4 years (2011–2014) (**Supplementary Figure S2c**). Furthermore, the shifts in microbial community function under

TABLE 1 | Significance tests of the effects of clipping and year on the overall microbial community structures across 5 years and in each year by the non-parametric multivariate analysis of variance.

	16S rRNA		ITS		GeoChip	
	R^2	*P*	R^2	*P*	R^2	*P*
Year	0.169	**0.002**	0.183	**0.001**	0.481	**0.001**
Clipping	0.025	0.352	0.028	0.179	0.028	**0.020**
Year × clipping	0.103	0.208	0.096	0.313	0.228	**0.001**
2010 clipping	0.178	0.183	0.176	0.287	0.291	0.115
2011 clipping	0.143	0.506	0.142	0.434	0.306	**0.026**
2012 clipping	0.178	0.210	0.221	0.058	0.731	**0.034**
2013 clipping	0.125	0.632	0.121	0.855	0.256	**0.049**
2014 clipping	0.132	0.605	0.110	0.756	0.241	**0.027**

Bold values indicate P < 0.05.

clipping were consistent in direction of the first DCA axis in 2011, 2013, and 2014. Unexpectedly, the shift in microbial community function under clipping in 2012 was abnormally bigger than those in the other years and opposite in directionality, possibly due to the strong perturbance of the other environmental factors or the infestation of unidentified fungi.

Linking Microbial Communities to Environmental Variables

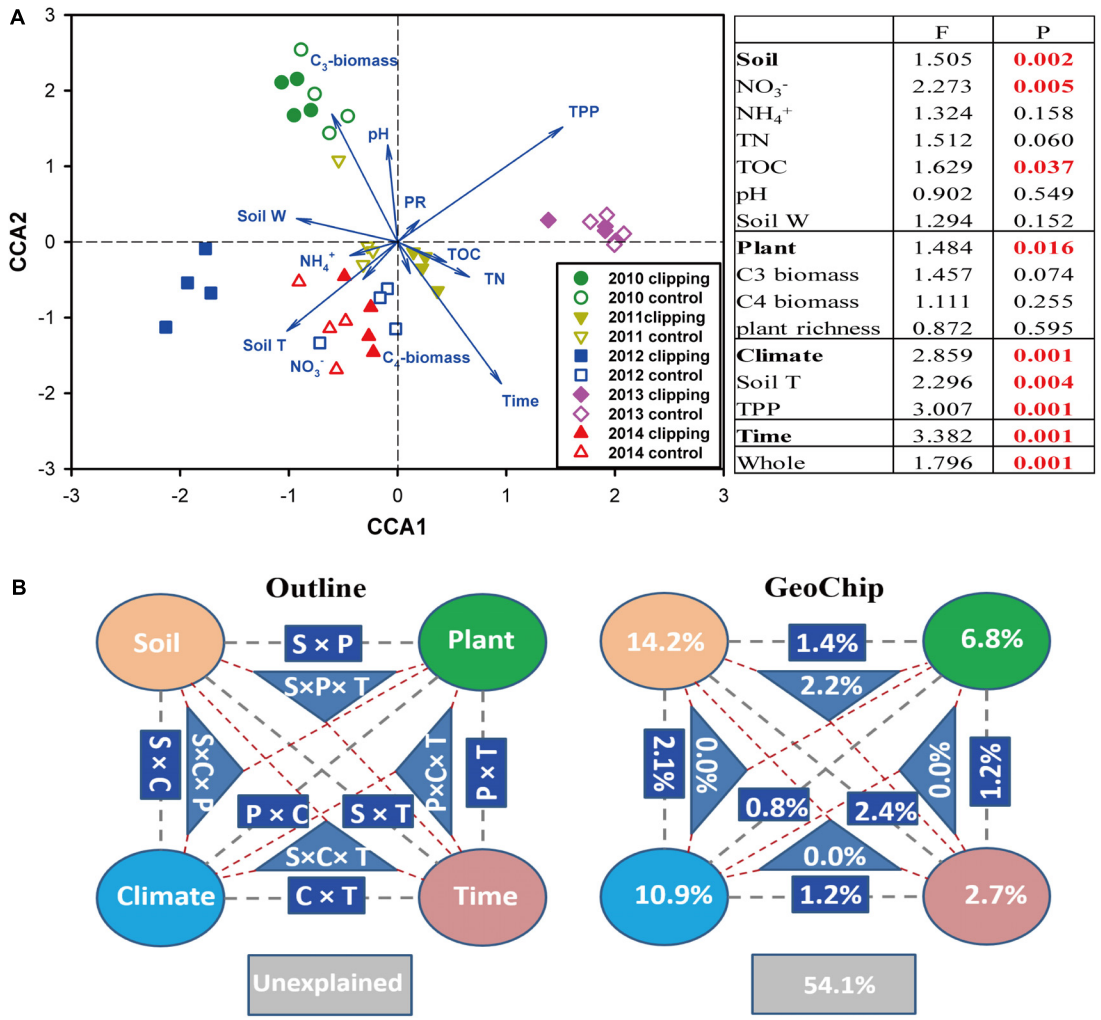
Canonical correspondence analysis and Mantel test were performed to discern the linkage between soil microbial phylogenetic and functional structures and environmental factors (**Figure 2** and **Supplementary Figures S5**, **S6**). The CCA results indicated that microbial functional structure was significantly (F = 1.796, P = 0.001) shaped by several soil, plant and climate variables as well as time (**Figure 2A**). Among these factors, time, precipitation, temperature and C_3 biomass exhibited more significant ($P < 0.05$) correlations with the variations of microbial functional structure. These soil, plant, climate variables and time exhibited significant correlations with taxonomic structure of bacterial community (F = 1.135, P = 0.026) but not fungal community (F = 0.966, P = 0.746) based on the CCA results (**Supplementary Figures S5a**, **S6a**). Furthermore, several key plant and soil variables also exhibited strong correlations with both functional and taxonomic community structures by Mantel tests (**Table 2**). For example, C_3 biomass and total biomass showed significant ($P < 0.05$) correlation with bacterial, fungal and functional community structures, and soil temperature showed significant ($P < 0.05$) correlation with bacterial and functional community structures. Importantly, HR exhibited significant correlations with bacterial community (P = 0.076), fungal community (P = 0.045), and microbial functional structure (P = 0.022) as revealed by Mantel tests (**Table 2**). These results indicated that the shifts of microbial communities were significantly ($P < 0.05$) correlated with clipping-induced changes of soil microclimate, soil respiration and aboveground plant productivity.

A partial CCA-based VPA indicated that these soil, plant, climate variables as well as time could explain more variations based on GeoChip data (42.4%, **Figure 2B**) than 16S rRNA gene (30.4%, **Supplementary Figure S5b**) and ITS (27.2%, **Supplementary Figure S6b**) sequencing data, suggesting that functional structure of microbial communities is more sensitive to detect clipping-induced environmental changes than taxonomic structure of microbial communities. Specifically, the variations in the community functional composition and structure were explained by soil (14.2%) and plant (6.8%) variables, time (2.7%) and their interactions (7.2%; **Figure 2B**). Soil temperature and precipitation alone could directly explain 10.9% of the variation in community functional structure (**Figure 2B**). These results indicated that temperature and precipitation were important environmental attributes that shape the microbial community under clipping treatment. Furthermore, clipping effects on some C-degrading and nitrogen cycling genes were significantly ($P < 0.05$) regulated by autumnal cumulative precipitation, as indicated that clipping-induced changes in some key genes for C degradation and N cycling were linearly ($P < 0.05$) or non-linearly ($P < 0.05$) increased along precipitation, including those for degrading starch, cellulose, hemicellulose and Vanillin/Lignin, denitrification and nitrification (**Figure 3**). Clipping effects on these genes in the driest year 2012 were substantially different from those in the other years (**Figure 3**). Therefore, the response of functional genes to clipping in 2012 possibly represented a feedback pattern under the extreme drought condition, which was greatly different from the long-term pattern of microbial functional changes under annual clipping.

Effects of Annual Clipping on Microbial Functional Genes

To understand how annual clipping affected functional processes of soil microbial communities, GeoChip data were further analyzed by focusing on C, N, and P cycling. The normalized signal intensities were calculated to evaluate the change of gene abundance under clipping in each year. Because the shift in microbial communities under clipping in 2012 appeared in stark opposition to all other years and 2012 correspondingly was a year with prolonged and wide-spread drought (Cook et al., 2014; Wolf et al., 2016), average normalized signal intensities across 5 years with the omission of the year 2012 were evaluated to obtain the long-term trends of C, N, and P cycling under annual clipping (**Figure 4**).

In the first year (2010), most C degradation genes were significantly ($P < 0.05$) decreased under clipping (**Supplementary Figure S7**). However, clipping increased the abundance of most C degradation genes in the second year (2011). Among these genes, 17 genes whose abundance significantly ($P < 0.05$) increased under clipping were those involved in the degradation of relatively labile C (e.g., starch, hemicellulose, pectin, and cellulose). For example, glucoamylase, involved in the degradation of starch, xylanase, which degrades hemicellulose, and cellobiase, which breaks down cellulose, all showed significantly ($P < 0.05$) higher signal intensities under clipping. Interestingly, clipping also significantly ($P < 0.05$) increased the abundance of five genes involved in the degradation of recalcitrant C (e.g., chitin, vanillin, and lignin) including those encoding chitinase and phenol oxidase (**Supplementary Figure S7**). In contrast, in 2012, the year with low precipitation, almost all of detected C degradation genes decreased in the relative abundance under clipping. In the last 2 years (2013 and 2014), very few genes associated with labile C degradation remained significantly increased under clipping, while most of the genes involved in recalcitrant C degradation, that had originally significantly increased in 2011, were again significantly ($P < 0.05$) increased under clipping (**Supplementary Figure S7**). These results suggested that the stimulation of clipping on the genes involved in recalcitrant C degradation was more persistent than genes involved in labile C degradation, and implied that the degradation of recalcitrant C might be triggered as the cumulative effect of annual clipping on microbial communities increased over time. Furthermore, the average signal intensities across 5 years with the omission



	F	P
Soil	1.505	**0.002**
NO_3^-	2.273	**0.005**
NH_4^+	1.324	0.158
TN	1.512	0.060
TOC	1.629	**0.037**
pH	0.902	0.549
Soil W	1.294	0.152
Plant	1.484	**0.016**
C3 biomass	1.457	0.074
C4 biomass	1.111	0.255
plant richness	0.872	0.595
Climate	2.859	**0.001**
Soil T	2.296	**0.004**
TPP	3.007	**0.001**
Time	3.382	**0.001**
Whole	1.796	**0.001**

FIGURE 2 | Constrained ordination analysis. **(A)** Canonical correspondence analysis (CCA) of GeoChip data and environmental variables. environmental variables: soil nitrate (NO_3^-), ammonia (NH_4^+), total organic carbon (TOC), total nitrogen (TN), soil pH, soil water content (soil W), aboveground C_3 biomass, C_4 biomass, plant richness (PR), soil temperature (Tm) and autumnal total precipitation (TPP). The insert table showed the significances of each or subsets of the environmental variables in explaining the variations of microbial community functional structure based on *F*-test. **(B)** CCA-based variation partitioning analysis (VPA) of microbial functional structure explained by soil geochemical properties (S), plant diversity (P), climate (C) variables and time (T). Each diagram represents the biological variation partitioned into the relative effects of each factor or a group of factors.

of the third year (year 2012) also indicated that annual clipping significantly ($P < 0.05$) increased the relative abundances of many genes involved in the degradation of labile and recalcitrant C (**Figure 4A**).

GeoChip 5.0M also has various probes for key enzymes in CO_2 fixation from four pathways: ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) in the Calvin cycle, propionyl-CoA carboxylase (PCC) in the 3-hydroxypropionate cycle, carbon monoxide dehydrogenase (CODH) in the reductive acetyl-CoA pathway and ATP citrate lyase (AclB) in the reverse tricarboxylic acid cycle. All of these key genes fluctuated greatly in different years, likely reflecting the interaction of annual clipping and annual temporal variation over time (**Supplementary Figure S8**). In 2010 the abundances of *rubisco*, *codh*, and *ppc* genes were significantly ($P < 0.05$) decreased under clipping, but these genes significantly ($P < 0.05$) increased under clipping in 2011. In the last 2 years, *rubisco* genes (2013 and 2014), *codh* genes (2014), and *ppc* genes (2014) were significantly increased under clipping (**Supplementary Figure S8**). The year of 2012 represents a unique set of environmental conditions and a strong response shift to clipping treatment by the microbial community was observed. In 2012, all of the key genes in C fixation incongruently decreased under clipping. However, the average signal intensities across 5 years without 2012 indicated that the abundances of *rubisco*, *codh*, and *ppc* genes were significantly ($P < 0.05$) increased under annual clipping (**Figure 4C**). These results suggested that CO_2 fixation might be enhanced under 5-years of clipping treatment, but further studies are needed to determine the impacts of the fixed C on the overall soil carbon flux.

TABLE 2 | Correlation analysis between microbial community structures and environmental variables by Mantel test[a].

environmental variables	16S rRNA		ITS		GeoChip	
	r	*P*	*r*	*P*	*r*	*P*
All variables	0.215	**0.015**	0.179	**0.034**	0.107	**0.099**
Time	0.136	**0.019**	0.295	**0.001**	0.222	**0.001**
NO_3^--N	0.049	0.286	−0.023	0.563	−0.082	0.791
NH_4^+-N	0.054	0.261	0.164	**0.047**	−0.111	0.927
TN	0.022	0.371	0.109	0.125	−0.106	0.939
TOC	0.032	0.336	0.107	**0.089**	−0.124	0.970
Soil pH	0.150	**0.026**	0.044	0.283	0.138	**0.047**
Soil T	0.116	**0.040**	0.046	0.243	0.101	**0.049**
Soil W	0.002	0.474	0.110	0.102	0.084	0.139
C_3 biomass	0.145	**0.036**	0.288	**0.001**	0.224	**0.005**
C_4 biomass	0.024	0.359	0.097	0.111	−0.002	0.462
Total biomass	0.133	**0.044**	0.237	**0.010**	0.215	**0.009**
Plant richness	−0.065	0.821	0.114	**0.052**	−0.038	0.679
Precipitation	0.042	0.190	0.091	**0.037**	0.201	**0.002**
HR	0.107	**0.076**	0.120	**0.045**	0.135	**0.022**
TR	0.100	0.163	−0.003	0.505	0.053	0.263

[a]*The significant value ($P < 0.10$) are indicated in bold. NO_3^--N, soil nitrate-nitrogen; NH_4^+-N, soil ammonium nitrogen; TN, soil total nitrogen; TOC, soil total organic carbon; soil W, soil water content; soil T, soil temperature; HR, soil heterotrophic respiration; AR, soil autotrophic respiration; TR, soil total respiration.*

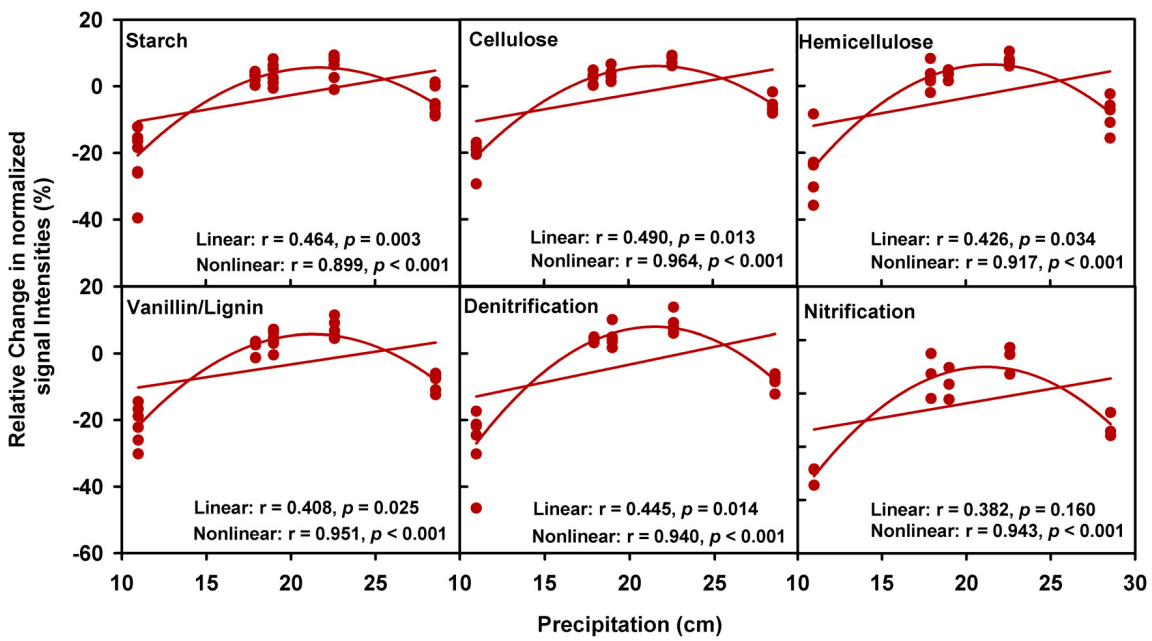


FIGURE 3 | Relationships between autumnal cumulative precipitation and clipping effects on C-degrading and N cycling gene groups. For all plots, the relative change of normalized signal intensities of detected genes by clipping in each year was presented in the *y*- axis as the clipping effects on functional genes [(clipping – control)/control]. In each plot, lines represent linear least squares regression fit and non-linear quadratic regression fit, respectively. The *r* values and significances were displayed for linear and non-linear fits. The genes in these plots are listed in **Supplementary Table S4**.

The abundances of N cycling genes involved in ammonification, anammox, assimilatory N reduction, denitrification, dissimilatory N reduction, N assimilation, nitrification, and nitrogen fixation were also shifted in different years (**Supplementary Figure S9**). The relative changes of average signal intensities of these years without 2012 were also analyzed to determine the long-term trend of N cycling under clipping. The abundance of most key genes involved in N cycling was significantly ($P < 0.05$) higher under annual clipping than control (**Figure 4B**). Specifically, the gene *ureC* and ammonium transporter gene (*gdh*) were significantly ($P < 0.05$) increased in clipped samples. Because the UreC protein can convert urea

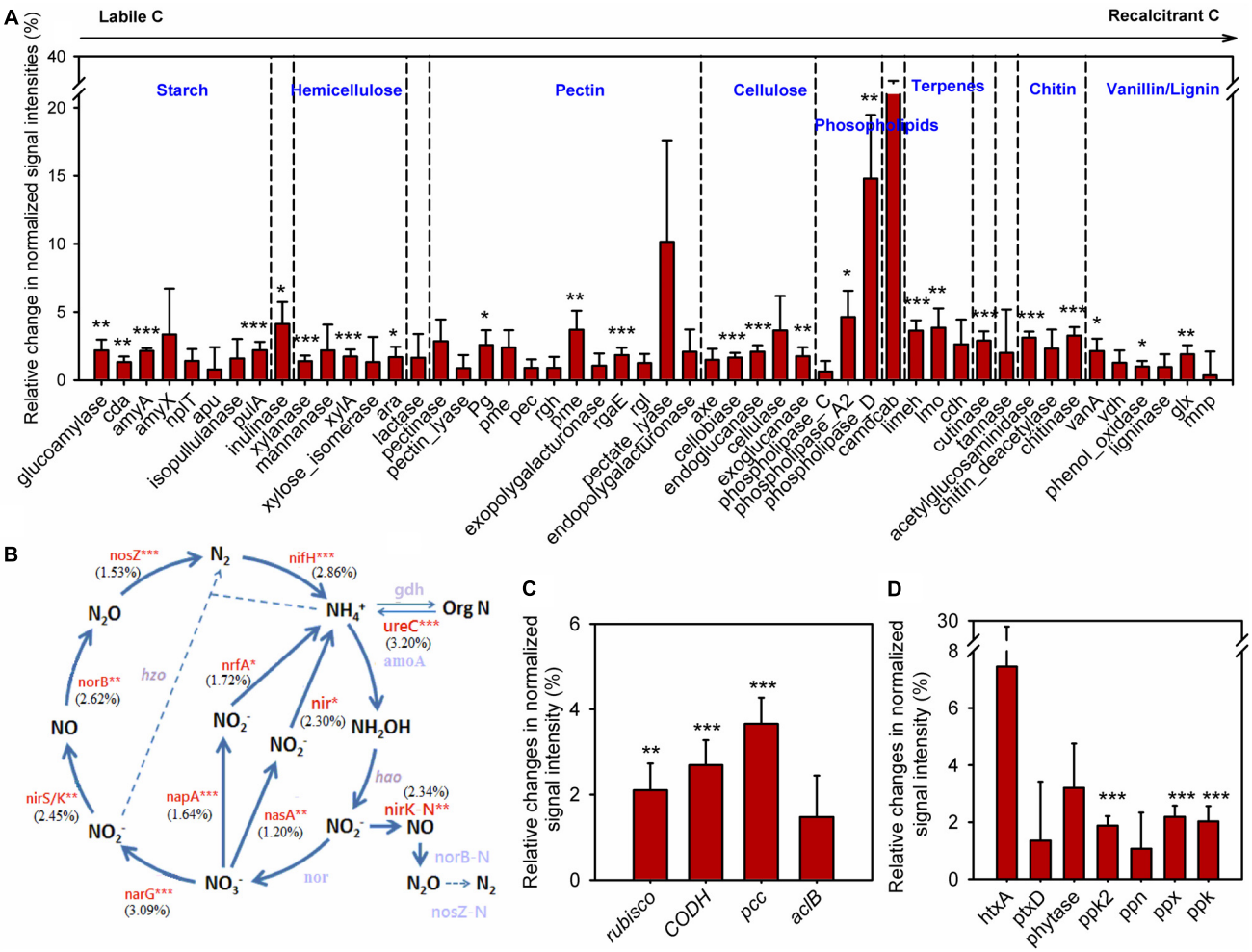


FIGURE 4 | Clipping effects on functional genes involved in biogeochemical cycling processes. **(A)** C degradation. The complexity of C is presented in order from labile to recalcitrant C. The average relative change of normalized signal intensity of detected genes by clipping across 5 years without 2012 samples was presented as the clipping effects on functional genes [(clipped – control)/control]. Error bars indicate standard error of the mean. Significance was tested by ANOVA as indicated by $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. **(B)** N cycling processes. The percentage changes in N cycling gene abundance under clipping were indicated in parenthesis. Genes where change in abundance was significant ($P < 0.05$) are labeled in red. Gray-colored genes were not significantly changed under clipping. **(C)** C fixation. **(D)** P utilization genes under annual clipping. The full names of the genes in this figure are listed in **Supplementary Table S4**.

into NH_4^+, and ammonium transporter proteins transport ammonium into microorganisms or plants, the combined effect of these two changes could potentially result in an increase in N mineralization but relatively stable NH_4^+ concentrations in the soil. Furthermore, *nirB* and *nasA*, involved in assimilatory N reduction, *norB*, *nirK*, *nirS*, and *narG* associated with denitrification, *napA* a dissimilatory N reductase, were also significantly ($P < 0.05$) increased under clipping treatment (**Figure 4B**). The combined effect of denitrification, assimilatory N reduction and dissimilatory N reduction could result in a rapid nitrate-nitrogen loss, which may be the reason why significantly decreased NO_3^- in soil were observed under clipping in 2012–2014 (**Supplementary Table S1**). In addition, the relative abundance of *nifH* for nitrogen fixation was significantly ($P < 0.05$) increased under 5-year clipping (**Figure 4B**). These significantly increased genes in nitrogen-cycling process may potentially lead to accelerating nutrient cycling rates under annual clipping.

GeoChip 5.0M contains seven enzymes involved in P utilization; exopolyphosphatase (Ppx), endopolyphosphatase (Ppn), and polyphosphate kinase (Ppk2) involved in phosphate degradation, polyphosphate kinase (Ppk) in polyphosphate biosynthesis pathways in prokaryotes, phosphonate dehydrogenase (PtxD) and phytanoyl-CoA dioxygenase (HtxA) responsible for phosphorus oxidation, and phytase associated with phytate degradation. All of these genes were also shifted in different years (**Supplementary Figure S10**). The abundance of *ppk*2 and *ppx* genes across 5 years, without 2012, were significantly ($P < 0.05$) increased under clipping, suggesting a possible increase of polyphosphate degradation with more available inorganic P in soil under annual clipping (**Figure 3**). Also, the abundance of *ppk* in clipped samples was significantly

($P < 0.05$) higher than in control samples (**Figure 4D**). Altogether, this indicated that P cycling potentials under annual clipping might also be accelerated in this ecosystem.

DISCUSSION

Plant tissue removal via grazing, mowing and/or clipping can significantly change ecosystem C fluxes, with consequent changes in plant-litter decomposition, soil microbial communities and nutrient cycling (Garibaldi et al., 2007; Klumpp et al., 2009; de Faccio Carvalho et al., 2010). As soil microbial community mediate important biogeochemical processes, such as C, N, P, and S cycling, understanding their responses to annual clipping is crucial for a robust prediction of soil C stocks and fluxes. In this study, we analyzed the potential taxonomic and functional responses of soil microbial communities to annual clipping. Our results showed that annual clipping markedly shifted the functional structures of soil microbial communities and relative abundances of some bacterial and fungal lineages over time, which generally support our three hypotheses.

Previous studies showed that clipping significantly affected the composition and productivity of plant communities (Ward et al., 2007; Wu et al., 2009), likely decreasing nutrient and C inputs from aboveground plants (Semmartin et al., 2008; Klumpp et al., 2009). Furthermore, clipping has been shown to increase soil temperature but decrease soil water content as did warming (Wan et al., 2002). Under clipping, an increase of root respiration and exudation was also observed (Bahn et al., 2006; Hamilton et al., 2008). Collectively, these shifts in plants and microenvironments under the cumulative effects of clipping are expected to progressively affect the structure and functional potential of soil microbial communities across a time span of several years. Our results generally supported this hypothesis. Consistent with those previous studies, we found annual clipping increased plant productivity, soil CO_2 efflux and microbial activity in most of years. More importantly, the microbial functional structure was not changed in the first year but altered significantly in the following 4 years by annual clipping (**Table 1**). Furthermore, statistical analyses proved that the changes of microbial community structure were significantly correlated with soil respiration, soil physical and chemical variables, and above-ground plant productivity. Besides, the relative abundance of some key bacterial and fungal phyla such as *Actinobacteria*, *Bacteroidetes*, *Zygomycota,* and *Ascomycota* were significantly altered across 5 years by annual clipping. These results demonstrated that the shifts of soil microbial communities under a long-term clipping can cumulatively affect certain soil ecosystem functions. However, certain studies suggested clipping or mowing reduced soil CO_2 efflux, microbial biomass and activity due to decreased canopy photosynthesis and lessened C supply from aboveground plant parts to roots, mycorrhizae and rhizosphere microorganisms (Zhang et al., 2005; Bahn et al., 2006; Shahzad et al., 2012). The disparity among studies may be caused by the different ecosystems studied and/or experimental designs including clipping and sampling time.

Microbial responses to global changes, such as warming, precipitation, and clipping may be greatly regulated by temporal background variations. Previous studies at the Jasper Ridge Global Change Experiment (JRGCE) showed that annual background variation of soil microbial communities was greater than even very significant treatment effects including warming, elevated CO_2, water addition, and N addition (Gutknecht et al., 2012). Another study also reported that temporal (seasonal and interannual) variation overshadows the responses of soil microbial communities to simulated global changes including drought and N addition (Matulich et al., 2015). Consistently, the taxonomic composition of both bacterial and fungal communities varied substantially from year to year in our study. No significant clipping effect was observed in the overall bacterial and fungal communities, most likely due to the large interannual background variation in soil microbial community overshadowing the response of bacterial and fungal communities to clipping (Matulich et al., 2015). Correspondingly, interannual background variations affected the relative abundance of bacterial and fungal phyla more significantly than annual clipping. Also, interannual background variation was more significant than the effect of clipping on soil microbial functional genes. The abundance of many functional genes involved in C fixation, C degradation, N cycling, and P utilization greatly fluctuated in different years. These results suggested that microbial responses to annual clipping were strongly shaped by temporal background variations.

More interestingly, we found a stark contrast in the functional community response to clipping when the ecosystem underwent an extreme drought disturbance as well as a significant correlation between precipitation and clipping-induced changes in some C-degrading or N cycling genes. Furthermore, precipitation was found to be one of the most important factors in explaining the variations of functional community structure in our study. Previous studies also reported that altered precipitation in different years can significantly change fungal and bacterial community structures (Schmidt et al., 2007; Castro et al., 2010). Precipitation can shift microbial biomass, community composition and activity directly by changing soil moisture as well as indirectly through shaping plant community, potentially with a lag (Schmidt et al., 2007; Castro et al., 2010). Provided these information, it may be that the effects of clipping on microbial functional activities are strongly associated with precipitation at the interannual scale.

In our study, no significant changes of the overall taxonomic structure of bacteria and fungi were observed in all years, whereas microbial functional structure was significantly shifted by clipping in the continuous four years. Furthermore, the variations of soil microbial functional structure were closely related to clipping-induced environmental changes, while the taxonomic variations were only poorly explained by environmental condition. No significant correlation was observed between taxonomic groups and functional gene groups. Such phenomena have been previously observed in the global ocean or in soil (Raes et al., 2011; Louca et al., 2016; Nelson et al., 2016). These results can be explained by an

elegant paradigm for microbial ecology, in which community function is strongly shaped by energetic and stoichiometric constraints (Raes et al., 2011), while the composition within functional groups is modulated by additional mechanisms. According to this paradigm, the functional responses of microbial communities to clipping can decouple with microbial taxonomic variations.

Whether the clipping-stimulated microbial community resulted in the significant changes of soil C and N cycling is another central hypothesis. Some studies showed that clipping reduced total soil CO_2 efflux composing of root respiration and mineralization of plant litter and recalcitrant SOM by 20–50% (Wan and Luo, 2003; Shahzad et al., 2012). In contrast, another study showed a TR increase under clipping treatment (Antonsen and Olsson, 2005). Significant increases in total and heterotrophic soil respirations by clipping were observed in our study. Theoretically, the increase of soil respiration could be due to the increase of microbial biomass and/or the variation of microbial community structure (Marschner et al., 2015; Bond-Lamberty et al., 2016). However, very limited amounts of samples were available from this long-term field experiment, thus we didn't investigate microbial biomass. In this study, we focused on the variation of community structure. Significant correlations between soil HR and the variations of bacterial, fungal and functional community structures suggested that the variation of community structure significantly modified soil C cycling, regardless of potential altered microbial biomass.

Although annual background variations (e.g., precipitation) strongly affected functional patterns of soil microbial community in different years, progressive changes of C degradation under clipping were still observed in our GeoChip data. In the first year, annual clipping as a strong disturbance to grassland ecosystem not only decreased soil microbial community functional diversity but also decreased most of gene abundances involved in C degradation. This may be a short-term response to the decrease of aboveground C input and the sudden changes of soil temperature and moisture under annual clipping. In the following 4 years, except 2012, the abundance of key genes involved in the degradation of labile and recalcitrant C increased under annual clipping, suggesting that reduced inputs of aboveground C under clipping did not suppress microbial activity, probably because of the offset by elevated belowground biomass through such processes as root exudation (Hamilton et al., 2008) and root decay (Belay-Tedla et al., 2009). There were no significant reductions in aboveground plant biomass in the clipped plots and, in fact, in the last 2 years there were significant increases in the clipped plots. This means that the plant growth rates were stimulated by annual clipping. A likely consequence of this, is enhanced root development and increased exudation by actively growing roots. Indeed, significant increase of root respiration under clipping, measured as AR, was observed in this grassland ecosystem. This may be the reason why soil total C did not significantly decrease under annual clipping. More importantly, the abundances of genes involved in the degradation of some recalcitrant substrates were consistently increased in the last 2 years under annual clipping, indicating that the recalcitrant C degradation may be triggered under annual clipping. Since the recalcitrant carbon in soil is much more abundant than labile carbon, even a small change in its decomposition rate could have significant effect on soil C storage (Davidson and Janssens, 2006). By this way, clipping land use practices may significantly affect future climate warming scenarios.

How clipping or mowing changes ecosystem N cycling is another important issue. A previous study showed that total N contents of soil at the Great Plains Apiaries, Oklahoma were significantly decreased by clipping, resulting in N-deficient soil conditions (Belay-Tedla et al., 2009), and another study showed that yearly clipping significantly decreased litter N contents, indicating a significant effect of N deficiency on plants (Cheng et al., 2010). Consistently, a significant decrease in NO_3^--N was observed under annual clipping in our study, suggesting that soil N dynamics were significantly altered. In our GeoChip data, average signal intensities across 5 years with the omission of the year 2012 indicated that annual clipping also stimulated the abundance of most key genes involved in N cycling, including ammonification, denitrification, N assimilation, and nitrogen fixation. The significant increases in the abundance of N cycling genes may result in a potential increase of nutrient cycling process rates. In high N cycling rates, N fixation and N mineralization through recycling N from SOM would compensate N loss by denitrification as well as enhance plant growth (Zhou et al., 2012). As a result, the total soil N may remain unchanged under annual clipping. However, the effects of long-term clipping on soil N dynamics may depend on the balance of the accumulation derived from the inputs from litter and root biomass decomposition, microbial N fixation and the consumption of N mineralization, denitrification, and plant uptake.

CONCLUSION

Despite the important roles of the soil microbial communities in carbon and nitrogen cycling, the responses of microbial community structure and function under long-term clipping are not fully understood. In this study, the functional structure of soil microbial community was significantly altered by 5 years of clipping and the relative abundance of bacterial and fungal lineages was also significantly changed under annual clipping. Furthermore, annual clipping significantly increased the abundance of genes involved in the degradation of labile and recalcitrant C, nitrogen cycling and phosphorus utilization in the long-term scale. The shifts in microbial community structure and function were significantly correlated with soil microclimate, C and nutrient concentrations, respiration and plant productivity. Interestingly, the effects of clipping on microbial functional activities may be heavily associated with precipitation at the interannual scale. Annual clipping-induced changes in microbial community structure and function may be important in predicting long-term land-use responses to global change.

DATA ACCESSIBILITY

DNA sequences of 16S rRNA gene and ITS amplicons were deposited under NCBI project accession no. PRJNA331185. OTU table and OTU representative sequences are available: http://ieg.ou.edu/4download/. Microarray data (GeoChip 5.0) are available: http://ieg.ou.edu/4download/. Soil physical and chemical attributes, plant biomass and richness and soil respirations: online Supplementary Materials.

AUTHOR CONTRIBUTIONS

All authors contributed intellectual input and assistance to this study. The original concept and experimental strategy were developed by JZ, YL, and JT. Field management was carried out by MY, JF, XG, XZ, LH, FL, LW, and JVN. Sampling collections, DNA preparation, and MiSeq sequencing analysis were carried out by XZ, XG, JF, MY, and LH. Soil chemical analysis was carried out by XZ, XG, and MY. Various statistical analyses were carried out by XG, YQ, DN, and ZS. Assistance in data interpretation was provided by XL and ZH. All data analysis and integration were guided by JZ. The paper was written by XG with help from ZH and JZ. Considering their contributions in terms of site management, data collection, analyses, and/or integration over the last 6 years, XG and XZ were listed as co-authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00954/full#supplementary-material

FIGURE S1 | Average air temperature **(a)** and cumulative precipitation **(b)** over the entire autumn (September to November) in the experiment site. The air temperature and cumulative precipitation were obtained online from the Oklahoma Mesonet Station (Washington Station). Air temperature data were collected at 1.5 m above the soil surface, and precipitation data were collected at the soil surface.

FIGURE S2 | Detrended correspondence analysis (DCA) of 16S rRNA gene **(a)** and ITS **(b)** amplicon sequences and GeoChip hybridization **(c)**. Symbols correspond to year of sampling. Colors of the symbols represent sample treatments with filled symbols representing clipped samples and hollow symbols representing control samples. Arrows represent the direction of change under clipping treatment in different years.

FIGURE S3 | The composition of bacterial community under clipping and control in 5 years based on 16S rRNA gene sequencing. The phylum *Proteobacteria* was represented by Alpha-, Beta-, Gamma-, and Delta-divisions. The insert table showed the significances of each phylum affected by clipping and sampling year based on ANOVA test. Red indicated $P < 0.05$.

FIGURE S4 | The composition of fungal community under clipping and control in 5 years based on ITS sequencing. The insert table showed the significances of each phylum affected by clipping and sampling year based on ANOVA test. Red indicated $P < 0.05$.

FIGURE S5 | Constrained ordination analysis of 16S rRNA gene amplicon sequences. **(a)** Canonical correspondence analysis (CCA) of 16S rRNA gene amplicon sequences and environmental variables. **(b)** CCA-based variation partitioning analysis (VPA) of bacterial community structure explained by soil geochemical properties (S), plant diversity (P), climate variables (C), and time (T). Details are described in **Figure 2**.

FIGURE S6 | Constrained ordination analysis of ITS amplicon sequences. **(a)** Canonical correspondence analysis (CCA) of ITS amplicon sequences and environmental variables. **(b)** CCA-based variation partitioning analysis (VPA) of fungal community structure explained by soil geochemical properties (S), plant diversity (P), climate variables (C), and time (T). Details are described in **Figure 2**.

FIGURE S7 | The relative changes of normalized signal intensities of detected C-degradation genes under clipping in different years. The complexity of C is presented in order from labile to recalcitrant C. Error bars indicate standard error of the mean. Significance is tested by ANOVA as indicated by $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. The full names of the genes in this figure are listed in **Supplementary Table S4**.

FIGURE S8 | The relative changes of normalized signal intensities of detected C fixation genes under clipping in different years. Error bars indicate standard error of the mean. Significance is tested by ANOVA as indicated by $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. The full names of the genes in this figure are listed in **Supplementary Table S4**.

FIGURE S9 | The relative changes of normalized signal intensities of detected N cycling genes under clipping in different years. Error bars indicate standard error of the mean. Significance is tested by ANOVA as indicated by $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. **(A)**, ammonification; **(B)**, anammox; **(C)**, assimilation; **(D)** assimilatory N reduction; **(E)**, denitrification; **(F)**, dissimilatory N reduction; **(G)**, N assimilation; **(H)**, nitrification; **(I)**, nitrogen fixation. The full names of the genes in this figure are listed in **Supplementary Table S4**.

FIGURE S10 | The relative changes of normalized signal intensities of detected P utilization genes under clipping in different years. Error bars indicate standard error of the mean. Significance is tested by ANOVA as indicated by $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. The full names of the genes in this figure are listed in **Supplementary Table S4**.

TABLE S1 | Soil geochemical variables, plant biomass and soil respirations under control and clipping in each year.

TABLE S2 | The overall microbial community diversity under clipping and control detected by ITS and 16S rRNA gene sequencing data and GeoChip data.

TABLE S3 | The significance tests of bacterial and fungal lineages affected by clipping in different years by ANOVA.

TABLE S4 | The enzyme/protein encoded by the functional genes shown in **Figures 3**, **4**, and **Supplementary Figures S7**–**S10**.

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Response of Nitrifier and Denitrifier Abundance and Microbial Community Structure to Experimental Warming in an Agricultural Ecosystem

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Soil microbial community plays an important role in terrestrial carbon and nitrogen cycling. However, the response of the soil nitrifier and denitrifier communities to climate warming is poorly understood. A long-term field warming experiment has been conducted for 8 years at Luancheng Experimental Farm Station on the North China Plain; we used this field to examine how soil microbial community structure, nitrifier, and denitrifier abundance respond to warming under regular irrigation (RI) and high irrigation (HI) at different soil depths (0–5, 5–10, and 10–20 cm). Nitrifier, denitrifier, and the total bacterial abundance were assessed by quantitative polymerase chain reaction of the functional genes and 16S rRNA gene, respectively. Bacterial community structure was studied through high throughput sequencing of the 16S rRNA gene. Under RI, warming significantly ($P < 0.05$) increased the potential nitrification rate and nitrate concentration and decreased the soil moisture. In most of the samples, warming increased the ammonia-oxidizing bacteria abundance but decreased the ammonia-oxidizing archaea (AOA) and denitrifier (*nirK*, *nirS*, and *nosZ* genes) abundance. Under HI, there was a highly increased AOA and 16S rRNA gene abundance and a slightly higher denitrifier abundance compared with RI. Warming decreased the bacterial diversity and species richness, and the microbial community structure differed greatly between the warmed and control plots. The decrease in bacterial diversity was higher in RI than HI and at the 0–5 cm depths than at the 5–10 and 10–20 cm soil depths. Warming led to an increase in the relative abundance of Actinobacteria, Bacteroidetes, and TM7 but a decrease in Acidobacteria, Alphaproteobacteria, Deltaproteobacteria, Nitrospira, and Planctomycetes. The greater shift in microbial community structure was observed only in RI at the 0–5 cm soil depth. This study provides new insight into our understanding of the nitrifier and denitrifier activity and microbial community response to climate warming in agricultural ecosystems.

Keywords: warming, irrigation, potential nitrification rate, nitrifier, denitrifier, microbial community

INTRODUCTION

The soil microbial community plays an important role in terrestrial nutrient cycling; many biological processes involved in nitrogen (N) cycling in terrestrial ecosystems are altered due to climate warming (Mosier, 1998; Rustad et al., 2001), and these changes are likely to result in altered plant productivity and atmospherically active gases (Mosier, 1998; Barnard et al., 2006). However, due to the complexity of the microbial community in soil, how climate warming affects the activity, abundance, and structure of microbial community is poorly understood (Rui et al., 2015).

Warming can directly affect soil bacterial physiology and indirectly affect microbial activity through changing plant and soil properties (Rui et al., 2015). For example, an increase in temperature may lead to a shift in community structure and can enhance the predominance of thermally adapted microorganisms (Bradford et al., 2008). Previous long-term warming experiments have shown that warming alone (Zhou et al., 2012; Rui et al., 2015) and in combination with precipitation (Castro et al., 2010) could alter the microbial community structure in the soil. Moreover, warming is responsible for moderate natural drought and decreased microbial diversity, with significant changes in community composition (Sheik et al., 2011). Xu et al. (2016) reported that simulated warming and drying conditions are responsible for altering the nitrifier and denitrifier community in vegetable soil. However, these field experiments mainly focused on grasslands, grass prairies, alpine forest, and vegetable soil to explain how the community composition of N cycling microorganisms was altered by simulated warming. In addition, the feedback response of microorganisms involved in nitrous oxide (N_2O) emission caused by warming and drought differed between various ecosystems (Singh et al., 2010). No study is available regarding the effects of simulated warming on communities involved in N cycling (i.e., nitrifier and denitrifier) and microbial community structure under an agricultural ecosystem, especially intensely fertilized wheat fields in the North China Plain. Understanding the effects of climate warming on the abundance of nitrifiers and denitrifiers, which carry out key processes such as nitrification and denitrification, is important because these processes influence soil inorganic N concentrations, nitrate leaching, and the production of N_2O (Barnard et al., 2006).

In the alpine forest and polar regions, warming can increase the soil moisture content due to glacier and permafrost melting (Walther et al., 2002) and thereby can enhance the activity of microbial communities involved in nitrification and denitrification. However, these observed changes are different from those in upland agricultural ecosystems, where climate warming is often accompanied by decreased soil moisture (Liu et al., 2016). Soil-warming experiments can offer an opportunity to elucidate the response of a microbial community to climate warming. Previous study from our group has reported that the experimental warming decreased N_2O emissions (Liu et al., 2016), possibly due to drier soil conditions which may be unfavorable for denitrifying activity. However, that study only provides evidence regarding the impact of climate warming on N_2O emission from the soil; the response of nitrifiers and denitrifiers involved in N_2O emission and microbial community composition to climate warming was not investigated.

In the present investigation, we aimed to elucidate the response of nitrifier and denitrifier abundance and microbial community structure to climate warming in an agriculture ecosystem. We hypothesized that (i) nitrifier and denitrifier abundance would respond differently to climate warming, as an increase in soil temperature would increase nitrifier abundance and decrease denitrifier abundance, and (ii) the microbial community structure would respond differently between regular irrigation (RI) and high irrigation (HI), as well as at different soil depths, to climate warming. To test these hypotheses, sampling was performed from the ongoing long-term (for 8-year) warming experiment with wheat cultivation in RI and HI plots at different soil depths. We assessed the nitrifier and denitrifier abundance by quantitative polymerase chain reaction (Q-PCR) and the microbial community structure by 16S rRNA gene sequencing.

MATERIALS AND METHODS

Site Description

The soil sampling site was located at the Luancheng Experimental Farm Station (37° 53′N, 114° 41′E, 50 m above sea level) of the Chinese Academy of Sciences, Hebei Province, China. Detailed information about the experimental site has been described previously (Liu et al., 2016). In brief, this long-term warming experiment began in 2008. Six pairs of infrared heaters (2 m × 0.02 m in size) with a rated power of 1000 W were installed 2 m above the ground at the center of six plots. The plot allocation treatments were randomized. The plot size was 4 m × 4 m, and the effective radiation area was 2 m × 2 m. Another six pairs of the same framework and heaters without power were placed in plots next to the heating plots at a distance of approximately 1 m to mimic the shadow effect of the heater frames. The crop under cultivation was winter wheat. The soil at the experimental site was classified as sandy loam with soil pH 8.1 (1:2.5 with H_2O), organic matter 15.1 g kg^{-1}, and total N 1.1 g kg^{-1} at a 0–20 cm soil depth.

Design of the Field Study and Soil Sampling

An experiment was conducted with simulated warming (temperature increased approximately 1.5 °C on average over 7 years at a 5 cm soil depth) and its control (no warming) under RI and HI (i.e., the treatments were designated as warmed and control under RI and HI; Liu et al., 2016). The fertilizer dose (N fertilizer, 315 kg N ha^{-1} $year^{-1}$; P fertilizer, 65 kg P ha^{-1} $year^{-1}$) used in this experiment was the same for all treatments, as reported previously (Liu et al., 2016). In case of irrigation, RI and HI plots were irrigated with 60 and 90 mm, respectively, at the same time. Irrigation was applied one time before the soil sampling (first week of April 2016). The high irrigation treatments included in this study are based on the conclusion from our previous field experiment, which states that high irrigation overrides the warming impact on denitrifying activity (Liu et al., 2016).

Soil sampling was performed at 0–5 (5), 5–10 (10), and 10–20 cm (20 cm) soil depths within effective radiation area in triplicates (April 2016). Three soil cores were randomly taken from each triplicate plot by auger (3.2 cm diameter) and mixed together to get a composite sample. The soil samples were transported to the lab in an icebox, sieved through a 2 mm sieve and stored at 4 °C for biochemical analyses and −80 °C for gene abundance and microbial community composition analyses.

Soil Temperature, Moisture, and Mineral N Concentration

T-type thermocouple lines were placed in the soil at the center of all plots to automatically monitor the soil temperature at 0–5 cm every hour, which was recorded by a data logger (CR 10X, Campbell, CA, United States). The volumetric soil water content at 0–5 and 10–20 cm depths in each plot was measured manually by time-domain reflectometry.

Five grams of soil were extracted with 50 mL of 2 M KCl and shaken at 150 rpm for 30 min. The filtered solution was then analyzed for ammonium (NH_4^+) and nitrate (NO_3^-) using a spectrophotometer (UV-2450, Shimadzu, Japan). The NH_4^+ concentration was estimated by indophenol blue method (Page et al., 1982), 8 mL of filtrate was transferred to the 50 mL glass tube, and then 5 mL of phenol–nitroprusside solution and 5 mL of alkaline hypochlorite solution were added, mixed thoroughly, and waited for 1 h at room temperature to develop a blue color. The optical density was measured at 625 nm. For NO_3^- estimation, filtrate solution was directly used to measure the absorbance at 210 nm (A_{220}) and 275 nm (A_{275}) wavelength. The concentration of NH_4^+ and NO_3^- in the sample was calculated by plotting against a standard curve.

Potential Nitrification Rate

The potential nitrification rate (PNR) was used in the study as an index for the size of active nitrifier populations in the soil. In brief, for each sample, three subsamples (5 g of fresh soil) were incubated in 50 mL falcon tubes containing 20 mL of phosphate buffer solution including 1 mM $(NH_4)_2SO_4$. Potassium chlorate ($KClO_3$) was added to the tubes at a final concentration of 10 mM to inhibit the nitrite (NO_2^-) oxidation. The suspension was incubated in the dark at 25 °C for 24 h, and nitrite was extracted with 5 mL of 2 M KCl. After filtration, the optical density of the supernatant was analyzed for the presence of NO_2^- at 540 nm with N-(1-naphthyl) ethylenediamine dihydrochloride (Kurola et al., 2005). PNR was calculated as the linear accumulation in concentrations of NO_2^- between time 0 and 24 h.

Soil DNA Extraction

Soil samples from all treatments were selected for functional gene quantification by Q-PCR and microbial community structure analysis through 16S rRNA gene sequencing. Soil total nucleic acids were extracted using an E.Z.N.A.®Soil DNA Kit (Omega Bio-tek, Inc., Norcross, GA, United States) according to the manufacturer's instructions. The quality and quantity of the extracted DNA were examined with agarose gel (1%) electrophoresis and a NanoDrop spectrophotometer (NanoDrop ND-2000c Technologies, Inc., Wilmington, DE, United States). Extracted DNA was stored at −20 °C until further analysis.

Quantitative PCR Assay of Functional Genes

Quantitative polymerase chain reaction was performed to quantify 16S rRNA gene and functional genes involved in nitrification (*amoA* for bacteria and archaea) and denitrification (*nirS*, *nirK*, and *nosZ*). The *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes were quantified using primers as follows: amoA-1F/amoA-2R for bacterial *amoA* (Tourna et al., 2008; Jin et al., 2010), Arch_amoAF/Arch_amoAR for archaeal *amoA* (Francis et al., 2005), F1aCu/R3Cu for *nirK* (Hallin and Lindgren, 1999), cd3aF/R3cd for *nirS* (Michotey et al., 2000; Throback et al., 2004); nosZ-F/nosZ-1622R for *nosZ* (Kloos et al., 2001; Throback et al., 2004), and 1369F/1492R for 16S rRNA gene (Suzuki et al., 2000). Standard curves were constructed using a 10-fold series dilution of the plasmids for seven gradients carrying the respective target genes. The Q-PCR reaction was performed in a 25 μL volume, containing 2 × SYBR Premix Ex Taq (Takara Biotech, Dalian, China), 1 μM of each primer (for functional genes), 2 μM of each primer and 3 μM of probe for 16S rRNA gene, and 1 μL template DNA (20 ng μL^{-1}). The Q-PCR program consisted of an initial cycle of 95 °C for 2 min, 40 cycles of 30 s at 95 °C for denaturation (15 s for 16S rRNA gene), 40 s for annealing (53/60 °C for archaeal/bacterial *amoA*, 57 °C for *nirK*, 56.8 °C for *nirS*, 59 °C for *nosZ* and 60 s, 56 °C for 16S rRNA gene), 30 s at 72 °C for extension, and 10 s at 85 °C for collection of the fluorescent signals. Melting curves were generated for functional genes with continuous fluorescence acquisition from 57 to 95 °C at the rate of 0.5 °C per 10 s. After Q-PCR, the gene copy numbers were normalized by the amount of soils based on the dilution rates and the volumes of the DNA used for Q-PCR.

16S rRNA Gene Amplicon Sequencing

Microbial community structure was analyzed through sequencing of the 16S rRNA gene of samples from all treatments and all soil depths. Bacterial DNA was amplified with a set of primers targeting hypervariable V3–V4 region (approximately 460 bp) of 16S rRNA gene with attached overhang adapters (FwOvAd-341F: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACG GGNGGCWGCAG; ReOvAd-785R: GTCTCGTGGGCTCGGA GATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC; Yasir et al., 2015). In a 25 μL volume, reaction mixtures contained 2 × premix Ex Taq™ (Takara Biotechnology, Dalian, China), 5 μM of each primer, and 1 μL DNA template (20 ng μL^{-1} concentration). The reaction conditions were an initial cycle of 95 °C for 3 min; 23 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C; and a final extension at 72 °C for 10 min. The PCR products were visualized on agarose gels to confirm successful amplification and then purified with AMPure XP beads (Beckman Coulter, Inc., Brea, CA, United States) to remove residual primers and primer dimers following the manufacturer's protocol. Then, using a subsequent eight-cycle PCR, Illumina sequencing adapters and dual-index barcodes

were added to each amplicon. After purification on AMPure beads, the libraries were then normalized according to the Nextera XT (Illumina) protocol. The pooled samples were sent to Shanghai Jiao Tong University, Shanghai, China, and sequenced on a MiSeq platform (Illumina, San Diego, CA, United States).

Bioinformatics Analysis

The quality of the sequences was inspected with the fastQC program[1]. The paired-end reads were merged using FLASH (version 1.2.11) (Magoč and Salzberg, 2011) with the default settings, except that the maximum overlap length was set to 170. The low-quality merged sequences were then removed using fastx_toolkit software[2], and only the sequences with more than 80% of the bases that had quality scores higher than 20 were kept. Any sequences with ambiguous bases (N) and sequences outside 414–506 bp (460 ± 10%) in length were discarded for further analysis. Then, the sequences were pooled in one file and input into the Quantitative Insights into Microbial Ecology (QIIME) software suite. The subsampled open-reference workflow was used for Operational Taxonomic Unit (OTU) classification and taxonomy assignment, and OTU picking was performed using uclust (Edgar, 2010) with the default cutoff value (97%). The OTU table was subsampled (rarefied) and the alpha diversity (Shannon–Wiener index) was calculated based on the rarefied OTU tables (Magurran, 1988). The rarefication curves were plotted and presented as Supplementary Figure 2. Principal coordinate analysis (PCoA) was performed using the weighted UniFrac distance matrix between the samples in QIIME pipeline. Sequencing data were deposited into the European Nucleotide Archive under the accession number PRJEB22187.

Statistical Analyses

Statistical analyses were conducted with Statistix 8.1 and SPSS20.0 software. Analysis of variance (one-way ANOVA), Tukey's honestly significant difference (HSD, at $P < 0.05$), and unpaired *t*-test ($P < 0.05$) were performed to assess the significant effect of warming on soil physico-chemical parameters, abundance of functional genes, total bacterial gene, and microbial community structure among the treatments and at all soil depth. A two-way ANOVA analysis and HSD (at $P < 0.05$) analysis were performed to assess the main and interactive effect of warming and irrigation on abundance of functional genes and total bacterial gene. SPSS20.0 was used to assess the Pearson's correlation between PNR, nitrate concentration, and gene abundance [ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA)].

RESULTS

Soil Temperature, Moisture, and Mineral N Concentration

Warming increased the soil temperature by 1.6 °C in RI and 0.8 °C in HI (**Table 1**). In addition, warming decreased the soil moisture content at 5 and 20 cm soil depths compared to the control plots, and the decrease in soil moisture was higher in the RI treatment than in the HI treatment (**Table 1**).

Warming increased the NH_4^+ concentrations at all soil depths than in control, but this increase was not significant. Warming also increased NO_3^- concentrations in both RI and HI, but the increase was significant ($P < 0.05$) only in RI (**Table 2**). Moreover, the concentration of mineral N was higher at the 5 cm depth and decreased with soil depth.

Potential Nitrification Rate and Abundance of Nitrifiers and Denitrifiers

The PNR was higher in warmed than in control plots at all soil depths, but a significant difference ($P < 0.05$) was observed only in RI (**Table 2**). AOA abundance was slightly lower in warmed plots compared with control plots but was statistically at par (**Figure 1A**) in both irrigation treatments; however, the abundance was more than twofold higher in HI than in RI. In contrast, the AOB abundance was significantly ($P < 0.05$) higher in warmed than in control plots at a 5 cm soil depth in RI

TABLE 1 | Effect of warming on the monthly average value of soil temperature and moisture under the regular irrigation (RI) and high irrigation (HI) treatments.

	RI		HI	
Soil depth (cm)	**Warmed**	**Control**	**Warmed**	**Control**
Soil temperature (°C)				
5	12.7 ± 0.74a	11.1 ± 0.44a	12.4 ± 0.55A	11.6 ± 0.65A
Soil volumetric moisture (%)				
5	11.1 ± 0.94a	13.6 ± 1.7a	13.1 ± 1.05A	14.7 ± 1.12A
20	10.2 ± 1.29a	12.4 ± 1.75a	12.4 ± 1.27A	14.1 ± 2.35A

Identical letters in the same row indicate no significant difference at P < 0.05 [Tukey's honestly significant difference (HSD) post hoc test]. Values are expressed as the means and standard errors (n = 3).

TABLE 2 | Effect of warming on soil NH_4^+, NO_3^- concentrations and potential nitrification rate (PNR) activity under RI and HI at different soil depths.

	RI		HI	
Soil depth (cm)	**Warmed**	**Control**	**Warmed**	**Control**
NH_4^+ (mg-N kg^{-1})				
5	2.69 ± 0. 10a	2.10 ± 0.02b	4.57 ± 0.26A	4.67 ± 0.12A
10	1.43 ± 0.13a	1.35 ± 0.20a	2.02 ± 0.29A	1.73 ± 0.03A
20	1.42 ± 0.18a	1.10 ± 0.09a	1.34 ± 0.03A	1.28 ± 0.03A
NO_3^- (mg-N kg^{-1})				
5	139.8 ± 3.73a	57.4 ± 4.98b	91.2 ± 9.03A	80.4 ± 6.47A
10	126.3 ± 6.95a	43.3 ± 4.19b	74.9 ± 15.6A	61.9 ± 5.07A
20	117.9 ± 2.80a	38.5 ± 3.19b	57.2 ± 7.43A	45.3 ± 1.0A
PNR activity (mg of NO_2 kg^{-1} soil day^{-1})				
5	18.2 ± 0.93a	12.6 ± 2.44b	13.3 ± 0.30A	12.0 ± 0.42A
10	17.7 ± 3.86a	7.88 ± 2.54b	20.2 ± 2.32A	18.6 ± 0.23A
20	19.6 ± 1.17a	11.4 ± 3.62a	18.7 ± 0.91A	17.8 ± 0.10A

Different letters in the same row indicate a significant difference at P < 0.05 (Tukey's HSD post hoc test). Values are expressed as the means and standard errors (n = 3).

[1]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[2]http://hannonlab.cshl.edu/fastx_toolkit/

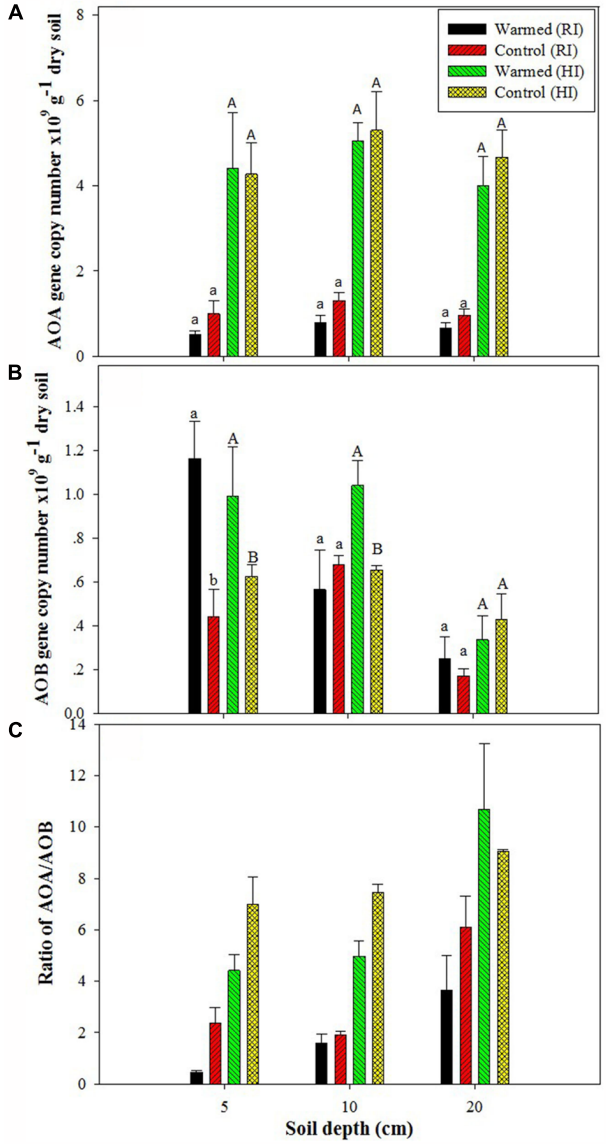

FIGURE 1 | Effect of warming on ammonia-oxidizing archaea (AOA) **(A)**, ammonia-oxidizing bacteria (AOB) gene copy number **(B)**, and AOA/AOB ratio **(C)** under the regular irrigation (RI) and high irrigation (HI) treatments at different soil depths. Different letters indicate a significant difference at $P < 0.05$ (unpaired *t*-test). The error bar indicates the standard error of the mean ($n = 3$).

and at 5 and 10 cm at HI; there was no significant difference between control and warmed plots at the 20 cm soil depth (**Figure 1B**). AOB abundance was higher in 5 and 10 cm soil than at 20 cm, whereas AOA did not show any decrease in abundance with soil depth. The ratio of AOA to AOB decreased in warmed plots compared with control plots at all soil depths, except at 20 cm in HI (**Figure 1C**). PNR ($r^2 = 0.74$, $P < 0.01$) and nitrate ($r^2 = 0.82$, $P < 0.001$) showed a positive correlation with AOB abundance, whereas PNR ($r^2 = -0.59$, $P < 0.05$) exhibited a negative correlation with AOA. Two-way ANOVA analysis showed that warming had a significant correlation with AOB abundance alone ($P < 0.001$) and with irrigation ($P < 0.05$) at the 5 cm soil depth; however, the AOA abundance had a significant ($P < 0.001$) correlation only with irrigation at all soil depths, and there was no significant correlation between warming and AOA abundance (Supplementary Table S1).

In the case of denitrifying genes, we observed a significant impact of soil warming on the *nirK*, *nirS*, and *nosZ* gene abundance. The abundance of the *nirK* (**Figure 2A**) and *nirS* (**Figure 2B**) genes was higher in control than in warmed plots at all soil depths and in both irrigation treatments, but the significant ($P < 0.05$) decrease was mostly observed under RI. The *nosZ* abundance was decreased in warmed compared with control plots at 10 and 20 cm soil depths under RI (**Figure 2C**). The relative abundance (normalized to total 16S rRNA gene copies) of functional genes showed a similar trend with denitrifying gene abundance in warmed and control plots (Supplementary Figure 1). Warming and irrigation alone had a significant effect on *nirS*, *nirK*, and *nosZ* abundance, and there was no interactive effect of warming with irrigation on these genes, except for *nirS* and *nosZ* at 20 and 10 cm, respectively (Supplementary Table S1). Warming had no significant effect on 16S rRNA gene abundance under both irrigation treatments, but the higher irrigation increased 16S rRNA gene abundance by more than twofold compared with RI (**Figure 2D**). Irrigation had a significant correlation with 16S rRNA gene abundance at all soil depth ($P < 0.001$); however warming alone and with irrigation had no significant correlation with 16S rRNA gene abundance (Supplementary Table S1).

Assessment of the Microbial Diversity and Community Structure

In total, 2,751,268 sequences were generated, resulting in 21,343–57,433 sequences per sample. Quality control steps removed around 55% low-quality reads and ended up with 1,224,231 high-quality reads, which were analyzed using QIIME pipeline. After OTU picking, the singletons were removed, and then the OTU table was rarefied to have 12,071 sequences in each sample. In the control and warmed plots, the Proteobacteria, Actinobacteria, and Acidobacteria were the most abundant phyla followed by Planctomycetes, Chloroflexi, Bacteroidetes, and Gemmatimonadetes, and minor sequences were related to the Firmicutes, TM7, Verrucomicrobia, and Nitrospirae (**Figure 3**). Soil warming strongly influenced the abundance of bacterial taxa and a large proportion of phyla were significantly responded to warming in RI treatment (especially at 5 cm soil depth); however, some phyla, such as Acidobacteria, Actinobacteria, Firmicutes, and Gemmatimonadetes, showed significant differences in their relative abundance between warmed and control under HI treatment. We observed a significant increase in the relative abundance of Actinobacteria ($P < 0.05$), which was compensated by a tendentious decrease in Acidobacteria and Proteobacteria (**Figure 3** and Supplementary Table S2). Warming significantly ($P < 0.05$) increased abundance of Actinobacteria under both irrigation treatments except at the 20 cm soil depth under HI; while TM7 increased and Verrucomicrobia decreased significantly ($P < 0.05$) in relative abundance in warmed plot

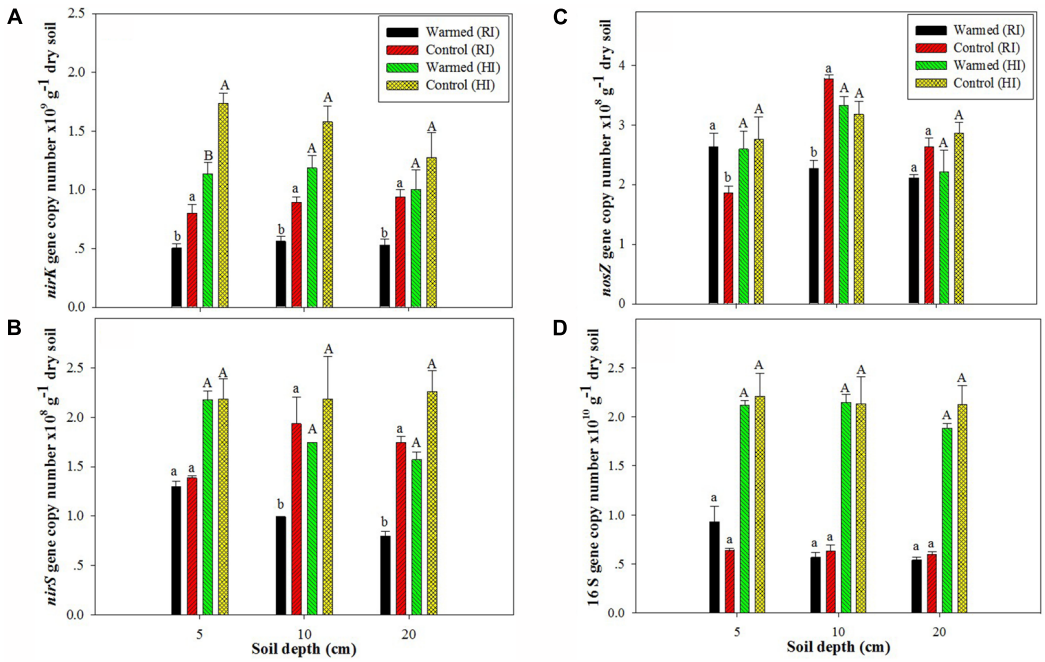

FIGURE 2 | Effect of warming on the abundance of *nirK* **(A)**, *nirS* **(B)**, and *nosZ* **(C)** and on the 16S rRNA gene **(D)** at different soil depths under RI and HI. Different letters indicate a significant difference at $P < 0.05$ (unpaired *t*-test). The error bar indicates the standard error of the mean ($n = 3$).

compared to control plot under RI at all soil depths. Order Actinomycetales (Actinobacteria) also showed higher ($P < 0.05$) relative abundance in the warmed plots than in control plots at all soil depths under both irrigation treatments, except at the 20 cm soil depth under HI (Supplementary Table S2). However, Acidobacteria showed a lower relative abundance in warmed plots compared with control plots under both irrigation treatments, but significant decrease was observed in 5 cm soil depth. Gemmatimonadetes, Nitrospirae, and Planctomycetes showed significantly lower relative abundance in warmed compared with control plot at 5 cm soil depth under RI treatment. Firmicutes and Gemmatimonadetes showed a significantly lower relative abundance in warmed compared with control plots under HI at most of the soil depths (Supplementary Table S2). Class Solibacterales (Acidobacteria) also showed a lower abundance ($P < 0.05$) in warmed compared with control plots under both irrigation treatments except at 20 cm where the differences were not significant. Nitrospirales (Nitrospirae) showed a significantly ($P < 0.05$) lower abundance in warmed compared with control plots at the 5 cm in RI and the 5 and 20 cm soil depths in HI treatment. Among the Proteobacteria phyla, relative abundance of Alpha-, Gamma-, and Delta-Proteobacteria was generally lower in warmed plot than control plots. Order Rhodospirillales (Alphaproteobacteria) and Syntrophobacterales (Deltaproteobacteria) showed a lower relative abundance in warmed compared with control plots under RI (significant at 5 cm soil depth). Most of the significant differences in the relative abundances occurred under RI. This meant that the community structure changes caused by warming could be override by high rate irrigation. Soil depth was also influencing the taxon relative abundance, larger proportion of taxa at the 5 cm soil depth (i.e., surface layer) was significantly influenced by warming compared with the 10 and 20 cm soil depths under RI, while the trend was not as obvious in HI (**Figure 3** and Supplementary Table S2).

The heatmap of the microbial community displays the relative abundances of dominant bacterial genera either increased or decreased in response to warming under RI and HI at different soil depths (**Figure 4**). A pairwise comparison showed that the relative abundance of genera was strongly influenced by warming and a large proportion of genera were either increased or decreased significantly in relative abundance in RI (especially at 5 cm soil depth) when compared with HI treatment (**Figure 4** and Supplementary Table S2). *Arthrobacter*, *Rubrobacter*, *Cellulomonas*, *Cohnella*, *Mycoplana*, *Janthinobacterium*, and *Lysobacter* were significantly enhanced by warming compared with the control under RI treatment, while *Arthrobacter*, *Rubrobacter*, *Cohnella*, and *Janthinobacterium* were significantly enhanced by warming under HI. However, some genera such as *Nitrospira*, *Rhodoplanes*, *Planctomyces*, and *Gemmata* were decreased in relative abundance by warming compared with the control under RI, and *Bacillus*, *Gemmata*, and *Pseudomonas* (except at 20 cm soil depth) showed a similar response to warming under HI. These results suggested that the significant change in abundance of most bacterial communities in response to soil warming was observed under the RI and was mainly limited to the 5 cm soil depth.

Shannon–Wiener and Chao1 indexes were calculated to assess the bacterial diversity and richness. Warming decreased the bacterial diversity compared with the control, and the decrease

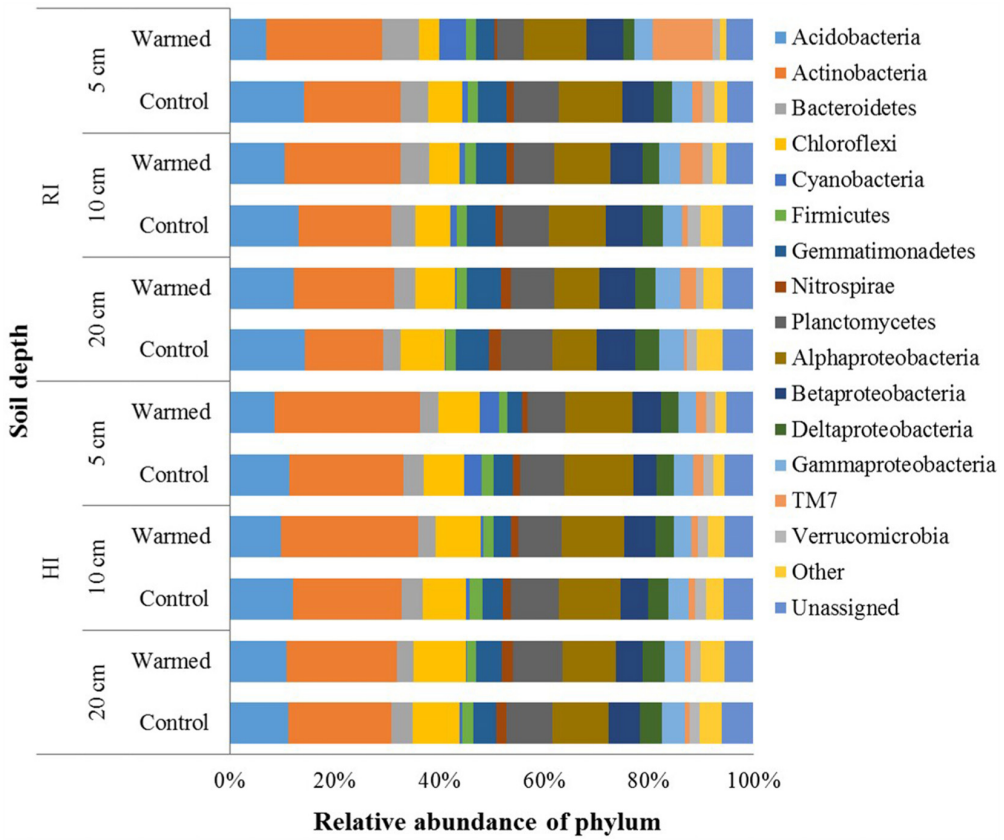

FIGURE 3 | Relative abundance of the dominant bacterial phyla in warmed and control plots at different soil depths under RI and HI irrigation.

was much higher in RI than HI; furthermore, the decrease in diversity was significant ($P < 0.05$) at the 5 cm soil depth in RI (**Figure 5A**). The warmed plot had a lower richness compared to the control, with higher OTU number in control than in warmed plots in both RI and HI treatments. The comparison of the rarefaction curve (Supplementary Figure 2) showed a similar result to the diversity index (**Figure 5A**) and species richness (Chao1, Supplementary Table S3). PCoA analysis (weighted UniFrac) of the bacterial community for the control and warmed plots showed that the difference between bacterial communities in the warmed and the control plots was less in HI than in RI treatment (**Figure 5B**). In RI, the bacterial community was clearly different between warmed and control plots at 5 cm soil depth; the difference was bigger in the 5 cm than at 10 and 20 cm soil depth. These results suggested that the dissimilarity in bacterial community caused by warming could be overridden by increased irrigation.

DISCUSSION

Microbial Community Responding to Soil Warming

Temperature has long been known a determinant for the growth and physiology of microorganisms and may be a determining factor for niche space competition among physiologically similar organisms (Sheik et al., 2011). The microbial communities analyzed in this study showed that the relative abundance of Actinobacteria, Bacteroidetes, and TM7 bacteria was positively correlated with soil warming, whereas that of Proteobacteria, Acidobacteria, Chloroflexi, Firmicutes, Nitrospirae, and Verrucomicrobia was negatively correlated to soil warming under both irrigation treatments, except that Bacteroidetes, Chloroflexi, and Firmicutes showed opposite trend with warming under high irrigation (**Figure 3** and Supplementary Table S2). The increase of Actinobacteria and decrease of Acidobacteria in relative abundance with soil warming were in agreement with the previous observations (Kuffner et al., 2012; Rui et al., 2015). The response of Actinobacteria to warming might be related to spore-forming ability which could be an advantage over other phyla likely to persist in warmed drier soil (Hayden et al., 2012). Actinobacteria are among the most important litter decomposers (k-selected) in soil and might be favored for soil organic matter (SOM) decomposition over other microbes because of adaptation to warming (Kopecky et al., 2011). Warming significantly increased the relative abundance of *Actinomycetales* (dominant order) and *Arthrobacter* (dominant genus) (Supplementary Table S2) which were previously reported their involvement in SOM decomposition and recalcitrant carbon degradation, respectively

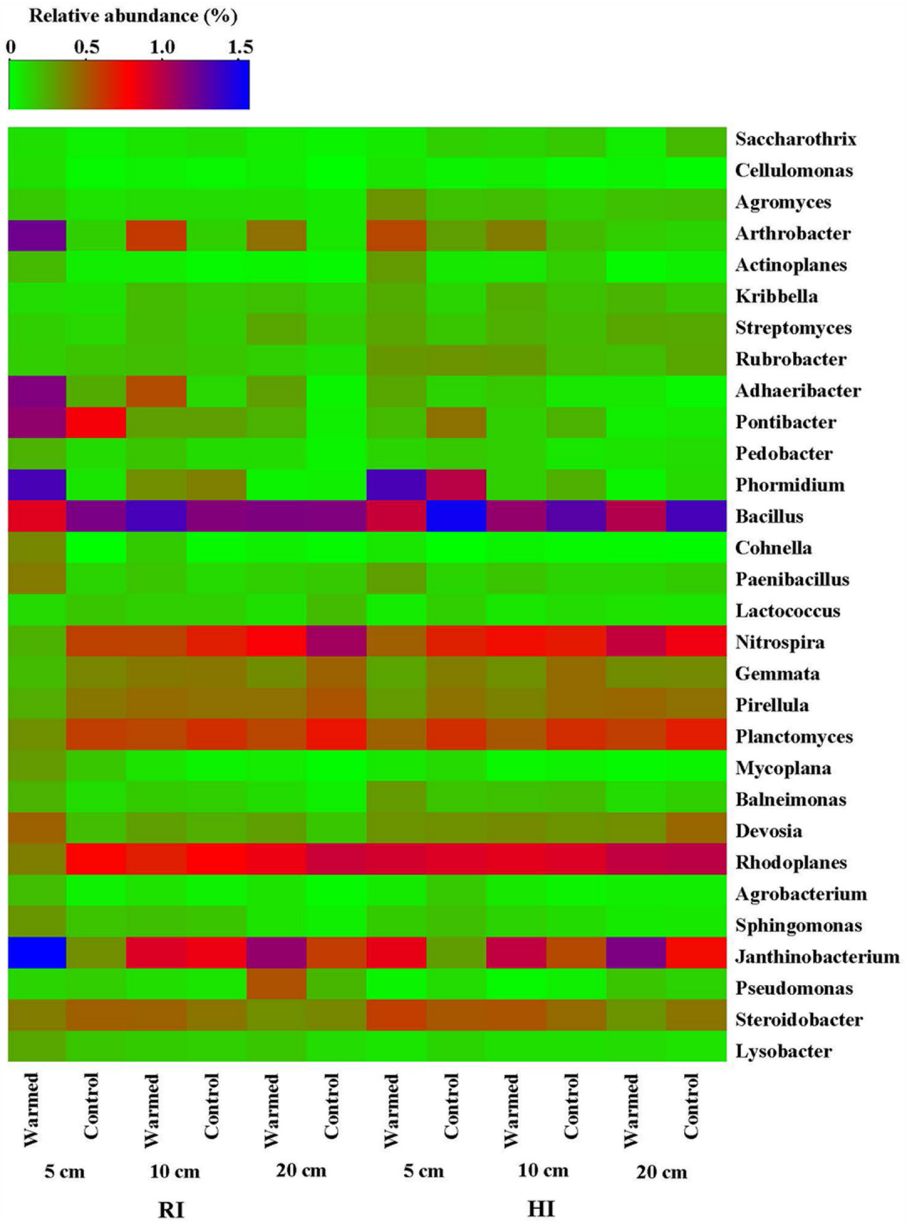

FIGURE 4 | Heatmap of the bacterial distribution of different communities from warmed and control samples at the genus level (most abundant genera either increased or decreased in response to warming were selected). The row represents the relative abundance of each bacterial genus, and the column stands for each sample at different soil depths under RI and HI treatment. The relative abundance of each bacterial genus is depicted by color intensity with the legend indicated at the top of the figure. The relative abundance for each genus in different samples is colored in shades of green (low relative abundance) to red and purple to blue (high relative abundance).

(Ferreira et al., 2008; Bengtson et al., 2012). The Proteobacteria and Acidobacteria showed lower abundance in warmed plot compared to control plots. A decrease in Proteobacterial phyla in warmed plot might to due to drier soil environment caused by soil warming, as Proteobacterial phyla found more responsive to wet environment than dry environment (Castro et al., 2010). Among the Proteobacteria, the relative abundance of Deltaproteobacteria (order Syntrophobacterales) and order Rhodospirillales (Alphaproteobacteria) was significantly decreased in response to soil warming, which could be due to soil drier condition in warmed plot than in control plots. Previous studies also reported the significant decrease in the relative abundance of order Rhodospirillales in response to long-term soil warming (Deslippe et al., 2012; DeAngelis et al., 2015). Acidobacteria are generally considered as oligotrophic organisms which grow well in lower carbon availability (Fierer et al., 2007). Previous study reported higher relative abundance of Acidobacteria in dry environment than wet environment, as

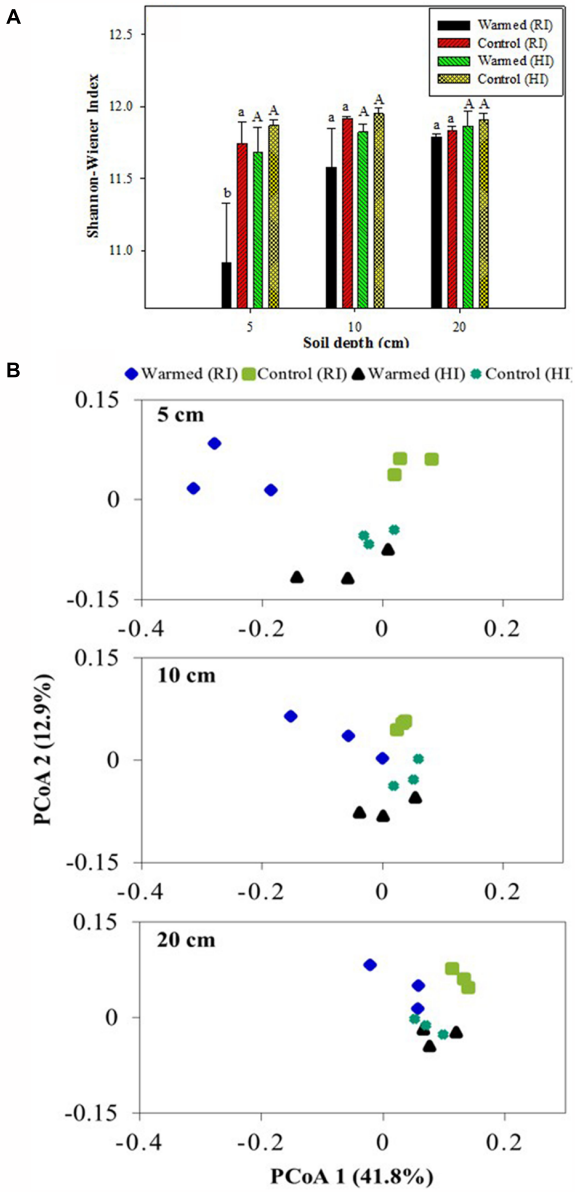

FIGURE 5 | Shannon-Wiener index **(A)** and weighted UniFrac principal coordinate analysis (PCoA, **B**) of the bacterial communities based on OTUs of the 16S rRNA gene from the warmed and control plots under RI and HI at different soil depths. One PCoA ordination was performed for all treatments, but the three depths (5, 10, and 20 cm) are shown separately for clarification. Identical letters indicate no statistically significant difference at $P < 0.05$ (Tukey's HSD *post hoc* test). The error bars indicate the standard errors of the means ($n = 3$).

dry environment slowed turnover of carbon in the particulate organic matter pool, which can reduce substrate availability and lead to more oligotrophic conditions (Garten et al., 2009; Castro et al., 2010). This is in contrast with our result, as we have found lower Acidobacteria abundance in warmed plot than in control plot. The decrease in abundance of Acidobacteria in our study might be due to higher dominance of Actinobacteria as these two groups are likely to share similar niches (Sheik et al., 2011). The phyla Gemmatimonadetes and Verrucomicrobia were lower and TM7 was higher in relative abundance in the warmed plot than in the control (**Figure 3** and Supplementary Table S2). The scarcity of the cultured representative of Gemmatimonadetes and Verrucomicrobia and TM7 phyla makes it difficult to ascertain their anticipated role in the ecosystem. However, owing to their significantly prompt response to warming, further research on their ecology and role in the environment is necessary. Our result indicated that different species might respond to climate warming at different rates and in different directions, resulting in an increase or decrease in the relative abundance of certain taxa.

Warming showed stronger effects on bacterial abundance, bacterial diversity, and community structure at surface layer than subsurface soil layers. The bacterial abundance (16S rRNA gene copy numbers) from the most of the samples was decreased slightly in response to warming. Other studies have also reported the decrease in bacterial abundance in response to warming in soil (Allison and Treseder, 2008; Castro et al., 2010; Hayden et al., 2012). Warming decreased bacterial diversity compared to control plot. The decrease in bacterial diversity might be due to the warmer and drier soil environment; it has been reported that warming treatment and soil water content strongly influenced bacterial population size and diversity in grassland soil (Sheik et al., 2011). The greater effect of warming on structuring of bacterial communities at surface layer (5 cm) than subsurface layers (10 and 20 cm soil depth) (**Figure 5B**) suggests that the effect of warming declined with soil depth. A declining effect of soil microbial communities over depth has also been noted by others (Rinnan et al., 2007; Deslippe et al., 2012).

Nitrifiers Responding to Soil Warming

For nitrifier abundance, AOB abundance showed a significant response to warming when compared with AOA. Although the warming caused a drier condition and that condition could be restrictive to AOB growth (Chen et al., 2013), the AOB abundance was higher in warmed plot than in control, which might be due to the adaptive tendency of AOB to drier conditions. This result supported by Xu et al. (2016), who showed that under the simulated warmer and drier condition, the AOB community displayed rapid and significantly higher growth rate than that of AOA, with the population abundance being one order of magnitude higher than the control. Moreover, the previous study found that soil warming for +0–5 °C significantly increased the AOB abundance under N fertilization in boreal forest soil (Long et al., 2012). As in our study, warming that increased the soil temperature by 1.6 °C would significantly affect AOB abundance. Previous studies reported the soil water content and temperature could influence microbial activity (nitrification rate) and nitrifying community structure in soil (Avrahami and Bohannan, 2007; Gleeson et al., 2008; Tourna et al., 2008; Szukics et al., 2010). In our study, though the gene abundance of ammonia oxidizing bacteria was higher in the warming treatment, the relative abundance of *Nitrospira* (nitrite oxidizing bacteria) was significantly lower in the warmed

plots compared to the control, especially at the surface layer (Supplementary Table S2). In the previous study, Rui et al. (2015) also found a low abundance of *Nitrospira* at a high temperature. The lower abundance of *Nitrospira* could be due to its sensitivity to drier conditions and, also outcompetition with other AOB species (i.e., *Nitrosomonas*) under high oxygen and substrate (NO_3^-) concentrations (Xu et al., 2016). Compared with AOB, the relative abundance of AOA was slightly lower in the warmed than the control plots. However, the AOA abundance substantially increased under HI irrigation, which means that AOA community was more responsive to high water content. Szukics et al. (2010) also reported that the AOA community rapidly adapted to high water content and lower temperature, while AOB community increased with increasing temperature. The decreased ratio of AOA to AOB in the warmed plot was mainly due to the increase in AOB growth at elevated soil temperature. The previous study also indicated that the ratio of AOA to AOB significantly decreased under the warmer and drier condition (Xu et al., 2016), which corroborated that the AOB community more rapidly adapted to the warmer and drier condition than did AOA community.

Denitrifiers Responding to Soil Warming

Our study observed a significantly higher nitrate concentration in the warming treatments. Despite the higher nitrate concentration, the denitrifier abundance was lower in warming than in control plot. The decrease in denitrifier abundance might be due to drier soil condition produced through soil warming (Keil et al., 2015). Higher temperature leads to a higher evapotranspiration demand and thus a higher vapor pressure deficit, which could produce drier soils (Liu et al., 2013). The previous study concluded the reduction in soil water content by soil warming produces an oxic condition, which may be unfavorable for denitrification activity (Liu et al., 2016). A previous study reported that the low moisture and a higher oxygen concentration inhibited activities of denitrification enzymes in the soil (van Spanning et al., 2007). Warming increased nitrate and ammonium concentration than in control, which is consistent with previous observations (Liu et al., 2016). Xu et al. (2016) reported lower denitrification activity from warmed soil even though there was higher nitrate and ammonium concentrations compared to the control treatment, and concluded that warming could accelerate N mineralization and nitrification processes, not denitrification. This was consistent with our results of higher nitrifier abundance and lower denitrifier abundance in response to soil warming. These results were also in agreement with previous study by Barnard et al. (2006), who found that soil drying due to warming lowered the denitrification potential in soil. The denitrification in soil is mainly performed by facultative aerobic heterotrophic bacteria from diverse phylogenetic branches; therefore, it is hard to draw general conclusions about how warming influence the denitrifier composition, in spite of that the typical denitrifier genus *Bacillus* (Verbaendert et al., 2011) was found decreased with compared to control in the relative abundance in HI. Therefore, a combined higher temperature and drier scenario may affect denitrifier activity and/or abundance in soil.

The microbiome is an integral part of the soil, which is important for maintaining ecosystem function. A sound understanding of how these microbial communities respond to disturbances such as climate warming is limited. The study on how projected climate warming affects soil nitrifiers and denitrifiers community from wheat field soil is critically important for managing and minimizing the impact of climate change. The results from this study clearly demonstrated that the simulated temperature rise and drier soil condition could affect both the population abundance of nitrifiers and denitrifiers and the total bacterial community structure in soil. In summary, our observation revealed that experimental warming increased the soil temperature and decreased the soil moisture. Increased temperature significantly increased the PNR activity, NO_3^- concentration, and AOB abundance in the soil but decreased (not significantly) the AOA abundance. Warmer and a drier soil condition tended to reduce the denitrifier abundance. Warming decreased the bacterial diversity and species richness and enhanced the relative abundance of species that have key roles in the decomposition of SOM. In RI, warming clearly yielded a significant shift in the microbial community structure, compared with HI, whereas the application of higher irrigation overrode the warming effect on the microbial community structure. Furthermore, warming had a pronounced effect on the microbial community structure at the surface layer (5 cm) compared with the deep soil layers (10 and 20 cm soil depths). Taken together, these results suggest that a projected warmer and drier climate change scenario would alter the population abundance of nitrifiers–denitrifiers and the microbial community structure (especially at surface layer), which, in turn, could affect the nitrogen turnover in the agricultural ecosystems.

AUTHOR CONTRIBUTIONS

TW, CH, and BL: conceived and designed the experiments. TW: performed the experiments. TW, SC, RS, and JL: analyzed the data. TW: wrote the paper. RS, CH, and BL: provided comments and improvements to the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00474/full#supplementary-material

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PERSPECTIVE
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Strategies to Maintain Natural Biocontrol of Soil-Borne Crop Diseases During Severe Drought and Rainfall Events

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In many parts of the world, agricultural ecosystems are increasingly exposed to severe drought, and rainfall events due to climate changes. This coincides with a higher vulnerability of crops to soil-borne diseases, which is mostly ascribed to decreased resistance to pathogen attacks. However, loss of the natural capacity of soil microbes to suppress soil-borne plant pathogens may also contribute to increased disease outbreaks. In this perspectives paper, we will discuss the effect of extreme weather events on pathogen-antagonist interactions during drought and rainfall events and upon recovery. We will focus on diseases caused by root-infecting fungi and oomycetes. In addition, we will explore factors that affect restoration of the balance between pathogens and other soil microbes. Finally, we will indicate potential future avenues to improve the resistance and/or recovery of natural biocontrol during, and after water stresses. As such, our perspective paper will highlight a knowledge gap that needs to be bridged to adapt agricultural ecosystems to changing climate scenarios.

Keywords: extreme weather events, climate change, crop, pathogen, disease suppression, soil microorganisms, antagonistic interactions

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INTRODUCTION

Climate change is expected to increase the exposure of agricultural ecosystems to extreme drought and rainfall events (IPCC, 2012; Fischer and Knutti, 2016), which can result in severe decreases in crop yields (Challinor et al., 2014; Obidiegwu et al., 2015; Challinor et al., 2016; Eurostats, 2016). It will, therefore, be a great challenge to maintain sufficient food production for the growing human population. Next to direct decreases in crop yields due to unfavorable growth conditions, additional problems may be caused by a reduced resistance of agricultural crops to soil-borne plant pathogen attacks after drought and rainfall events (Ramegowda and Senthil-Kumar, 2015; Dikilitas et al., 2016). The coincidence of extreme weather events and higher vulnerability of crops to pathogen attacks can be due to a decrease in the plant immune response (for a detailed review on this topic see Ramegowda and Senthil-Kumar, 2015) and/or an altered pathogen pressure.

Root-infecting fungi and oomycetes are two major groups of pathogens causing problems in agricultural crops at a broad range of moisture levels (Duncan and Kennedy, 1989; Dixon and Tilston, 2010; Thompson et al., 2013). For example, high water content increases the ability of motile zoospores of plant pathogenic oomycetes to reach roots (Malajczuk and Theodorou, 1979;

Judelson and Blanco, 2005). In contrast, drought increases the amount of drought resistant microorganisms. Fungi are often more resistant to drought than bacteria (Barnard et al., 2013; Meisner et al., 2013; de Vries et al., 2018) and many fungal pathogens, such as species belonging to *Fusarium* or *Verticillium* genera, are often involved in increased pathogen pressure during drought, (Dikilitas et al., 2016). Hence, the types of pathogens that thrive under drought and wet conditions will differ.

A largely ignored potential mechanism of increased pathogen pressure after an extreme drought or rainfall event is the reduction of the natural capacity of soil to suppress pathogens. The legacy of an environmental stress, including water stress, can decrease the biological suppression of crop pathogens and therewith increase the vulnerability of crops for pathogen attacks (Ho and Ko, 1985; Lootsma and Scholte, 1997; van Agtmaal et al., 2015). Most soils show a certain level of suppression against pathogenic fungi and oomycetes, often referred to as general soil suppression (Garbeva et al., 2011). Competitive interactions in soil microbial communities are thought to be the major causal factor of general soil suppression (Garbeva et al., 2011). In addition, some soils show so-called specific suppression against one pathogenic species (Raaijmakers and Mazzola, 2016). The plant's response to increased pathogen abundance depends on the microbial community colonizing the roots and the plant's ability to tolerate water stress. The colonization of plant roots by soil microorganisms is influenced by the amount and composition of rhizodeposits (Philippot et al., 2013). Several root-colonizing microorganisms are known to improve the plants response to pathogens (Berendsen et al., 2012). In addition, several rhizosphere microorganisms can increase drought tolerance in plants (Ngumbi and Kloepper, 2016). However, there is limited information about interactions of plant-growth promoting microbes with pathogens during drought stress and upon recovery. In this perspectives paper, we propose that improvements to the maintenance and recovery of suppression of plant pathogens during and after drought and rainfall may prevent severe losses due to soil-borne pathogens. In addition, we will suggest areas for future research that improve our understanding of how extreme drought and rainfall events will affect interactions between pathogen suppressive microorganisms and crop pathogens.

ANTAGONISTIC INTERACTIONS BETWEEN PATHOGENS AND HETEROTROPHIC MICROBES

The suppression of pathogen infection on roots is caused by interactions with other soil microorganisms (van Os et al., 1999; Duran et al., 2017) and often occurs via the production of inhibitory secondary metabolites (Garbeva et al., 2011). Chemical compounds, such as antibiotics, that are produced during antagonistic interactions between competing heterotrophic microbes may also affect other biota in soils, including pathogens (Garbeva et al., 2011; Raaijmakers and Mazzola, 2012; Schulz-Bohm et al., 2017). Most secondary chemicals exuded by microorganisms can diffuse through the water-filled area of soil pores and, therefore, only interact with microbes that live in the water phase (Tyc et al., 2017). However, one group of secondary compounds, volatiles, is of special interest, as volatiles can diffuse through both the water-filled and air-filled soil pores thereby widening the spatial range of inhibition of pathogens (Schmidt et al., 2015; Tyc et al., 2017). As such, the impact of fluctuations of soil water content on the role of volatiles in pathogen suppression is of special interest (Peñuelas et al., 2014). Differences in moisture content will affect the composition of chemical compounds produced by soil microbes (Bastos and Magan, 2007; Hiltpold and Turlings, 2008). Waterlogged conditions after heavy rainfall will expel gasses from soil and reduce the movement of gasses in soil (Moyano et al., 2013). Volatiles will be especially involved in competitive interactions in the air-filled area of the pores in unsaturated soils (**Figure 1A**), whereas water soluble secondary metabolites will be the main compounds in antagonistic interactions during waterlogged conditions (**Figure 1C**). Therefore, the chemical and physical characteristics of secondary metabolites that are effective in suppressing interactions will be determined by soil moisture conditions (**Figure 1**).

There is increasing evidence that volatiles produced by soil microorganisms play an important role in the natural suppression of pathogens. For example, growth of three common plant pathogens was inhibited by volatiles emitted from 50 agricultural soils (van Agtmaal et al., 2018). Production of pathogen-inhibiting volatiles by bacteria has received particular attention in research (Garbeva et al., 2011; Schmidt et al., 2015; Schulz-Bohm et al., 2017). Research on suppression of fungal pathogens by bacterial volatiles has also indicated that composition of bacterial communities is an important determinant of the spectrum of volatiles produced. For example, loss of rare soil bacteria decreased volatiles that suppressed *in vitro* growth of the plant pathogen *Fusarium oxysporum* (Hol et al., 2015). In addition, the legacy of anaerobic disinfection, which is the anaerobic treatment of soil in between crop cover, reduced volatiles and pathogen suppression three months after recovery, via effects on the bacterial community composition (van Agtmaal et al., 2015). Differences in soil moisture can affect the composition of the microbial community (Barnard et al., 2015; Hartmann et al., 2017; Meisner et al., 2018) and, consequently, also the spectrum of inhibiting compounds. The question remains if these changes coincide with altered pathogen suppression.

BALANCE BETWEEN SOIL PATHOGENS AND HETEROTROPHIC SOIL MICROBES

Pathogen suppression will be influenced by the response of both heterotrophic microorganisms and pathogens to drought and waterlogged conditions as well as their ability to recover (**Figure 2A**). First, both pathogens and heterotrophic microorganisms have to survive the extreme conditions. This will likely depend upon the niche space for water availability as

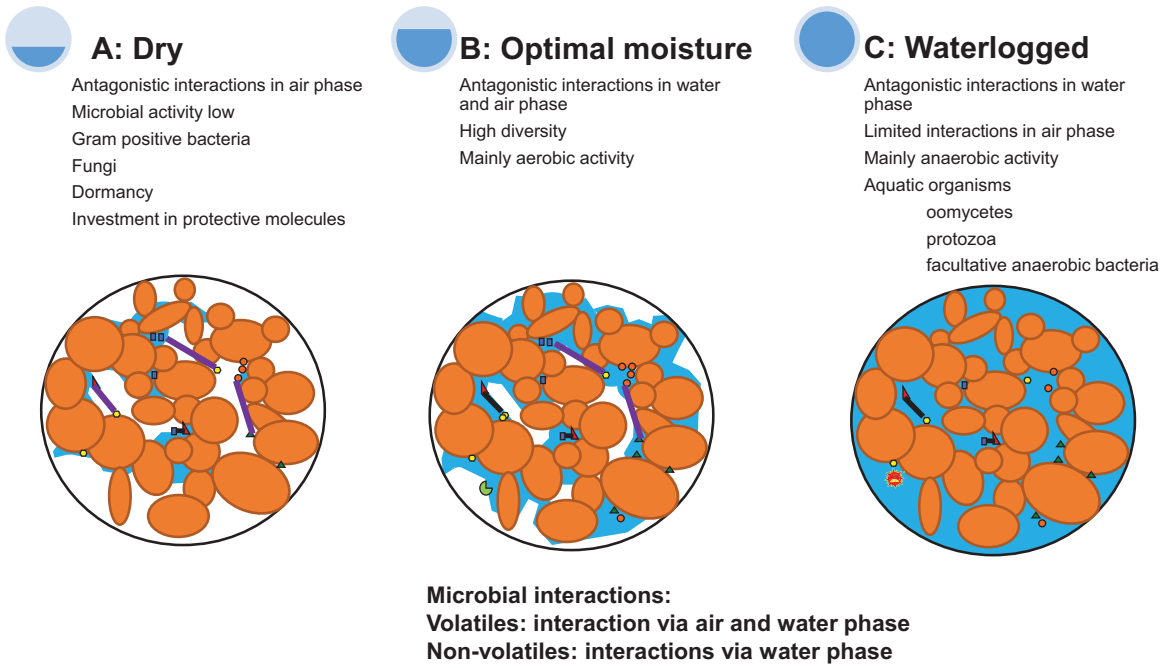

FIGURE 1 | The types of antagonistic interactions between pathogens and other soil microorganisms are influenced by water availability. Under dry conditions **(A)**, there is a big air phase and the interactions between microorganisms may occur mainly via volatile organic compounds in the air phase. However, the microbial activity of both resident and plant pathogens is low when moisture is limiting. Microorganisms that survive drought may invest in protective molecules or formation of dormancy structures. During optimal moisture conditions **(B)**, most microorganisms grow aerobically and interact via secondary chemicals, enzymes and volatiles in both the air and water phase. During waterlogged conditions **(C)**, interactions between microorganisms occur in the water phase of soils. Microorganisms that survive waterlogged conditions include organisms that can cope with anoxic conditions. Small triangles, squares, and circles reflect different soil microorganisms. The purple lines reflect microbial interactions that occur in the air phase and the black lines reflect interactions that occur in the water phase of soil. The blue areas indicate the water phase and the white areas the air phase. Figure adapted from Moyano et al. (2013).

microbial species, including pathogens, differ in their potential to maintain activity along a range of matric potentials (Whiting et al., 2001; Lennon et al., 2012). A wider niche space for a microorganism results in a higher chance of surviving the extreme conditions and, consequently, a higher chance to be present in the recovery phase. Soil microorganisms often experience anoxic conditions when exposed to waterlogged conditions. This can have an impact on the composition of microbes in the recovery phase (van Agtmaal et al., 2015). Microbes may also survive unfavorable conditions by going into dormancy (Manzoni et al., 2014; Shoemaker and Lennon, 2018), by producing protective molecules, such as osmolytes (Warren, 2014) or extracellular peptides (Or et al., 2007). Another strategy to survive is to have a thicker cell wall such as the thick peptidoglycan layer of Gram positive bacteria (Potts, 1994; Schimel et al., 2007).

Although there are many survival strategies to cope with drought and waterlogged conditions, cells of many soil microorganisms are irreversibly damaged (Nocker et al., 2012). For example, drying increases damage to DNA and enzymes (Dose et al., 1991; Potts, 1994). As a result, the active microbial biomass size is reduced upon recovery (Kieft et al., 1987; Lennon et al., 2012; Meisner et al., 2017). The partial elimination of microbes does result in an increase in the number of empty niches available upon recovery that both pathogens and other microbes can colonize. The success of colonization of empty niches by microbial species is determined by community assembly rules, such as priority effects. Priority effects describe the inhibitory or facilitative effects of early arriving species on next arriving ones (Fukami, 2015). Species that will recover faster from an extreme weather event will likely have a priority to become abundant first (Placella et al., 2012). In addition, dispersal due to movement of spores via wind or mixing of the content of soil pores during heavy rainfall and rewetting events can affect the composition of microbial species developing during recovery (Szekely and Langenheder, 2017).

The increased availability of easily available substrates upon recovery (Williams and Xia, 2009) due to increased necromass will act as a surplus of food sources for both pathogens and other microorganisms. This implies that the competitive pressure for energy resources is temporarily relieved. This is expected to coincide with a decrease in intensity of antagonistic interactions between microorganisms, including antagonistic interactions that suppress soil-borne plant pathogens. A similar condition can be created by adding easily available substrates to soils. For example non-mature compost can result in an increased infection by soil-borne pathogens (Hoitink and Grebus, 1994). Several factors can contribute to an increased risk for outbreaks

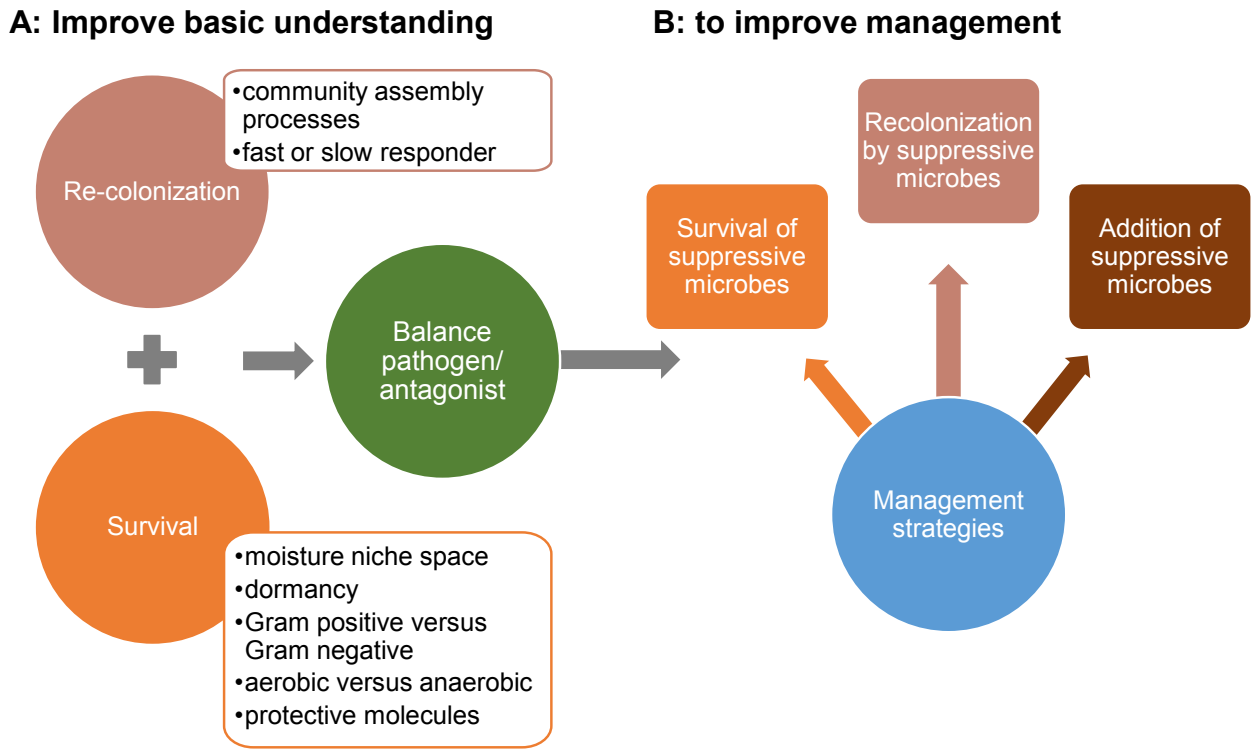

FIGURE 2 | Future research priorities are to improve basic understanding of microbial interactions that affect the balance between pathogens and antagonists upon their survival during exposure to extreme water stress and recolonization strategies during moisture stress and upon recovery **(A)** and use this basic understanding to improve management strategies that improve the pathogen suppression **(B)**.

of soil-borne pathogens during nutrient excess, namely (1) lower colonization of microorganisms that suppress pathogens (Hoitink et al., 1997); (2) decreased production of secondary metabolites due to investment of nutrients in growth and not in defense strategies (Coley et al., 1985; de Boer et al., 2003; Ghoul and Mitri, 2016); (3) reduced sensitivity of microorganisms, which are well fed, to inhibitory compounds, because they invest more in defense strategies (Garbeva et al., 2011). Thus, community assembly processes, the availability of labile nutrients and empty niches will influence the composition of the microbial communities during the recovery phase. Indeed, composition of microbial communities has often been observed to differ with different moisture treatments (Fierer et al., 2003; Drigo et al., 2017; Hartmann et al., 2017; Naylor and Coleman-Derr, 2017). In summary, water-related stress due to drought and rainfall events will change the interactions between microorganisms, which will affect the opportunities of pathogens to infect roots.

FUTURE RESEARCH TO IMPROVE AGRICULTURAL ADAPTATION TO CLIMATE CHANGE

Future research should take into account knowledge about microbial interactions, survival, and recovery of pathogens and antagonistic microorganisms during or after extreme water stress events to find strategies for increasing pathogen suppressive activities of microbes (Hawkes and Connor, 2017). Most important is to have insight in the key factors that affect the balance between heterotrophic soil microbes and pathogens. In this section, we will indicate knowledge gaps and management strategies that could be explored for the improvement of pathogen suppression upon the recovery of agricultural soil after drought or rainfall events.

Knowledge Needed to Improve Survival of Pathogen Suppressive Microorganisms

Survival of microorganisms is dependent on the moisture niche space and microbial traits (See "Balance Between Soil Pathogens and Heterotrophic Soil Microbes"). There are indications that drought is a natural selector for the microbial community, as microbial communities differ in soil with a legacy of drought, weeks to months after recovery (Bouskill et al., 2013; Meisner et al., 2018). Changes in the microbial community composition after a stress can affect the response of the microbial community to an additional drought stress. For example, microbial communities with a drought legacy seem to have a better ability to cope with an additional drought than microorganisms previously exposed to ambient conditions (Evans and Wallenstein, 2014). In addition, drought adapted

microbes can improve fitness of plant species exposed to dry conditions (Lau and Lennon, 2012; Ngumbi and Kloepper, 2016). Drought-adapted microbes do not only improve the drought tolerance of their host plant, but also of other plants (Rodriguez et al., 2008; Marulanda et al., 2009). Drought exposed microorganisms can also recover faster to other stresses (van Kruistum et al., 2018). However, the question remains if drought-tolerant microorganisms suppress pathogens.

Microorganisms that survive waterlogged conditions need to cope with a wide range of oxygen concentrations (Neira et al., 2015). For example, *Enterobacteriaceae* have been observed to maintain metabolic activity when going from oxic to anoxic conditions after a rainfall event (Degelmann et al., 2009). In addition, a legacy of waterlogged conditions, such as flooding can result in a reduced suppression of bulb-rot causing *Pythium* spp. (van Os et al., 1999). The anaerobic activity of microbes is releasing compounds like organic acids, organic sulfides, and ammonia that can be toxic to aerobic microbes. This is the reason why stimulation of anaerobic decomposition of incorporated organic material into agricultural soils is used as a method to kill aerobic pathogens (Strauss and Kluepfel, 2015). However, changes in microbial community composition due to anaerobic disinfestation can cause a drastic reduction of the pathogen suppressive capacity of soils that remains present months after recovery (van Agtmaal et al., 2015). This implies that pathogens that will survive waterlogged conditions can remain abundant in the recovery phase. However, it is unknown if microorganisms that survive anaerobic conditions can improve pathogen suppression upon a second rainfall event.

Strategies to Improve Re-colonization of Pathogen Suppressive Microbes

Management strategies should focus on ways to improve re-colonization of empty niches by microbes that suppress pathogens, as this would allow for an earlier recovery of pathogen suppression. One way of improving recovery is the addition or manipulation of organic material, as the 'carrying capacity of substrate' has been suggested to regulate species composition, their abundance, and activity and therewith regulates the suppression of pathogens (Hoitink et al., 1997). Soil with higher carbon content can maintain higher moisture levels during droughts (Ng et al., 2015) and higher microbial biomass (Hueso et al., 2012). Accordingly, the addition of organic material may improve survival and create patches of microbes that can colonize empty niches upon recovery. However, difference in decomposition stage of the organic material can be important to consider. Early stages of the breakdown of organic material have many easily available substrates and are low in supporting pathogen suppression. In contrast, later stages with more recalcitrant substrates may have higher pathogen suppression (Hoitink et al., 1997; Bonanomi et al., 2010; Berg and McClaugherty, 2014). Differences in decomposition stage may explain why organic amendments can have different effects on the microbial biomass after recovery (Bapiri et al., 2010; Lado-Monserrat et al., 2014; Ng et al., 2015). As such, there are many avenues for future studies to identify if and how patches of organic material affect pathogen suppression during the recovery phase.

Pathogen suppression could also be managed by the addition of specific microorganisms or complete microbial communities (O'Hanlon et al., 2012). For example, the addition of a forest fungus (*Penicillium* WPTIIIA3) can increase yields of winter wheat when this species is exposed to drought and *Fusarium* pathogens (Ridout and Newcombe, 2016). This strategy would be beneficial when knowledge of the specific pathogen and pathogen suppressive microorganism is available (Borneman and Becker, 2007). However, added single strains need to establish and overcome the colonization resistance of the soil microbiome (van Veen et al., 1997; de Boer, 2017), which can be difficult due to the high diversity of soil microbial communities (van Elsas et al., 2012; Bashan et al., 2014). Thus, it can be difficult to overcome the colonization resistance of the resident community when all niches are filled with other microbes. These difficulties can change when extreme weather events result in empty niches for the introduced microorganism to establish. Therefore, the addition of beneficial microorganisms in the recovery phase may be successful as they can colonize empty niches and can be worthwhile to be investigated (Adam et al., 2016). The addition of beneficial microbes could potentially be combined by rewetting with water spraying systems during the recovery from drought conditions. An alternate strategy could be to engineer microbial communities that benefit host plants under climate change, suppress pathogens and are able to colonize, and survive in the soil environment (Oyserman et al., 2018). These beneficial microorganisms could belong to the group of plant growth promoting microorganisms as they have the ability to both improve the plants physiological response to drought in sterile soils (Mayak et al., 2004; Timmusk et al., 2014) and can act as disease control agent (Kloepper et al., 2004). However, future studies should identify plant growth promoting microorganisms that can both improve drought resistant and disease resistance in crops (Coleman-Derr and Tringe, 2014; Ngumbi and Kloepper, 2016).

CONCLUSION

We conclude that the higher sensitivity of crops to infections by soil-borne pathogens during and after extreme weather events is in part due to loss of the pathogen suppressive capacity of soils. Therefore, adaptation of agricultural ecosystems to changing climate scenarios should include improvements of pathogen suppression of soil during and after extreme drought and rainfall events. However, basic knowledge about effects of extreme weather events on microbial interactions, survival of microorganisms that induce pathogen suppression as well as recovery of the pathogen suppression appears not to be addressed in literature. This knowledge is needed to develop management strategies that improve pathogen suppressive soils (**Figure 2**). Management strategies should focus on improving survival and

early recolonization of pathogen-suppressing microorganisms during the recovery phase after extreme weather events. Improved survival may be achieved via the natural selection of soil microorganisms to cope with drought or waterlogged conditions (selection by repeated stress) or via the addition of organic materials (survival spots). The challenge will be to find a strategy that allows to manage both drought and waterlogged conditions as the microorganism that respond to drought will differ from the ones that survive waterlogged conditions. In addition, improved and faster recovery of pathogen suppressive microorganisms can be managed by the addition of pathogen suppressive microorganisms. As such, there are many research directions to improve our understanding of pathogen suppression during and upon recovery to the drought and rainfall events. This understanding is needed to adapt agricultural ecosystems to changing climate scenarios.

AUTHOR CONTRIBUTIONS

AM and WB conceived and designed the ideas for the article and wrote the manuscript.

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Differentiated Mechanisms of Biochar Mitigating Straw-Induced Greenhouse Gas Emissions in Two Contrasting Paddy Soils

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Straw returns to the soil is an effective way to improve soil organic carbon and reduce air pollution by straw burning, but this may increase CH_4 and N_2O emissions risks in paddy soils. Biochar has been used as a soil amendment to improve soil fertility and mitigate CH_4 and N_2O emissions. However, little is known about their interactive effect on CH_4 and N_2O emissions and the underlying microbial mechanisms. In this study, a 2-year pot experiment was conducted on two paddy soil types (an acidic Utisol, TY, and an alkaline Inceptisol, BH) to evaluate the influence of straw and biochar applications on CH_4 and N_2O emissions, and on related microbial functional genes. Results showed that straw addition markedly increased the cumulative CH_4 emissions in both soils by 4.7- to 9.1-fold and 23.8- to 72.4-fold at low (S1) and high (S2) straw input rate, respectively, and significantly increased *mcrA* gene abundance. Biochar amendment under the high straw input (BS2) significantly decreased CH_4 emissions by more than 50% in both soils, and increased both *mcrA* gene and *pmoA* gene abundances, with greatly enhanced *pmoA* gene and a decreased *mcrA*/*pmoA* gene ratio. Moreover, methanotrophs community changed distinctly in response to straw and biochar amendment in the alkaline BH soil, but showed slight change in the acidic TY soil. Straw had little effect on N_2O emissions at low input rate (S1) but significantly increased N_2O emissions at the high input rate (S2). Biochar amendment showed inconsistent effect on N_2O emissions, with a decreasing trend in the BH soil but an increasing trend in the TY soil in which high ammonia existed. Correspondingly, increased *nirS* and *nosZ* gene abundances and obvious community changes in *nosZ* gene containing denitrifiers in response to biochar amendment were observed in the BH soil but not in the TY soil. Overall, our results suggested that biochar amendment could markedly mitigate the CH_4 and N_2O emissions risks under a straw return practice via regulating functional microbes and soil physicochemical properties, while the performance of this practice will vary depending on soil parent material characteristics.

Keywords: paddy soil, biochar, straw return, CH_4, N_2O, functional genes

INTRODUCTION

Global warming caused by the continued increase in anthropogenic greenhouse gas (GHG) emissions is expected to exert a severe impact on the stability of natural ecosystems and sustainable development of human society (Smith and Fang, 2010). The mitigation of GHG emissions remains a formidable challenge in the quest to slow climate change. The respective global warming potentials of methane (CH_4) and nitrous oxide (N_2O) are 23- and 298-fold higher than that of carbon dioxide (Munoz et al., 2010), and contribute around 17 and 6% to radiative forcing, respectively (WMO, 2017). Paddy soil is one of the important sources of atmospheric CH_4 and N_2O, with average annual emissions of 7.22–8.64 Tg and 88.0–98.1 Gg in China, respectively (Xing, 1998; Li et al., 2004; Liu et al., 2010). A strategy to mitigate CH_4 and N_2O emissions in rice paddies is imperative.

Crop residues have been widely applied in agriculture as a source of nutrients to improve soil fertility, and showed significant effects on improving soil organic C stocks (Li et al., 2010), reducing environmental pollution associated with straw burning (Kharub et al., 2004; Romasanta et al., 2017), and regulating carbon and nitrogen cycling (Lugato et al., 2006). However, the application of crop residues can increase the production of atmospheric GHGs (Zou et al., 2005; Ma et al., 2008; Hang et al., 2014). For example, the global warming potential was significantly enhanced by straw incorporation from a rice paddy field, with CH_4 increase by 3–11 times in straw-contained soils compared to the control (Ma et al., 2007). Therefore, it is crucial to find a method to mitigate the emissions of GHGs induced by straw application in rice paddy fields.

Biochar, a carbon sequestrating and recalcitrant material, is produced by the pyrolysis of plant residues under a zero or limited oxygen condition (Cao et al., 2011), possessing the characteristics of a high pH, high cation exchange capacity (CEC), and a high hydrophilic characteristic, large porosity and surface area (Lehmann et al., 2011). Biochar has been applied to soil as an optional amendment to improve soil fertility and grain yields via the promotion of nutrient turnover (Zhang et al., 2010; Kang et al., 2016). Soil with a low fertility or pH value can be improved with biochar amendment (Bakar et al., 2015). Biochar amendment can also regulate CH_4 and N_2O emissions from rice paddy soils (He et al., 2017). For example, CH_4 emissions were suppressed by 39.5% by adding biochar to a paddy soil under elevated temperature and CO_2 (Han et al., 2016). However, another study found that N_2O emissions significantly decreased following biochar addition, while CH_4 emissions increased, probably resulting from an improvement in microbial growth due to the supply of additional C (Singla and Inubushi, 2014). Either no effect (Brassard et al., 2016) or stimulation (Yu et al., 2013) of biochar-induced GHG emissions have also been observed, illustrating an apparent dependence on biochar and soil properties (Singla and Inubushi, 2014). However, the mechanism remains unclear as to how the soil interacts with biochar with respect to CH_4 and N_2O emissions.

CH_4 and N_2O emissions in paddy soils reflect the balance of production and consumption processes which are associated with microbial activities in soil (Yan et al., 2000; Bodelier, 2015). For example, soil organic matter decomposed by various microorganisms is ultimately utilized by the methanogenic archaea with the production of CH_4, which can be consumed by the methanotrophic proteobacteria as a sole source of carbon and energy before release to the atmosphere (Bridgham et al., 2013). N_2O emission from soil is also dependent on the balance of N_2O reduction and production processes and is influenced by multiple factors. However, there are no consistent conclusions on the influence of biochar amendment on soil microbial communities involved in CH_4 and N_2O production and consumption. Improved abundance of N_2O-reducing bacteria has been observed after biochar amendment, promoting the reduction of N_2O to N_2 during denitrification thus decreasing N_2O emissions (Harter et al., 2014). Similarly, several other studies reported that biochar amendment reduced N_2O emissions by increasing nitrous oxide reductase encoding gene (*nosZ*) due to soil pH increases (Van Zwieten et al., 2014; Xu et al., 2014). Conversely, increased N_2O emissions stimulated by biochar amendment in a rice paddy soil was found to be correlated with the increased bacterial ammonia monooxygenase encoding *amoA* gene, but not with nitrous oxide reductase encoding gene (*nosZ*) and nitrite reductase encoding genes (*nirK* and *nirS*) (Lin et al., 2017).

Among the environmental and edaphic factors influencing the microbial processes, the soil C/N ratio plays a pivotal role in controlling the shifts among key functional microbial processes with separate redox conditions (Kraft et al., 2014). Remarkably, a higher C/N ratio would favor anammox or dissimilatory nitrate reduction to ammonium (DNRA) while a lower ratio would contribute to denitrification (Tiedje et al., 1982; Kraft et al., 2014; Shan et al., 2016). Furthermore, the soil redox potential (*Eh*) and pH are also essential factors largely deciding the availabilities of electron transfer for microbial-mediated processes and microbial metabolism (Kralova et al., 1992; DeAngelis et al., 2010). Otherwise, soil carbon dynamics are highly relevant with the growth of microorganisms involved in GHGs (Wang et al., 2017). Therefore, the amount of straw addition could regulate GHGs emissions not only via influencing the availability of soil organic C (Wu et al., 2013; Liu et al., 2014; Zhang et al., 2014; Zhong et al., 2017), but also via adjusting the soil C/N ratio, and different amounts of straw returns exerted different effects on soil microbial activities and GHGs emissions (Naser et al., 2007). Furthermore, individual rather than interactive effects of straw and biochar amendments on CH_4 and N_2O emissions were the focus of earlier studies (Shen et al., 2014; Ly et al., 2015; Thammasom et al., 2016). Consequently, more studies are required to estimate the influence of biochar amendment on CH_4 and N_2O emissions under different rates of straw incorporation, and the processes controlling the gaseous emissions should be identified. Therefore an experiment involving straw and biochar amendments was conducted in two types of paddy soils to evaluate the dynamics of CH_4 and N_2O emissions in this study. Two rice straw levels were applied to construct different soil C/N ratio, and the effects of biochar on CH_4 and N_2O emissions were monitored. Microbial functional genes involved in the production and consumption of CH_4 and N_2O were analyzed.

The specific objectives were to: (1) Evaluate the effects of the biochar addition on the CH_4 and N_2O emissions in rice paddy soils under different rates of straw incorporation; (2) Quantify the responses of different functional microbial groups to biochar and straw amendments under two contrasting soil types and evaluate whether the difference in microbial groups might explain the variation in CH_4 and N_2O formation and release from the soils.

MATERIALS AND METHODS

Soil Information and Pot Experiment Setup

The paddy soils were originally collected from Taoyuan (TY, 111.48° E, 28.90° N), Hunan Province, and Binhai (BH, 119.84° E, 34.01° N), Jiangsu Province, rice production areas in Southeast China. The soils were classified as an Inceptisol and an Utisol, respectively, according to the USDA Taxonomy. Fresh soils were air dried to 30–40% maximum field capacity and then passed through a 2 mm sieve, followed by a homogenous mixing before being used for the pot experiment.

The pot experiment was located outdoors in a farm field which received natural day light and ambient temperature in the suburb of Beijing. The experimental design involved two rice straw levels with or without biochar addition, i.e., five treatments: (1) S0, no addition of rice straw (control); (2) S1, 0.33% (w:w) rice straw addition (equal to all aboveground biomass return); (3) S2, 0.66% (w:w) rice straw addition; (4) BS1, 0.33% (w:w) rice straw addition plus 2.0% (w:w) biochar (equal to 45 t ha^{-1}); and (5) BS2, 0.66% (w:w) rice straw addition plus 2.0% (w:w) biochar. The rice straw used in the experiment was collected from the area where soil samples were collected, and ground into a powder before use. Biochar was pyrolytically produced from maize straw feedstock under 450°C, and was purchased as a commercial product from Liao Ning Golden Future Agriculture Technology Co., Ltd., with a pH of 9.2, and total carbon, nitrogen, and phosphorus of 679, 9.4, and 7.8 g kg^{-1}, respectively. Three replicate pots (26 cm in diameter and 30 cm in height) were setup for each treatment, and each pot contained 10 kg soil (dry weight). For the rice growing season in 2016, before pots were filled, straw or straw plus biochar were thoroughly mixed with the soil according to the treatment, and phosphorus and potassium were applied as a basal fertilizer mixture for all treatments at 90 and 180 kg ha^{-1} P_2O_5 and K_2O, respectively. All pots were flooded for 10 days and then two rice seedlings were transplanted to each pot at day 10 to avoid seedling burnt. Nitrogen fertilizer (72 kg N ha^{-1} as urea) was dissolved in 200 ml deionized water and applied into surface water of each pot before 1 day rice was transplanted. The remaining urea fertilizer (108 kg N ha^{-1} N) was applied after tillering at day 60. The soils were continuously flooded to a depth of 2.5 cm except for 2 weeks of drainage during tillering (from day 43 to day 58, corresponding to days 33–48 after rice transplanting). After the rice growing season in 2016, pots were preserved *in situ* and covered with tarpaulins to reduce anthropogenic disturbance. In spring 2017, tarpaulins were removed and all pots were flooded for 1 month before rice straw and basal fertilizers were applied into soils. The water and fertilizers regime, rice transplanting and daily management were the same as those in 2016, except that biochar was no longer added.

Gas Sampling and Measurement

The soil N_2O and CH_4 fluxes were measured using the static chamber method during the whole rice growing season at 2- or 3-day intervals from 12 June 2016 to 21 August 2016 and from 8 March 2017 to 19 July 2017. A transparent Plexiglass chamber of 30 or 60 cm in height was affixed by a water-filled groove to the top edge of the soil column to ensure an air-tight system. An electrical fan was attached on the top of the chamber to mix the gas in the headspace. On each sampling day, gas collecting was conducted between 10 and 11 a.m., and gas was collected from each pot at 15 min and 30 min after chamber was sealed. For each time, 30 ml of gas was taken from the chamber using a syringe connected with a three-way valve, and then stored in a glass cylinder for next measurement. Gas samples were measured by using a gas chromatograph (Agilent 7890B, Santa Clara, CA, United States) equipped with a flame ionization detector (FID) and an electron capture detector (μECD), and the gas sample (20 ml) was fed into the GC using a syringe manually. Gas fluxes were calculated using a linear regression analysis.

$$F = \rho \times (P/101.3) \times (V/A) \times (\Delta c/\Delta t) \times 273/(273 + T)$$

Where: F was the flux of N_2O or CH_4 (μg N_2O-N m^{-2} h^{-1} or μg CH_4-C m^{-2} h^{-1}), ρ was the density of the trace gas at 0°C and 101.3 KPa (kg m^{-3}), P was the atmospheric pressure of the experimental site (KPa), V was the volume of chamber (m^3), A was the surface area of the chamber, $\Delta c/\Delta t$ was the rate of N_2O or CH_4 accumulation in the chamber (μg m^{-3} h^{-1}), T was the chamber mean air temperature in Celsius.

Cumulative N_2O and CH_4 emissions (E, kg N ha^{-1} for N_2O, kg C ha^{-1} for CH_4) were calculated by the following equation:

$$E = \sum_{i=1}^{n} (F_i + F_{i+1})/2 \times (t_{i+1} - t_i) \times 24$$

Where: F was the gas flux (μg N_2O-N m^{-2} h^{-1} or μg CH_4-C m^{-2} h^{-1}), n was the gross number of gas measurement, i was the time of sampling, $(t_{i+1} - t)$ represented the days between the two conjoint gas measurements.

Soil Sampling and Physicochemical Analysis

Soil samples were taken after gas sampling at day 18, day 58, and day 120, corresponding to rice seedling, tillering, and heading stages, respectively. A soil core (4 cm in diameter) at a depth interval of 0–5 cm was collected from each pot at each sampling time, the core being 10 cm distant from the rice plant. Finally, three cores in each pot were sampled equidistant along the edge of the soil columns to minimize the disturbance. *In situ Eh* measurements were made at rice-transplanting and before soil sampling days by using a PRN-41 soil *Eh* meter (DKK, TOA,

Tokyo, Japan). After a homogenous mixing, soil subsamples were stored at 4°C and −40°C for physicochemical determinations and molecular analyses, respectively.

Soil pH was measured in a soil and water suspension (1:2.5 w/w) using a glass electrode. Soil moisture was measured as loss in weight after oven drying at 105°C to constant weight. NH_4^+ and NO_3^- were extracted with 1 M KCl solution and determined by using a continuous flow analytical system (AA3, SEAL analytical, Germany). Soil dissolved organic carbon (DOC) was extracted with 0.5 M K_2SO_4 and determined by a TOC analyzer (Multi N/C 3100, Analytik Jena, German). Soil total carbon (TC) and total nitrogen (TN) were measured by an Elemental analyzer (Vario EL III-Elementar, Germany).

DNA Extraction and Quantitative PCR

Total DNA was extracted from 0.3 g freeze-dried soil by using a Power Soil DNA Isolation Kit (Mo Bio, Carlsbad, CA, United States) under the guidance of the manufacturer's instructions, and the quality of the extracted soil DNA was checked by an agarose gel electrophoresis. All the extracted DNA products were stored at −40°C for the next analysis.

Real-time PCR was conducted on an IQ2 system (Bio-Rad Laboratories Inc., Hercules, CA, United States). The abundances of microbial functional genes related to N_2O emission (archaeal and bacterial *amoA*, *nirK*, *nirS*, and *nosZ* genes), and methanotrophs *pmoA* gene (methane monooxygenase encoding gene) and methanogens *mcrA* gene (methyl coenzyme M reductase encoding gene) were quantified using a SBYR Green assay with the primer pairs and thermal cycle programs as listed in **Supplementary Table 1**. The qPCR reactions were executed in a 25 μl mixture containing 12.5 μl SYBR Green Premix Ex Taq (TaKaRa Bio Inc.), 1 μl of each primer for *nirK*, *nirS* and *nosZ* (clade I) genes at 10 μM, 2 μL of each primer for archaeal and bacterial *amoA*, *nosZ* (clade II), *mcrA* and *pmoA* genes at 10 μM, and 2 μl of DNA template (1–20 ng). A negative control without DNA template was also conducted in all the qPCR runs. Melting curves aiming to ensure the reaction specificity were conducted at the end of each PCR run. QPCR results were accepted when melting curve is under a single peak, and the amplification efficiencies were in the range between 86.3% and 110.0% with a R^2 value greater than 0.95. To engender a standard curve for qPCR, the amplifications of target genes were performed with the same primer sets mentioned above, following a cloning sequencing. The plasmids DNA containing the correct insert were extracted, purified and quantified, following a 10-fold dilution series as standards for qPCR. Soil DNA samples, standards and negative controls were all included in triplicates in each run.

High-Throughput Sequencing Analysis of *pmoA* and *nosZ* Genes

To explore the influence of different treatments on microbial community, all soil samples collected at the seedling stage were subjected to high-throughput sequencing analysis for *pmoA* and *nosZ* I genes, and the soils from S0, S2, and BS2 treatments were selected for survey on the variation of *nosZ* I gene containing community over time. The *pmoA* and *nosZ* I genes were amplified with the primers and PCR conditions listed in **Supplementary Table 1** in triplicates. And a unique barcode of 6 bp in length were attached in the forward primer at the 5′ end to distinguish the amplicons from different soil samples. Metabarcoded amplicons were purified and sequenced by Illumina Miseq PE300 (Illumina Inc., San Diego, CA, United States).

The sequencing-read data sets were processed using QIIME 1.90 (Caporaso et al., 2010) standard operation pipeline. The raw data was demultiplexed according to the barcode of each sample. Usearch (version 10.0) program (Edgar, 2013) was used to achieve the mergence between the forward and reverse reads, followed by the trimming barcodes from sequences, demultiplexing and quality filter of sequence. Then, filtering chimera, clustering Operational Taxonomic Unit (OTU) at 97% sequence identity and picking out representative sequences from each OTU (Edgar, 2013) were all operated in the same program. Further, the representative sequences were compared to the public databases, GenBank, by using the National Center for Biotechnology Information (NCBI[1]) BLASTn to guarantee the maximum sequence similarity was a *pmoA* or *nosZ* gene. The annotation for taxonomic information of the methanotrophs and *nosZ* gene containers were conducted based on the Fungenes database[2] and further confirmed by blasting the representative sequence of each OTU against the NCBI GenBank database. To correct the sampling effort, OTUs resampling were rarefied at minimum number of sequences (5,689 reads for *pmoA* gene and 8,356 reads for *nosZ* gene) per sample for downstream analysis.

Statistical Analysis

Statistical analyses were conducted with SPSS software (version 19, IMB, Inc., United States). Spearman's correlation was used to determine the relationships among the N_2O and CH_4 emissions, soil properties and abundance of microbial functional genes at different rice growing stages. Repeated measures ANOVA was applied to assess the difference of soil properties and gas emissions in different rice growing stages and treatments. One-way analysis of variance (ANOVA) was performed to test for differences in gas emissions, soil characteristics and abundance of microbial functional genes, while significant difference was defined as $P < 0.05$.

Mothur (Schloss et al., 2009) was operated to analyze the alpha and beta diversity. Beta diversity was characterized by Bray-Curtis dissimilarity matrices based on OTU matrices. Cluster analysis was performed with UPGMA (Unweighted Pair Group Method with Arithmetic Mean) using Bray–Curtis distance measures. To identify the critical parameters driving the community diversity of denitrifier, canonical correlation analysis (CCA) were performed using community ecology vegan package of R software (3.2.4). The envfit function (999 permutations) was used to identify the environmental variables, which significantly contributed to the soil microbial community variance.

[1] http://www.ncbi.nlm.nih.gov

[2] http://fungene.cme.msu.edu/index.spr

Nucleotide Sequence Accession Numbers

The representative sequences retrieved in this study were deposited in the GenBank database and assigned accession numbers from MH909699 to MH909751 for *pmoA* gene, from MH909601 to MH909698 for *nosZ* gene.

RESULTS

Soil Physicochemical Properties

The TY soil had an initial $pH_{(H_2O)}$ of 5.7, DOC at 89.07 mg kg^{-1}, total N at 2.20 g kg^{-1}, while the BH soil had an initial $pH_{(H_2O)}$ of 7.6, DOC at 33.28 mg kg^{-1}, and total N at 1.30 g kg^{-1}. During the whole rice growing season in 2016, soils properties were significantly impacted by the straw and biochar amendments. For the TY soil, straw addition (S1 and S2) significantly increased DOC by 2.7–42.4%, but showed no significant impact on the soil C/N ratio over the rice growth stage, in comparison with no straw control (S0) ($P < 0.05$) (**Table 1**). When compared with straw addition alone (S1 and S2), the TY soil pH significantly increased by 0.5–0.8 unit, DOC by 38.16–40.90% and C/N ratio by 24.07–46.16% with the biochar amendment (BS1 and BS2) at day 18, and similar significant increases of soil pH, DOC, and C/N were also observed at day 58 and day 120 (**Table 1**, $P < 0.05$). For the BH soil, there was no significant effect on the soil C/N ratio and pH with straw addition alone (S1 and S2), but soil DOC increased by 0.90- to 1.22-fold at day 18 (**Table 1**), in comparison with S0 treatment. The C/N ratio significantly increased by 24.10–28.87% and TN by 4.17–23.85% with the biochar amendments (BS1 and BS2) at day 18, compared with treatments without the biochar amendment (S1 and S2). Notably, soil pH increased over time in both soils from 6.1 to 7.6 in TY and from 7.7 to 8.7 in BH (**Table 1**) due to the occasional drainage during the heading stage. Both the rice growing stages and treatments showed significant impacts on soil properties, such as DOC and *Eh,* and there was no significant interaction of the treatments and rice growth stages on the NH_4^+, TC and C/N ratio (**Table 2**).

Similar trends of soil DOC variation were found in both soils (**Figure 1A**). Soil DOC was greatly increased after straw addition during the seedling stage, while it decreased at the tillering stage. Soil DOC was also increased by the biochar amendment over all rice growth stages, when compared with that in control, except for a decrease at the tillering and heading stages in the BH soil (**Figure 1A**), which might have been caused by adsorption on the biochar. The dynamics of soil redox potential (*Eh*) was generally consistent in the two paddy soils (**Figure 1B**). The *Eh* was generally low during the flooding period, and sharply increased through the drainage. Straw incorporation reduced the *Eh* in both soils during the flooding stage, which ranged from −104.7 to −15.2 mV in the TY soil, and from −103.6 to −13.7 mV in the BH soil. A lower *Eh* in both soils was recorded at the seedling stage with biochar amendment, which was nearly 36–272 mV lower than treatments without biochar incorporation (S1 and S2) (**Figure 1B**). Moreover, the difference in *Eh* between the treatments with and without biochar amendment became smaller following drainage.

CH_4 Emissions From Rice Paddy Soils

The methane fluxes showed significant differences among treatments and varied over the rice growing season (**Supplementary Figure 1**). In general, the transient and cumulative CH_4 emissions in the TY soil were much lower than those in the BH soil. During the rice growing season in 2016, CH_4 emissions were more concentrated in the seedling stage in both soils (**Figure 2** and **Supplementary Figure 1**), which accounted for 64.5–93.4% of cumulative methane emissions in all treatments except the control (S0) (**Figure 2**).

CH_4 emissions significantly increased in both soils with straw addition ($P < 0.01$), and the response in the high straw rate (S2) was greater than in the low straw rate (S1) (**Figure 2** and **Supplementary Figure 1**). The S2 treatment had the highest cumulative CH_4 emissions among all the treatments with 448 kg C ha^{-1} in TY and 1,075 kg C ha^{-1} in BH in 2016. In contrast, the cumulative CH_4 emissions significantly decreased to 207 kg C ha^{-1} in the TY soil and 489 kg C ha^{-1} in the BH soil under biochar amendment at the high straw input level (BS2) ($P < 0.05$). However, no significant difference in cumulative CH_4 emissions was detected between with and without biochar amendment at the low straw input level, i.e., S1 and BS1 (**Figure 2**). Furthermore, the data of CH_4 emissions collected in the rice growing season in 2017 were highly consistent with that in 2016, showing a significant increase by rice straw addition and a significant suppression by biochar amendment in the high straw incorporated soils (**Figure 2** and **Supplementary Figure 1**, $P < 0.05$).

N_2O Emissions From Rice Paddy Soils

A similar trend of N_2O flux among all the treatments was observed in the two soils during the rice growing season. For both soils in the 2016 rice growth season, the N_2O flux was pronounced at the start of the continuously flooding period, and quickly decreased within a week (**Supplementary Figure 2a**). No marked variation was found in the following drainage and re-flooding periods, except for a peak flux in the BH soil at the 55th day, which might be due to the alternation of the water regime caused by a rainfall event.

Generally, the N_2O flux in the TY soil was slightly lower than that in the BH soil in 2016 (**Supplementary Figure 2a**). During the whole rice growing season in 2016, cumulative N_2O emissions in the TY soil were significantly lower than those in the BH soil (**Figure 2**). For the TY soil, nearly 55.4–92.8% of the cumulative N_2O was emitted at the seedling stage. Moreover, the cumulative N_2O emissions significantly increased with straw addition in treatment S2 by 1.94-fold ($P < 0.05$), while little effect was seen in treatment S1. The cumulative N_2O emissions increased by 0.88- to 1.51-fold with biochar addition, compared with no biochar incorporation (**Figure 2**). For the BH soil, 91.2–99.8% of the cumulative N_2O emissions originated at the seedling and tillering stages. The cumulative N_2O emissions in S2 treatment was significantly higher than that in control (S0, $P < 0.05$), while a decreasing trend was observed with biochar amendment in treatment BS2 (**Figure 2**). For both TY and BH soil, N_2O emissions showed no significant

TABLE 1 | Physiochemical parameters in two paddy soils over rice growth stages in 2016.

Days after straw addition	Treatment	pH	*Eh*-surface (mV)	*Eh*-subsurface (mV)	DOC (mg kg^{-1})	NH_4^+ (mg kg^{-1})	NO_3^- (mg kg^{-1})	TN g kg^{-1}	C/N
18 days	TY-S0[a]	6.4 ± 0.1B[b]	12.9 ± 31.4A	75.7 ± 125.7A	61.98 ± 18.33B	49.74 ± 3.74A	0.24 ± 0.15A	2.14 ± 0.08B	9.53 ± 0.13C
(seedling	TY-S1	6.1 ± 0.02C	19.3 ± 43.3A	38 ± 85.1A	66.19 ± 5.45B	51.19 ± 4.07A	–	2.18 ± 0.10B	9.64 ± 0.25C
stage)	TY-S2	6.4 ± 0.13B	6.9 ± 59.6A	157 ± 83.4A	88.25 ± 31.81AB	50.6 ± 13.62A	1.47 ± 2.15A	2.2 ± 0.05AB	10.03 ± 0.09C
	TY-BS1	6.9 ± 0.13A	−105.3 ± 40.3B	132 ± 26.6A	93.26 ± 13.39AB	52.91 ± 9.43A	0.85 ± 1.11A	2.14 ± 0.12B	11.96 ± 1.27B
	TY-BS2	6.9 ± 0.07A	−90 ± 40.7B	136 ± 91.9A	121.93 ± 31.02A	45.61 ± 13.14A	0.11 ± 0.16A	2.34 ± 0.05A	14.66 ± 1.44A
	BH-S0	7.9 ± 0.22a	180.8 ± 12.4a	30.3 ± 52.5a	46.10 ± 4.68c	8.71 ± 6.88a	9.93 ± 5.65a	1.11 ± 0.02c	19.43 ± 0.36b
	BH-S1	7.8 ± 0.11a	3.8 ± 32.7b	−224.7 ± 140.3b	87.48 ± 12.78abc	12.75 ± 3.02a	0.84 ± 0.53b	1.44 ± 0.14ab	17.99 ± 0.26b
	BH-S2	7.7 ± 0.05a	−6.1 ± 38.3b	−169.7 ± 67.6b	102.53 ± 33.93ab	6.93 ± 4.69a	0.32 ± 0.34b	1.3 ± .15bc	18.8 ± 0.32b
	BH-BS1	7.8 ± 0.06a	−32.1 ± 12.9b	−132.7 ± 47.2b	60.23 ± 35.64bc	7.50 ± 0.54a	0.27 ± 0.19b	1.5 ± .14ab	23.18 ± 1.78a
	BH-BS2	7.8 ± 0.07a	−278.2 ± 48.1c	−171.7 ± 50.9b	131.14 ± 8.45a	4.45 ± 3.57a	0.31 ± 0.28b	1.61 ± 0.204a	23.33 ± 1.62a
58 days	TY-S0	6.5 ± 0.34B	510.9 ± 33.8A	438.3 ± 88.5A	61.90 ± 1.10B	26.84 ± 2.63A	4.38 ± 1.87A	2.02 ± 0.05B	9.66 ± 0.05B
(tillering	TY-S1	6.5 ± 0.13B	439.8 ± 127.7A	494.3 ± 41.6A	63.57 ± 5.55B	25.12 ± 0.35A	2.03 ± 0.42AB	2.04 ± 0.06A	10.25 ± 0.24B
stage)	TY-S2	6.5 ± 0.12B	536.8 ± 10.9A	507.3 ± 38.6A	69.00 ± 10.23AB	26.34 ± 2.63A	1.85 ± 0.73AB	2.01 ± 0.12B	9.96 ± 0.11B
	TY-BS1	7.2 ± 0.41A	435 ± 67*A*	501.5 ± 215.7A	81.84 ± 2.01A	30.37 ± 4.73A	1.30 ± 1.81B	2.19 ± 0.09A	13.8 ± 0.94A
	TY-BS2	7.0 ± 0.01A	488.7 ± 33.9A	452.7 ± 203.3A	79.26 ± 11.7A	30.06 ± 4.61A	0.23 ± 0.13B	2.18 ± 0.08A	14.57 ± 1.39A
	BH-S0	8.4 ± 0.12b	434.7 ± 61.5a	423 ± 176.7a	47.06 ± 15.71a	33.12 ± 3.86a	5.23 ± 1.77c	1.12 ± 0.05c	19.42 ± 0.56b
	BH-S1	8.4 ± 0.1b	358.3 ± 117.3ab	334.7 ± 181.7a	34.73 ± 3.84a	33.06 ± 5.14a	7.03 ± 0.37bc	1.41 ± 0.12ab	17.98 ± 0.4b
	BH-S2	8.6 ± 0.07a	205.6 ± 174.6b	279.3 ± 140.3a	39.91 ± 2.90a	29.06 ± 4.07a	11.74 ± 2.05a	1.38 ± 0.17b	18.17 ± 0.19b
	BH-BS1	8.7 ± 0.04a	336.6 ± 24.2ab	563 ± 11*a*	32.91 ± 5.42a	26.83 ± 2.09a	6.52 ± 3.02bc	1.42 ± 0.14ab	23.17 ± 1.39a
	BH-BS2	8.6 ± 0.03a	206.6 ± 132.9b	229.7 ± 105.6a	39.41 ± 5.57a	29.53 ± 2.42a	9.36 ± 1.21ab	1.62 ± 0.08a	22.37 ± 1.33a
120 days	TY-S0	7.1 ± 0.14C	435.3 ± 24.6A	318 ± 199.3B	124.11 ± 7.62B	43.10 ± 2.20A	1.36 ± 2.06A	2.04 ± 0.04BC	9.85 ± 0.16B
(heading	TY-S1	7.2 ± 0.34BC	380.6 ± 69.1A	161 ± 36.7AB	137.37 ± 25.1AB	41.05 ± 2.73A	0.93 ± 0.51A	2.15 ± 0.10AB	9.58 ± 0.13B
stage)	TY-S2	7.2 ± 0.12BC	352.9 ± 74.8A	227.7 ± 17.9B	148.29 ± 19.82A	43.61 ± 1.30A	0.63 ± 0.11A	2.01 ± 0.03C	9.69 ± 0.25B
	TY-BS1	7.5 ± 0.04AB	446.3 ± 22.9A	385.7 ± 112.3A	174.01 ± 27.15A	41.64 ± 18.14A	0.16 ± 0.17A	2.05 ± 0.11BC	13.34 ± 1.19A
	TY-BS2	7.6 ± 0.19A	437.8 ± 76.9A	465.7 ± 206.4B	144.46 ± 11.16A	31.38 ± 2.56A	0.05 ± 0.04A	2.26 ± 0.02A	13.79 ± 0.46A
	BH-S0	8.3 ± 0.15bc	164.9 ± 7.9b	−10.3 ± 112*b*	102.80 ± 13.49a	32.09 ± 3.24b	1.57 ± 1.54a	1.1 ± 0.06b	19.81 ± 0.35b
	BH-S1	8.5 ± 0.07a	234.7 ± 93.6ab	129 ± 30.8ab	75.80 ± 10.85ab	29.59 ± 2.63b	0.52 ± 0.06a	1.45 ± 0.109a	18.46 ± 0.61b
	BH-S2	8.4 ± 0.14abc	164.8 ± 60.8b	186.3 ± 78.3a	68.05 ± 12.12b	37.34 ± 2.79a	0.26 ± 0.23a	1.25 ± 0.15ab	18.93 ± 0.29b
	BH-BS1	8.5 ± 0.12ab	321.1 ± 50.3a	210.3 ± 58.9a	80.33 ± 7.14ab	40.25 ± 3.19a	0.33 ± 0.06a	1.49 ± 0.12a	22.52 ± 1.85a
	BH-BS2	8.2 ± 0.05c	251.9 ± 75.1ab	118 ± 112.2ab	100.94 ± 26.30a	41.55 ± 2.31a	1.97 ± 2.17a	1.5 ± 0.16a	21.81 ± 1.38a

[a]*Treatment: TY represents acidic Utisol from Taoyuan, BH represents alkaline Inceptisol from Binhai; control without straw and biochar (S0), with straw at low (S1) and high (S2) rate, and their counterpart plus biochar (BS1 and BS2).* [b]*Mean values ± SD, n = 3. The captical letters indicate significant differences in parameters among treatments for TY soils at the individual stage, while the lowercase letters are used for BH soils, analyzed by Duncan's multiple range test (P < 0.05). "–" means the concentration below detecting limit.*

TABLE 2 | Repeat measures ANOVA of rice growing stages and treatments on soil properties and gases emissions.

Soil	Items	Stage (rice growing)	Treatment	Stage × Treatment
TY				
	DOC	$P < 0.001$ [a]***	$P = 0.005$ **	$P = 0.264$ ns
	pH	$P < 0.001$ ***	$P < 0.001$ ***	$P = 0.278$ ns
	Eh	$P < 0.001$ ***	$P < 0.001$ ***	$P < 0.001$ ***
	NH_4^+	$P < 0.001$ ***	$P = 0.556$ ns	$P = 0.727$ ns
	NO_3^-	$P = 0.034$ *	$P = 0.027$ *	$P = 0.163$ ns
	TC	$P = 0.409$ ns	$P < 0.001$ ***	$P = 0.216$ ns
	TN	$P < 0.001$ ***	$P = 0.018$ *	$P = 0.028$ *
	C/N ratio	$P = 0.184$ ns	$P < 0.001$ ***	$P = 0.264$ ns
	Cumulative CH_4 emission	$P < 0.001$ ***	$P < 0.001$ ***	$P < 0.001$ ***
	Cumulative N_2O emission	$P < 0.001$ ***	$P < 0.001$ ***	$P = 0.331$ ns
	CH_4 flux	$P = 0.022$ *	$P = 0.026$ *	$P = 0.043$ *
	N_2O flux	$P = 0.011$ *	$P = 0.240$ ns	$P = 0.509$ ns
BH				
	DOC	$P < 0.001$ ***	$P = 0.037$ *	$P < 0.001$ ***
	pH	$P < 0.001$ ***	$P = 0.213$ ns	$P = 0.004$ **
	Eh	$P < 0.001$ ***	$P < 0.001$ ***	$P < 0.001$ ***
	NH_4^+	$P < 0.001$ ***	$P = 0.414$ *ns*	$P = 0.801$ ns
	NO_3^-	$P < 0.001$ ***	$P = 0.014$ *	$P < 0.001$ ***
	TC	$P = 0.255$ ns	$P = 0.001$ ***	$P = 0.354$ ns
	TN	$P = 0.175$ ns	$P = 0.013$ *	$P = 0.074$ ns
	C/N ratio	$P = 0.543$ ns	$P < 0.001$ ***	$P = 0.470$ ns
	Cumulative CH_4 emission	$P < 0.001$ ***	$P < 0.001$ ***	$P < 0.001$ ***
	Cumulative N_2O emission	$P < 0.001$ ***	$P = 0.249$ *ns*	$P = 0.005$ **
	CH_4 flux	$P < 0.001$ ***	$P < 0.001$ ***	$P < 0.001$ ***
	N_2O flux	$P = 0.008$ **	$P = 0.593$ *ns*	$P = 0.424$ ns

[a]****For the effect, *, **, *** denote significant difference at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. The ns means no significant difference.*

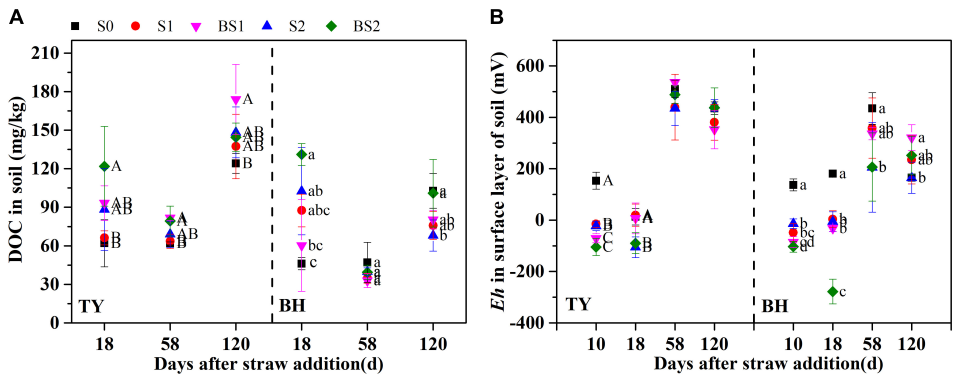

FIGURE 1 | Soil dissolved organic carbon contents **(A)** and redox potential in surface layer of paddy soil **(B)** among the treatments during rice growing season in 2016. Error bars present standard deviation of means ($n = 3$). The different letters (capital letter for the TY soil and lowercase for the BH soil) indicate significant difference among treatments at each stage, which is analyzed by Duncan's multiple range test ($P < 0.05$).

difference among treatments in the year 2017 (**Supplementary Figure 2b**).

Abundances of Methanogens and Methanotrophs in Rice Paddy Soils

The abundances of *mcrA* and *pmoA* genes, encoding the key enzymes functioning in the generation of CH_4 and the consumption of CH_4, respectively, were quantified to estimate the dynamics of methanogens and methanotrophs during the rice growing season. Generally, both *mcrA* and *pmoA* genes were more abundant in the TY soil (ranged from 1.43×10^8 to 1.60×10^9 copy genes g^{-1} dws for *mcrA*, and from 6.79×10^7 to 1.05×10^9 copy genes g^{-1} dws for *pmoA*) than those in the BH soil (ranged from 1.27×10^7 to 5.15×10^8 copy genes g^{-1} dws for *mcrA* and from 5.05×10^6 to 3.38×10^8 copy genes g^{-1} dws for

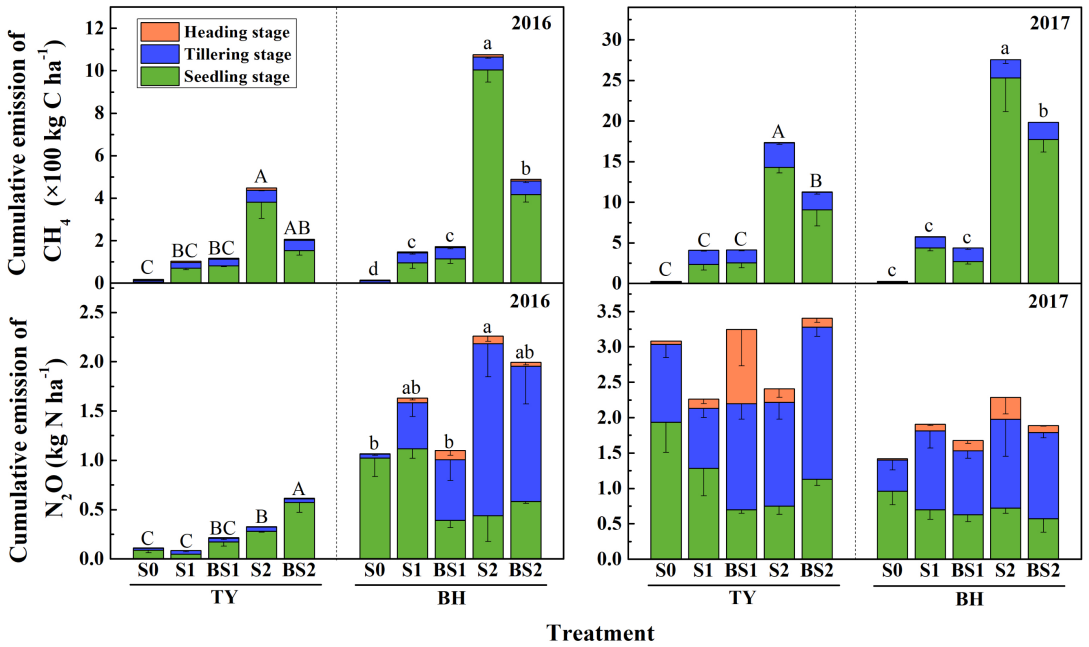

FIGURE 2 | Cumulative emissions of CH_4 and N_2O over the rice growing season in 2016 and 2017. Colors in column denote different stage: the seedling stage in green, the tillering stage in blue and the heading stage in orange. Error bars present standard deviation of means (n = 3). The different letters (capital letter for the TY soil and lowercase for the BH soil) among different treatments indicate significant difference of the cumulative emissions during the whole rice growing season, which was analyzed by Duncan's multiple range test ($P < 0.05$).

pmoA), and decreased over time in both soils (**Figures 3A,B**). The *mcrA* gene abundance generally increased with straw addition alone (S1 and S2) and high straw level plus biochar (BS2), and showed statistically significant differences at tillering and heading stages for both soil types (**Figure 3A**, $P < 0.05$). Interestingly, the *mcrA* gene abundance in biochar amendment under low straw input (BS1) showed no significant difference with control (S0), but was generally lower than S1 treatment (**Figure 3A**, $P < 0.05$), which could be due to the suppressive influence of the biochar amendment under low straw input.

Compared with the control (S0), straw addition at low and high rates (S1 and S2) showed no significant promotion or suppression effects on the *pmoA* gene abundances in both TY and BH soils, except that *pmoA* gene abundance was significantly higher in S1 than in S0 at tillering in the TY soil (**Figure 3B**). By contrast, biochar amendment under high straw input (BS2) showed a visible promotion on the *pmoA* gene abundance in both TY and BH soils at seedling and tillering stage, when compared with S0 and S2 treatments. A distinct augment of *pmoA* gene abundance by 53.3–123.9% was observed in the BS2 treatment at the seedling and tillering stages, when compared with S2 in both soils (**Figure 3B**). Consequently, biochar plus straw amendment (BS1 and BS2) generally decreased the ratio of *mcrA* to *pmoA* gene abundance in comparison with straw addition alone (S1 and S2) (**Figure 3C**).

Correlation analysis showed that *mcrA* gene abundance was positively correlated with the CH_4 flux (r = 0.514, $P < 0.01$ for the TY soil, r = 0.730, $P < 0.01$ for the BH soil), while negatively correlated with soil pH and *Eh* in both soils (**Supplementary Table 2**). The *pmoA* gene abundance showed no significant correlation with the CH_4 flux in both soils, but was negatively correlated with *Eh* ($r = -0.665$, $P < 0.01$) was observed in the TY soil, but not in the BH soil (**Supplementary Table 2**). Otherwise, the ratio of *mcrA* to *pmoA* gene abundance was positively correlated with the cumulative CH_4 emission (r = 0.476, $P < 0.01$ for the TY soil, r = 0.299, $P < 0.05$ for the BH soil (**Supplementary Table 2**). All these suggested that the *mcrA* gene abundance, compared with *pmoA* gene abundance, was more closely related to the dynamics of CH_4 flux, and the ratio of *mcrA* to *pmoA* gene abundance also could be a good indicator for CH_4 flux.

Abundances of N_2O-Related Functional Genes

The functional genes relevant to the N_2O production and consumption were analyzed in this study (**Figure 4**). The abundance of ammonia-oxidizing archaea (AOA) and bacteria (AOB) *amoA* genes were both lower in the TY soil (ranged from 5.29×10^5 to 1.59×10^6 copy genes g^{-1} dws for AOA and from 1.44×10^5 to 6.48×10^5 copy genes g^{-1} dws for AOB) compared with that in the BH soil (ranged from 2.65×10^6 to 1.57×10^7 copy genes g^{-1} dws for AOA and from 2.03×10^6 to 1.75×10^7 copy genes g^{-1} dws for AOB), with slight change over the crop growth stages (**Figures 4A,B**). Besides, no significant variations of AOA and AOB *amoA* gene abundances were observed among all the treatments regardless of rice growth stage in both soils, except for a significant promotion of AOA abundance in the BS2

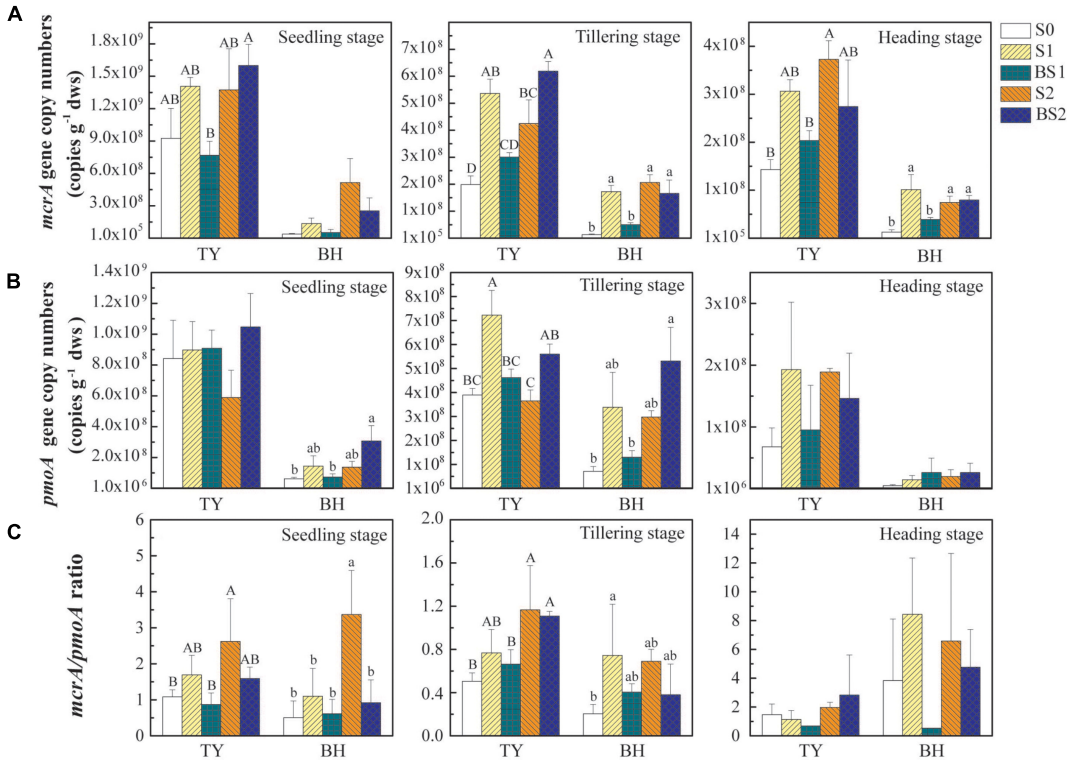

FIGURE 3 | The abundance of *mcrA* **(A)** and *pmoA* **(B)** genes and the *mcrA*/*pmoA* ratio **(C)** at the seedling, tillering, and heading stages in 2016. Error bars present standard deviations of means ($n = 3$). The different letters (capital letter for the TY soil and lowercase for the BH soil) indicate significant difference among different treatments at each sampling point, which was analyzed by Duncan's multiple range test ($P < 0.05$).

treatment at the seedling stage in the BH soil (**Figures 4A,B**). Generally, both straw addition and biochar incorporation (S1, S2, BS1, and BS2) had little impact on AOA and AOB *amoA* gene abundances for both soil types over time, when compared with the control (S0).

Similarly, straw addition and biochar application showed little effect on the *nirK* gene abundance, except for a significant increase by 1.51-fold in BS2 treatments in the BH soil at the seedling stage ($P < 0.05$), when compared with the control (S0) (**Figure 4C**). In general, the abundance of the *nirS* gene (ranged from 1.22×10^8 to 9.48×10^8 copy genes g^{-1} dws) was much greater than that of *nirK* gene (ranged from 7.28×10^6 to 3.28×10^7 copy genes g^{-1} dws) ($P < 0.01$). Straw addition alone (S1, S2) or biochar amendment (BS1, BS2) had little effect on the *nirS* gene abundance compared with the control (S0) for both soils over time, except for significant increases by 87.6% and 65.8% under the treatments S1 and BS2 in the TY soil at the tillering stage, respectively (**Figure 4D**, $P < 0.05$).

The *nosZ* gene, as an index of the nitrous oxide-reducing bacteria, consisted of two distinct clades (clade I and clade II). The abundance of *nosZ* clade I was higher in both soils at tillering compared to the seedling stage, followed by a decrease at the heading stage (**Figure 4E**). Straw addition alone (S1, S2) had no significant effect on the abundance of the *nosZ* I gene for both TY and BH soils. Biochar amendment (BS1, BS2) showed no obvious influence on the *nosZ* I gene abundance in the TY soil, while BS2 treatment significantly increased the *nosZ* I gene abundance in the BH soil at the seedling and tillering stages (**Figure 4E**, $P < 0.05$), during which N_2O emissions peaked. The enhanced *nosZ* I gene abundance could be responsible for the suppression of N_2O emissions in BH soil. The abundance of *nosZ* II gene showed slight variation over time, and generally decreased in straw addition treatments (S1 and S2) in relative to S0 in both soils (**Figure 4F**). Biochar amendment under low straw addition (BS1) significantly buffered the straw-induced decrease of *nosZ* II gene in the BH soil at tillering and heading stages, but showed no significant effect in the TY soil, which further explained the suppression of N_2O emissions in BH soil.

Community Similarity of the Methanotroph and N_2O-Reducing Bacteria

The methanotroph community at seedling stage and *nosZ* gene containers community at seedling, tillering and heading stages were characterized by Miseq sequencing. After resampling, 5,689 *pmoA* gene reads and 8,356 *nosZ* gene reads from each sample were selected for alpha- and beta-diversity analysis. Alpha diversity of both methanotroph and *nosZ*-containing bacteria showed no significant differences among treatments in both soils. However, the alpha diversity of *nosZ*-containing bacteria was generally much higher in the

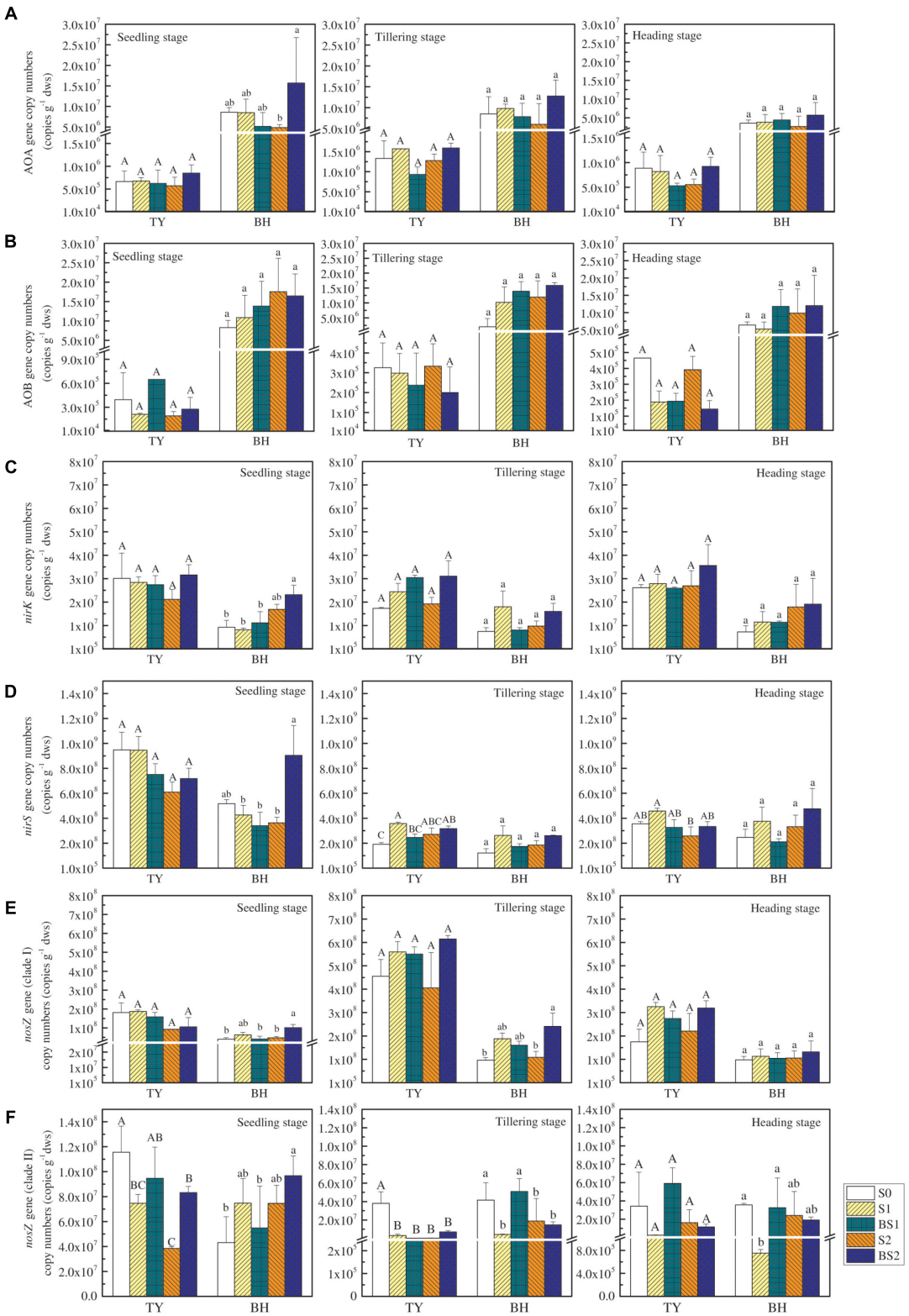

FIGURE 4 | The abundance of N_2O-related functional genes at the seedling, tillering and heading stages in 2016. The copy numbers of archaeal *amoA*, bacteria *amoA*, *nirK*, *nirS*, *nosZ* I, and *nosZ* II genes are exhibited in **(A–F)**, respectively. Error bars present standard deviations of means ($n = 3$). The different letters (capital letter for the TY soil and lowercase for the BH soil) indicate significant difference among different treatments at each sampling point, which was analyzed by Duncan's multiple range test ($P < 0.05$).

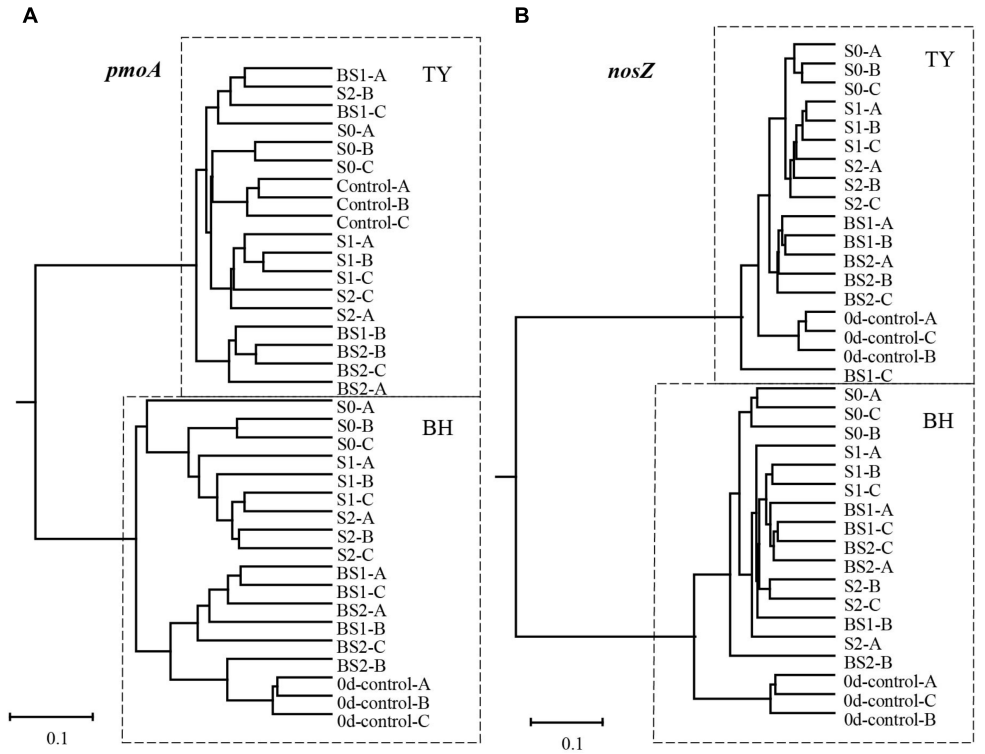

FIGURE 5 | UPGMA dendrogram contrasted from Bray–Curtis distance matrix of *pmoA* **(A)** and *nosZ* I **(B)** gene sequences at day 18 during the seedling stage. 0d-control represents the background of original soil at the beginning of pot experiment, and A, B, C mean three replicate pots.

BH soil than in the TY soil (**Supplementary Table 3**, $P < 0.05$).

The UPGMA clustering analysis for the beta-diversity of methanotroph and *nosZ* gene container communities at the seedling stage showed a clear separation between the two soil types (**Figure 5**). Within each soil type, samples generally clustered among treatments, with clear separation between treatments with and without biochar amendment for methanotroph community in the BH soil, and for *nosZ* gene container in the TY soil (**Figure 5**). These results suggested that community structure of methanotroph and *nosZ* gene containing denitrifiers were largely influenced by the addition of biochar than that of the straw, depending on the soil type.

Canonical correlation analysis based on the OTU matrix was performed to examine the influence of soil environmental factors on the community composition of *nosZ* gene containing denitrifiers. On the CCA plots, well separation of *nosZ* gene community among the three rice growth stages but slight aggregation among treatments were observed (**Figure 6**). The *x*-axis explained 21.18% and 10.25% of the variation of *nosZ* gene community in TY and BH soils, and the *y*-axis explained 5.18% and 6.18% of variation, respectively. Monte Carlo tests showed that *Eh*, TN and TC were factors significantly influencing *nosZ* gene containing community in the TY soil, and together explained the variation by 11.85%. For the BH soil, *Eh*, TC, TN, C/N, pH and NH_4^+ significantly influenced *nosZ* gene containing community and together explained 24.05% of variation (**Figure 6**). For both soils, *Eh* generally accounted for the greatest impact on *nosZ* gene containing community (**Supplementary Table 4**).

Community Composition of Methanotroph and N_2O-Reducing Bacteria Under Different Treatments

Analysis of methanotroph communities based on *pmoA* gene further showed that the TY soil and the BH soil possessed different methanotroph communities, with the TY soil dominated by Type II methanotrophs and the BH soil by type I methanotrophs (**Figure 7**). For the TY soil, *Methylocystis* (36.53–47.09%) and *Methylosinus* (38.93–44.46%) of Alphaproteobacteria (type II) were the dominant group and showed no marked variations among the five treatments, while the proportion of unclassified_*Methylococcaceae* belonging to type I increased from 3.54% in control (S0) to 8.27% in S2 and 11.78% in BS2 treatment, respectively. For BH soil, type II methanotrophs (*Methylocystis* and *Methylosinus*) accounted for 25.08–37.01% of the methanotroph community and type I methanotrophs accounted for 53.13–63.55%, with 3.93–13.67% of unclassified among the five treatments in day 18 (**Figure 7**). Compared with the S0, S1, and BS1 treatments, the proportion of type II methanotrophs decreased by 14.7–40.12% while the proportion of *Methylobacter* (type I methanotroph) significantly increased by 21.30–53.13% with the high straw incorporation

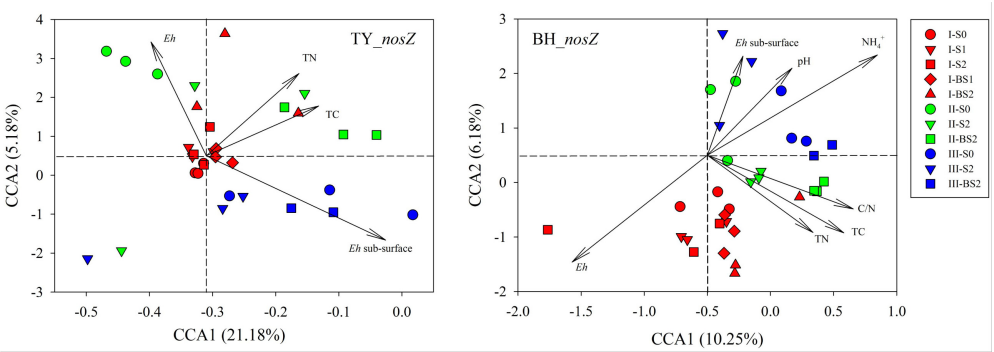

FIGURE 6 | Canonical correlation analysis for the *nosZ* gene containing denitrifier communities and soil properties. Three rice growth stages, seedling, tillering and heading, were included and labeled as I (red), II (green) and III (blue), respectively.

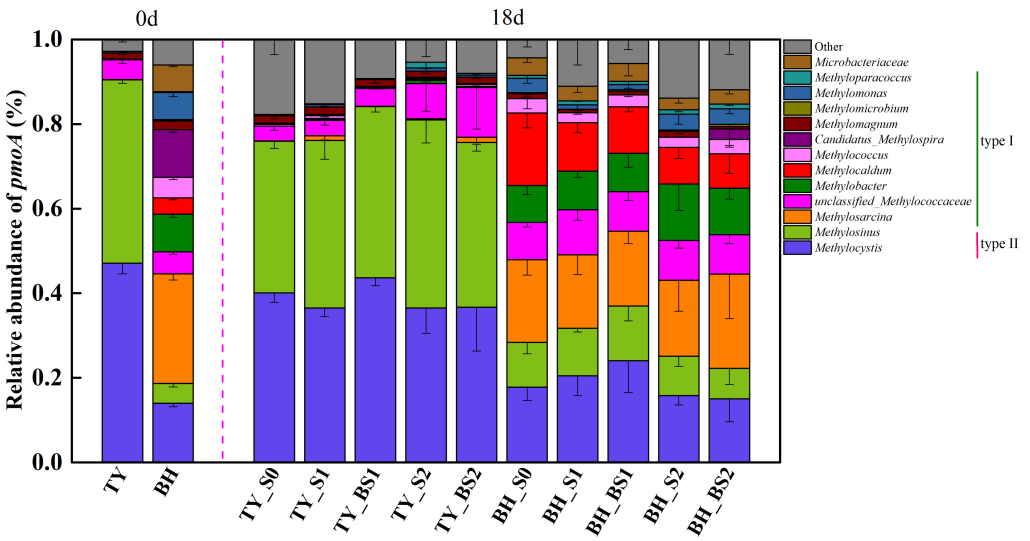

FIGURE 7 | Taxonomic distribution of *pmoA*-based methanotrophs at the genus level at day 18 during the seedling stage. Other includes the sequences with a relative abundance less than 0.9% and the unclassified sequences at genus level. Mean ± SD, $n = 3$. 0d represents the original soil used in this experiment.

(S2 and BS2). Straw addition and biochar amendment (S1, S2, BS1, and BS2) both decreased the relative abundance of *Methylocaldum* by 33.26–52.61% compared with the control (S0). Besides, a distinct increased (by 14.03%) of *Methylosarcina* was observed in the BS2 treatment with biochar amendment at high straw input. All these suggested that methanotrophs community responded to straw and biochar amendments more greatly in the BH soil than in the TY soil, coinciding with the separation of methanotrophs among the treatments in the UPGMA dendrogram.

As for typical *nosZ* genes, 1,362 OTUs at 97% similarity level were identified from 72 samples covering all treatments at seedling stage, and S0, S2, and BS2 treatments over three rice growth stages. The majority of *nosZ* gene reads were grouped into *Proteobacteria* (89.7%), with 3.48–18.05% unclassified (**Figure 8**). At the order level, the *nosZ* gene community was predominated by *Rhizobiales* in the TY soil with a proportion of 80.27%, while it was dominated by *Rhizobiales*, *Rhodospirillales,* and *Rhodobacterales* with similar proportions between 18.47% and 29.76% in the BH soil at day 0 (**Figure 8**). Flooding changed the *nosZ* gene community with *Rhizobiales* significantly decreasing from 80.27 to 44.61–46.98% in the TY soil, *Rhodospirillales* decreasing from 22.09 to 4.87–15.04% in the BH soil, and *Burkholderiales* increasing from 1.20–5.85% to 18.55–30.32% in both soils. After flooding, the *nosZ* gene containing denitrifiers showed no significant variation among all the treatments over the time in the TY soil (**Figure 8**). In contrast, the *nosZ* gene containing denitrifiers community in the BH soil showed visible variations among different straw and biochar treatments and greater variation over the three rice growth stages. Within treatments at day 18 (seedling stage), the proportion of *Burkholderiales* significantly increased by 6.78–50.28% in the straw addition alone treatment (S1 and S2), and 24.53–63.43% in the biochar amendment treatments (BS1 and BS2) compared with the control (S0) (**Figure 8**) in the BH soil. Compared with the S0 treatment, S2 treatment had a higher proportion

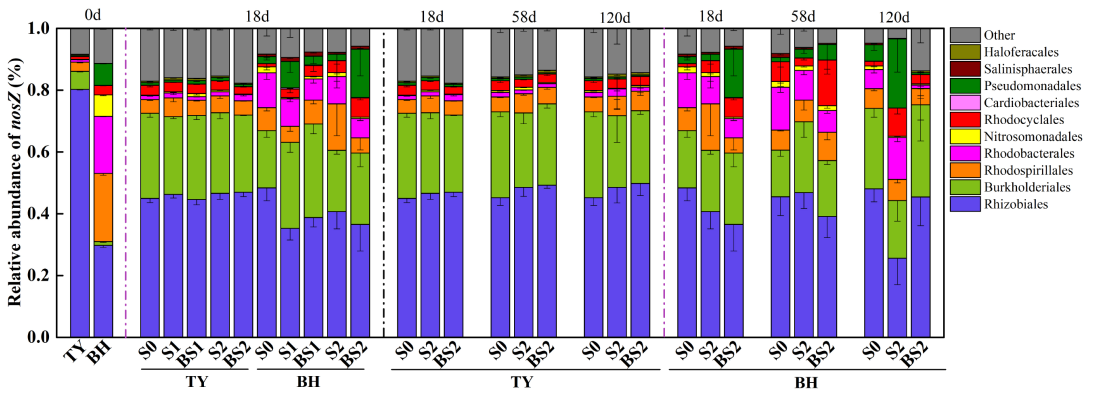

FIGURE 8 | Taxonomic distribution of *nosZ* I gene derived OTUs at the order level in paddy soils over time. Other includes the sequences with a relative abundance less than 0.8% and the unclassified sequences. Mean ± SD, n = 3. 0d represents the original soil used in this experiment.

of *Pseudomonadales* and *Rhodobacterales* but a relatively low proportion of *Rhizobiales* at day 120 (heading stage), while the proportions of these groups in BS2 treatment were closer to that in S0 treatment (**Figure 8**).

DISCUSSION

Effect of Straw Addition and Biochar Incorporation on CH_4 Emissions

In this study, the transient CH_4 and N_2O fluxes showed quite similar patterns between the two different soil types. Both the CH_4 flux and cumulative emissions peaked at the early rice growth period (seedling stage), and a significant increase in cumulative CH_4 emissions induced by the rice straw amendment and a greater increase with high straw rate input (S2 treatment) were observed (**Figure 2**). Correspondingly, a significantly higher DOC in the S2 treatment than in the S0 and S1 treatments (no straw and low straw rate) were detected in the early rice growth period (**Table 1**), indicating that more straw-driven C was probably transforming into CH_4 at the seedling stage. As suggested by previous studies, the increase of CH_4 emissions could be attributed to the additional substrate (e.g., H_2/CO_2 and acetate) provided for the methanogens via anaerobic decomposition of crop residues (Watanabe et al., 1995, 1998; Ma et al., 2007; Wang et al., 2016). The amounts of straw used in this study were equal (S1) and double (S2) to all aboveground biomass return, and the high straw level (S2) was set to stimulate the straw concentrated patches in field, as straw is generally surface returned to the field or incorporated into the plow layer by plowing thus cause highly concentrated patches. The similar case that the higher straw amount induced higher CH_4 and N_2O emissions observed in our pot experiments probably occurred in the field, so the amount of straw return to field should be taken into consideration in practice, even though biochar amendment could markedly mitigate the CH_4 emissions in straw incorporated soils.

On the other hand, soil *Eh* was extremely low in the BH soil (−104 to −14 mV in surface and −190 to −156 mV in subsurface) and the TY soil (−105 to −15 mV in surface and −165 to −146 mV in subsurface) 1 week after straw input (**Figure 1B** and **Supplementary Figure 3**), and significantly negative correlations were observed between soil *Eh* and CH_4 fluxes in both soils (**Supplementary Table 2**). This is consistent with previous studies which showed that soil *Eh* ranging from −230 to −150 mV greatly favored CH_4 emissions (Wang et al., 1993). Moreover, the soil *Eh* decrease was considered as another main reason of the enhanced CH_4 emissions after rice straw amendment, as more electron donors were provided for methanogen process under low *Eh* conditions (Tanji et al., 2003; Ma et al., 2008; Shen et al., 2014). Soil *Eh* therefore could be a sensitive indicator for CH_4 emission forecasting under flooded conditions.

By contrast, biochar amendment significantly decreased the CH_4 emissions under the high straw rate during the whole rice growing season in this study. Though sporadic studies found that biochar amendment increased CH_4 emissions in paddy fields (Knoblauch et al., 2011; Zhang et al., 2012), the majority of previous studies have shown that single biochar application could decrease CH_4 emissions effectively, and this was attributed to the increased soil pH induced by biochar (Tanji et al., 2003; Conrad and Klose, 2006; Liu et al., 2011; Shen et al., 2014; Ly et al., 2015; Thammasom et al., 2016). In this study, a significant pH increase by 0.5 unit with biochar amendment (BS2) was also observed in the acidic TY soil at the early period (at day 18 and day 58), which partially accounted for the decreased CH_4 emissions under biochar amendment with high straw input (**Table 1**). However, biochar amendment did not significantly increase pH in the BH soil at both straw input levels (**Table 1**), probably due to the alkaline property of the BH soil. Biochar-induced pH increase therefore could not explain the decreased CH_4 emissions in the BS2 treatment in the BH soil. Moreover, though biochar amendment with straw incorporation markedly decreased soil *Eh* for both soil types, while CH_4 emissions did not increase with decreasing *Eh* as observed in the straw incorporation treatments (S1 and S2). Biochar contains electroactive functional groups such as quinone/hydroquinone and has been shown to serve as electron acceptors or donors during the redox processes (Kluepfel

et al., 2014). It is also proposed as a "geoconductor" which could directly transfer electrons from char matrices to minerals (Sun et al., 2017). The depressed CH_4 emissions from biochar amended soil under high straw incorporation in this study therefore could be explained as a result of biochar competing for electrons with CO_2 thus disturbing the methanogenesis process. This also explained why the depression of biochar on CH_4 emissions was only obvious under high straw but not under low straw inputs, as the high straw input created a stronger redox condition (much lower *Eh* in BS2 than BS1, **Table 1**) and biochar could trap more electrons. All these suggest that biochar amendment together with straw incorporation is beneficial to mitigating CH_4 emissions from paddy soils, especially under high straw input conditions.

On the other hand, CH_4 emissions from soil are dependent on the balance of microbe-mediated methanogenesis and methane oxidation processes. Methane produced via methanogenesis under anaerobic conditions could be consumed by the methanotrophic bacteria via oxidizing CH_4 to CO_2, when O_2 was available (Bridgham et al., 2013). Some previous studies showed that straw incorporation enhanced CH_4 emissions with an increase in the abundance of the *mcrA* gene (Freitag et al., 2010; Cai et al., 2017). Consistently, straw addition generally increased the abundance of the *mcrA* gene, but showed no significant effect on the abundance of the *pmoA* gene in this study (**Figures 3A,B**). As a consequence, the *mcrA*/*pmoA* gene abundance ratio increased with straw incorporation, and both the ratio and the *mcrA* gene abundance were positively correlated with CH_4 emissions in both soils (**Supplementary Table 2**), which could be attributed to the stimulation of straw degradation and high available DOC for methanogens (**Figure 2** and **Table 1**). Biochar amendments under high straw input (BS2) showed no clear effect on the *mcrA* gene abundance but promoted the *pmoA* gene abundance, and consequently decreased the ratio of *mcrA*/*pmoA* significantly in both soils, when compared with that of S2 (**Figure 3C**). Therefore, it was the activated methanotrophs and the attenuated ratio of *mcrA*/*pmoA* that lead to the suppressed CH_4 emissions after biochar amendment at high straw incorporation.

Effect of Straw Addition and Biochar Incorporation on N_2O Emissions

For both TY and BH soils, straw amendment at the low rate (S1) caused no significant increase in N_2O emissions while the cumulative N_2O emissions increased significantly with straw addition at the high rate (S2) in this study (**Figure 2**). Ambiguous effects of straw amendment on N_2O emissions in paddy soils had been found in previous studies. For example, Ma et al. (2007) found that the N_2O emissions decreased by approximately 30% with straw incorporation, while significant N_2O emissions increased with straw addition was observed in other studies (Ma et al., 2009; Hu et al., 2016). The different effects of straw application on N_2O emissions were mainly due to the quality of the crop residues with various C/N ratios (Toma and Hatano, 2007). Incorporating crop residue with a high C/N ratio (>40) (like wheat straw) could enhance microbial N immobilization, which results in less available N for nitrification and denitrification (Vigil and Kissel, 1991; Millar and Baggs, 2005; Toma and Hatano, 2007; Rizhiya et al., 2011). In contrast, a lower C/N ratio of straw (like soybean stem, cabbage, and red clover) would provide more available N for denitrifiers and thus result in increased N_2O emissions (Baruah et al., 2016). Moreover, a negative correlation was detected between the N_2O emissions from crop residue incorporated soil and straw C/N ratio (Millar and Baggs, 2005), suggesting that the C/N ratio of incorporated straw might be a key factor influencing the N-cycling in paddy soils. In this study, no visible increase in N_2O emissions was observed under low straw input rate, and significantly higher N_2O emissions under high straw input rate were only observed in 2016, but not in 2017. The high C/N ratio at about 38 in the rice straw used in this study well explained the non-significant increase in N_2O emissions under low and high straw input in most cases. The higher N_2O emissions in S2 than in S0 treatment in 2016 could be attributed to the additional C and N substrate via straw decomposition under high straw input rate, while the effect of straw on N_2O emissions would be limited in the tested soils.

The cumulative N_2O emissions in biochar amendment treatments (BS1 and BS2) showed a decreasing trend in the alkaline BH soil. Conversely, N_2O emissions in BS1 and BS2 in the TY soil showed an increasing trend for two growth seasons and were statistically significant higher compared with straw incorporation treatments (S1 and S2) in 2016. Similarly, some previous studies reported that soil N_2O emissions decreased significantly following biochar amendment (Liu et al., 2012; Zheng et al., 2012; Saarnio et al., 2013), while some others showed a significant increase of N_2O emissions after biochar inputs (Verhoeven and Six, 2014). The inconsistent effect of biochar amendment on N_2O emissions might be explained by the soil properties (Cayuela et al., 2014). The above mentioned studies attributed the reduced N_2O emissions in the paddy fields with biochar amendment to soil aeration improvement after biochar application and the decrease of NH_4^+ availability due to the absorption by biochar (Lehmann et al., 2006; Zhang et al., 2010). These reasons could well explain the decreasing trend of N_2O emissions in biochar treatment in the BH soil in this study, but not for the TY soil with a converse trend. Some studies also suggested that the increase of soil pH in biochar-treated soils could enhance the activity of N_2O reductase within denitrifier microorganisms, and thus reducing the ratio of N_2O/N_2 (Yanai et al., 2007). Though soil pH increased significantly by more than 0.5 units in the TY soil under biochar amendments, biochar application did not decrease N_2O emissions but promoted N_2O emissions to some extent in this study. The possible explanation for such inconsistency could be: The TY soil contained much higher ammonia concentration (52.91 mg kg^{-1} in BS1 and 45.61 mg kg^{-1} in BS2) than the BH soil (7.50 mg kg^{-1} in BS1 and 4.45 mg kg^{-1} in BS2). The increased soil pH induced by biochar probably stimulated the nitrification and denitrification under such high ammonia condition, thus induced N_2O emissions in the TY soil. Some studies also suggested that biochar-induced increase of NH_4^+ or NO_3^--N content was the main reason for the increased N_2O emissions (Yoo and Kang, 2012; Shen et al.,

2014). Differently, our study did not observe significant increase NH_4^+-N content induced by pH improve in the TY soil with biochar amendment, as the high NH_4^+ -N background probably buffered it. These observations suggested that biochar-induced pH increase would not necessarily decrease N_2O emissions, but might increase N_2O emissions conversely when available N is high in soil environment. As biochar amendment might produce inconsistent effect on N_2O emissions in different soils, its extensive application requires appropriate estimation based on soil property.

A previous study found that the increased N_2O emissions were closely related with the significant increase in AOB abundance after biochar amendment in a paddy soil (Lin et al., 2017). However, in present study, no significant effects of straw and biochar addition on the abundances of AOA and AOB *amoA* genes were found during the rice growth stages (**Figure 4**), suggesting that nitrification was probably not the main process influencing the N_2O emissions in both soils. Meanwhile, straw addition showed little effect on the *nirK*, *nirS*, and *nosZ* I genes abundances, but showed depressive effect on *nosZ* II gene abundance in both soils. Previous studies have indicated that the *nosZ* II gene-containing denitrifier had higher affinity to N_2O than the *nosZ* I gene container and might be more responsible for the mitigation of N_2O emission (Jones et al., 2014; Yoon et al., 2016). The depression of *nosZ* II gene by straw addition in two soils partially explained the higher N_2O emissions in S1 and S2 treatment in relative to the control (S0). On the contrary, biochar amendment under high straw input significantly increased the *nirS* and *nosZ* I gene copy numbers, and biochar amendment under low straw input showed promotive effect on *nosZ* II gene in the BH soil (**Figure 4**). The increased *nosZ* gene abundance probably stimulated the transformation process from N_2O to N_2, and thus decreased N_2O emissions in the BH soil with biochar amendment. As the functional genes were quantified at DNA level and multiple genes were involved in the processes of N_2O production and consumption, the targeted genes and DNA-based analysis in this study might not sensitively indicate the microbial activity in N-cycling in this study. Further studies at RNA level and based on more functional genes like fungal, archaeal *nirK* and non-typical *nosZ* genes were necessary to reveal the microbial mechanism of N_2O emissions under straw and biochar amendments in future.

Effects of Straw and Biochar Addition on Functional Microbial Community

Generally, distinct dominant methanotrophs and *nosZ*-containing denitrifiers groups were found in the TY and BH soil. Particularly, type II methanotrophic groups (i.e., *Methylocystis* and *Methylosinus*) dominated in the TY soil, while the type I methanotrophs (i.e., *Methylosarcina*, *Methylobacter* and *Methylocaldum*) dominated in the BH soils (**Figure 7**). It has been suggested that both type I and type II were active methanotroph groups in different paddy soils, and that their distributions were mainly determined by the property of original soil types (Kolb et al., 2003; Ho et al., 2011, 2015). Generally, the type I methanotrophs possessed a lower affinity with CH_4 therefore preferred the condition with lower O_2 and high CH_4 concentrations, while the type II methanotrophs were more active in low CH_4 concentration environments (Dunfield et al., 1999; Macalady et al., 2002). Type I methanotrophs were also interpreted as r-type life strategy which could respond fast to environment change and devote to the oxidation of CH_4, while the type II were described as K-type life strategy possessing high competition ability under low nutrient conditions (Steenbergh et al., 2010). These characteristics well explained why straw addition resulted in a distinct shift of methanotrophs community in the BH soil but posed little effect in the TY soil in this study, as the BH soil and TY soil were dominated by type I and type II methanotrophs, respectively.

Specifically, the relative abundance of type I *Methylobacter* increased significantly in all straw addition treatments (S1, S2, BS1, and BS2) in the BH soil, corresponding to the significant decrease of nitrate in these treatments (**Figure 7** and **Table 1**). It has been found that the activities of *Methylobacter* can be strongly suppressed by extra NH_4^+ and NO_3^- supply (King and Schnell, 1994). Markedly decreased NO_3^- under straw incorporation treatments might relieve the suppression of nitrate and promoted the growth of *Methylobacter*, which well explained the enhanced proportion of *Methylobacter* under straw amendment condition.

On the other hand, biochar amendment in the high straw input treatment (BS2) greatly changed the community composition of methanotrophs, with *Methylosarcina* significantly increased (**Figure 7**). It has been reported that *Methylosarcina* and *Methylomonas* possibly required a certain O_2 and relatively higher concentration of CH_4 for methane oxidation (Lee et al., 2014). DNA-SIP experiment also demonstrated that *Methylosarcina* dominated under high CH_4 conditions (Zheng et al., 2014). As biochar could adsorb O_2 or CH_4, thus creating high-CH_4 hotspots (Brassard et al., 2016), which might contribute to the increased proportions of *Methylosarcina* in BS2 treatment. Moreover, significantly higher DOC and TN were detected in the BS2 treatment in this study (**Table 1**), which might contribute to the variation of methanotrophs community. Indirectly, the huge surface area and pores in biochar could provide habitats for microbial activities (Gul et al., 2015). All these difference in soil conditions induced by biochar amendment resulted in the change of methanotrophs community.

Similar to methanotrophs community, the community structure of *nosZ* gene containing bacteria responded to straw and biochar inputs differently in two soil types. For the TY soil, straw addition and biochar amendment showed little effect on the community composition of *nosZ* gene communities (**Figure 8**). Though straw and biochar additions significantly increased soil DOC, *Eh* in the subsurface of the TY soil was identified as the most significant environmental factor contributing to the shift of community structure in the TY soil in RDA analysis (**Figure 6**). Similarly, Richardson et al. (2009) found that denitrifiers containing *nosZ* gene were impressible to the dynamics of soil *Eh*. Contrastingly, the *nosZ* gene containing bacteria in the BH soil showed visible variations among different straw and biochar treatments. The relative abundances of *Rhizobiales* and *Nitrosomonadales* were

obviously decreased, while *Rhodocyclales* and *Burkholderiales* were increased under straw incorporation in comparison with control. Biochar application (BS1 and BS2) further enhanced the relative abundance of *Burkholderiales*, when compared with the straw input alone treatment (S1 and S2). In a DNA-SIP microcosm experiment, *Burkholderiales* and *Rhodospirllales* were identified as the predominant population under suitable N_2O reduction conditions, and were responsible for reduction of N_2O in rice paddy soils (Ishii et al., 2011). Another study also found that denitrifiers belonging to the orders of *Burkholderiales* and *Rhodocyclales* showed strong denitrifying activities in paddy soils (Ishii et al., 2009). The enhanced proportion of *Burkholderiales* and *Rhodocyclales* with biochar amendment might contribute to a more intensive N_2O consumption, thus led to the decreased N_2O emissions under biochar amendment in the BH soil.

CONCLUSION

2-year pot experiment in this study demonstrated that the rice straw amendment could significantly increase the cumulative CH_4 emissions in an acidic Utisol (TY) and an alkaline Inceptisol (BH) paddy soil, while biochar amendment could markedly mitigate the CH_4 emissions augmented by high straw incorporation in both soil types. These results could be explained by the straw-driven C and N substrate change, biochar-induced pH and *Eh* change, or electron competition etc., depending on the physiochemical characteristics of original soil type. Straw addition at high rate caused significant increase in N_2O emissions in both soils, while biochar amendment could decrease N_2O emissions in the BH soil but caused converse effect in the TY soil. The abundance of *mcrA* and *pmoA* genes related to the production and consumption of CH_4 changed in response to straw and biochar amendments well explained the variation of CH_4 emissions among the treatments. Straw and biochar amendment induced visible community change in methanotrophs and *nosZ* gene containing denitrifier in the alkaline BH soil, but slight change in the acidic TY soil. The BH soil and the TY soil possessed distinct microbial community, and straw and biochar amendments caused differentiated effect on soil property of two soil types, which together explained the interactive effect of straw plus biochar application on CH_4 and N_2O emissions in two contrasting paddy soils. Our pot experiment suggested that biochar amendment could effectively mitigate CH_4 and N_2O emissions risks induced by straw application in the tested soil types, while its extensive application into different soil types requires appropriate estimation based on soil physicochemical and microbial properties, and the amount of straw return should be taken into consideration in term of gross GHG emissions.

AUTHOR CONTRIBUTIONS

Y-QW was responsible to most of the pot experiments, laboratorial works, data processing, and article writing. RB had an important contribution to gas collecting, sampling, and some laboratorial activities. HJD provided essential helps for the article writing and revision. L-YM paid great efforts on the setup of pot experiments, and contributed to the gas and soil samplings. BH contributed to the bioinformatics analysis of sequencing data. J-ZH provided essential ideas to the experimental design and article writing. L-MZ provided essential ideas to the experimental design and paper writing, guidance for the experiments, and was responsible for pot experiments setup, article writing and revising.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02566/full#supplementary-material

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Organic Residue Amendments to Modulate Greenhouse Gas Emissions From Agricultural Soils

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Organic fertilizers have been shown to stimulate CH_4 uptake from agricultural soils. Managing fertilizer application to maximize this effect and to minimize emission of other greenhouse gasses offers possibilities to increase sustainability of agriculture. To tackle this challenge, we incubated an agricultural soil with different organic amendments (compost, sewage sludge, digestate, cover crop residues mixture), either as single application or in a mixture and subjected it to different soil moisture concentrations using different amounts of organic amendments. GHG fluxes and *in vitro* CH_4 oxidation rates were measured repeatedly, while changes in organic matter and abundance of GHG relevant microbial groups (nitrifiers, denitrifiers, methanotrophs, methanogens) were measured at the end of the incubation. Overall the dynamics of the analyzed GHGs differed significantly. While CO_2 and N_2O differed considerably between the treatments, CH_4 fluxes remained stable. In contrast, *in vitro* CH_4 oxidation showed a clear increase for all amendments over time. CO_2 fluxes were mostly dependent on the amount of organic residue that was used, while N_2O fluxes were affected more by soil moisture. Several combinations of amendments led to reductions of CO_2, CH_4, and/or N_2O emissions compared to un-amended soil. Most optimal GHG balance was obtained by compost amendments, which resulted in a similar overall GHG balance as compared to the un-amended soil. However, compost is not very nutrient rich potentially leading to lower crop yield when applied as single fertilizer. Hence, the combination of compost with one of the more nutrient rich organic amendments (sewage sludge, digestate) provides a trade-off between maintaining crop yield and minimizing GHG emissions. Additionally, we could observe a strong increase in microbial communities involved in GHG consumption in all amendments, with the strongest increase associated with cover crop residue mixtures. Future research should focus on the interrelation of plants, soil, and microbes and their impact on the global warming potential in relation to applied organic amendments.

Keywords: nitrous oxide, carbon dioxide, methane oxidation, agricultural soil, organic amendment, flux measurements, qPCR

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INTRODUCTION

The atmospheric concentrations of the main GHG carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) increased dramatically since the industrial revolution by 40, 150, and 20%, respectively (Stocker et al., 2013). Primarily, anthropogenic activities have increased the emission of CO_2, CH_4, and N_2O. An estimated part of ∼50% for CH_4 and ∼60% for N_2O originates from agricultural practices (Tian et al., 2016). Intensification of agricultural land used to meet the global food, feed, and bioenergy demand for the growing human population entails increasing reinvestment of climate neutral carbon compounds (residues) into agricultural systems to prevent decline of soil organic matter and subsequent soil quality and fertility. However, agricultural intensification through increased fertilization can lead to the loss of soil CH_4 uptake capacity (Bodelier and Steenbergh, 2014) and additionally causes an enhanced emission of N_2O by lowering the reduction of N_2O to N_2. Particularly, CH_4 uptake was 3–9 times weaker in agricultural than in unmanaged soils (Maxfield et al., 2008; Levine et al., 2011; Tate, 2015). Two major groups of fertilizers can be distinguished: organic fertilizer (e.g., compost, manure) and mineral forms (e.g., extracted from minerals or produced industrially) which both have been shown to strongly affect GHG emissions (Hallin et al., 2009; Syakila and Kroeze, 2011; Thangarajan et al., 2013; Shaaban et al., 2016). A common problem of mineral fertilizers is the loss of N and P by leaching (Kramer et al., 2006) and the decreased soil pH by repeated addition of N-fertilizer (Cheng et al., 2015) which by itself can give rise to enhanced N_2O emissions (Bakken et al., 2012). Organic amendments represent a more sustainable fertilization strategy as they convey more efficient retention of nitrogen and carbon compounds necessary for plant growth. These organic amendments, like composted cattle manure, biochar, or zeolite addition or crop residue addition can also lower the emission of N_2O, or increase its reduction to N_2 (Thomson et al., 2012; Thangarajan et al., 2013). However, regarding the GHG related, underlying microbiology under influence of fertilizer applications, knowledge is far from complete.

Recent novel insights led to the postulation that representatives of the newly discovered N_2O-reducing clade II can possibly turn soils into sinks of N_2O (Jones et al., 2014; Domeignoz-Horta et al., 2015). However, attempts to stimulate soil N_2O uptake by inoculation with a non-denitrifying *nosZ* clade II strain lowered the net potential emission but did not turn the soil into a sink of N_2O (Domeignoz-Horta et al., 2016b). While the soil sink function of N_2O still has to be verified, CH_4 uptake can be found in several soils thereby contributing to cooling side of the GHG balance, representing 6% of the total global methane sink (Kirschke et al., 2013; Tian et al., 2016). However, fertilizer effects on the CH_4 sink function in agricultural soils have received far less attention as compared to wetlands and well-aerated non-agricultural soils. This is due the very low or negligible methane uptake capacity in these soils as compared to grassland and upland forest soils (Mosier and Delgado, 1997; Veldkamp et al., 2013; Ciais et al., 2014). By converting natural soils into agricultural soils, up to a 7-fold reduction of CH_4 consumption was detected (Levine et al., 2011), taking up to 80 years to recover to pre-land use change levels. It has been demonstrated that the decrease in methane uptake in agricultural soils is due to the destruction of the soil physical structure (e.g., plowing, soil compaction), disrupting the methane gradients in the soil, which are proposed to be crucial for high affinity atmospheric methane oxidation. Next to this other agricultural practices (e.g., fertilization) have been demonstrated to have detrimental effects on atmospheric methane uptake (Bender and Conrad, 1992; Boeckx et al., 1997; Hiltbrunner et al., 2012). However, a recently published study (Ho et al., 2015) demonstrated strongly enhanced methane uptake rates after the addition of different organic amendments (e.g., compost, sewage sludge), to different agricultural soils. The observed rates of uptake were even comparable to the ones from well-aerated forest soils. Shackley et al. observed a similar effect upon addition of biochar which improved the GHG balance by reducing N_2O and CH_4 emissions from soil (Shackley et al., 2016). These findings are further supported by another study which showed that the use of organic fertilizers (in this case biochar and compost) influence microbial processes which resulted in alterations of soil nutrient cycles thereby affecting agricultural properties (Ye et al., 2016). Furthermore, the addition of plant-derived C compounds from external sources such as biochar or composts can increase soil C availability and may result in higher net CO_2 removals from the atmosphere (Paustian et al., 2016) thereby lowering the global warming potential (GWP) (Järveoja et al., 2016). Compared to fresh organic residues, mineralization of compost is slower after addition to soil, leading to a several fold greater mean residence time (Ryals et al., 2015). Ho et al. (2015) postulated that a well-balanced mix of different fertilizers could have a positive effect on GHG balance considering the creation of conditions for methanotrophs to take up atmospheric methane while at the same time keeping carbon dioxide and nitrous oxide emissions to a minimum by providing a greater variety of C- and N-compounds to the microorganisms. However, not all organic fertilizers are suitable to serve this purpose, since in single application only a few organic residues showed the capability to increase soil CH_4 uptake and keep CO_2 and N_2O emissions to a minimum (Ho et al., 2017). However, to develop a strategy to reduce GHG emission from agricultural soils without decreasing crop yield requires understanding of the underlying mechanisms of how organic fertilizers influence GHG. This study aims to answer the following research questions: What is the influence of a combination of organic amendments (compost, digestate, sewage sludge, and cover crop residues) on the GWP of agricultural soils? We hypothesize that methane uptake is stimulated while CO_2 and N_2O emissions are kept to a minimum compared to un-amended soil by application of mixes of organic amendment and mineral fertilizers. We test these hypotheses by performing soil incubations with various combinations of organic and mineral fertilizers and following GHG dynamics as well as soil chemistry and microbial functional gene abundance.

MATERIALS AND METHODS

Site Description, Soil Sampling, and Residues

The soil was collected in May 2017 at the research station of Wageningen University in Lelystad, the Netherlands (52°32′26.4″N, 05°33′34.7″E) representing a clay soil. The field was planted with onions and left fallow after harvest before sampling. Previously, soil physical-chemical properties have been determined (Ho et al., 2015). The upper 10 cm of the soils was collected in May 2017 from 1 × 1 m using a shovel. The soil was air-dried at room temperature before being sieved (2 mm). The residues included in this study comprised materials with a broad C:N ratio ranging from 4.85 to 22.39 (**Table 1**) and were selected based on their CH_4 uptake performance (compost and sewage sludge) (Ho et al., 2015) or their common usage as bio-based additives in agricultural soil. The residues were air-dried at 30°C, the sewage sludge (S), digestate (D), and the cover crop residues (in the following referred to as CC residues) powder mixture were crushed and ground (<2 mm) (Jaw Crusher Type BB-1/2, Aartselaar, Belgium). Both composts (C1 and C2) were broken down and sieved (<6 mm), while the CC residues were cut with a scissor to smaller pieces (<3–5 cm). Both the dried soils and residues were thoroughly mixed and sieved as per treatment prior to setup of the experiment to ensure standardized initial incubation conditions.

Experimental Setup for *in situ* GHG Flux Measurements

The soil (200 g dry weight) and residues were mixed with a spoon in a pot and put in an incubation bottle (500 mL volume), deionized water was added to 65 or 40% of soil water holding capacity, respectively. The residue addition to the soil corresponded to a rate of either 20-ton ha^{-1}, which is typically used in agricultural practice (Diacono and Montemurro, 2010), or 5-ton ha^{-1}, which is the maximum amount of cover crop biomass incorporated in agricultural fields in spring. Incubation was performed using three replicates for each treatment in a climate chamber at 15°C (mean annual temperature in the Netherlands is 10°C) in the dark for ∼1 month (for 28 days). Water loss, measured by weight, was compensated weekly. Periodically (0, 1, 3, 7, 14, 21, 28 d) methane, nitrous oxide and carbon dioxide fluxes were measured under ambient air by closing the bottles tightly with a lid for 3 h and measuring directly after closing, after 1.5 h and after 3 h. At every time point 20 mL of the headspace was withdrawn and stored in exetainers (5.9 mL) vials (Labco Limited, Lampeter, UK). The first 8 ml of sample was used to flush the exetainer, followed by 12 ml sample introduced into the exetainers creating a 2 bar overpressure. Introduction of the sample (1 ml) into the GC was by an autosampler (TriPlus RSH, Thermo Fisher Scientific, Bleiswijk, The Netherlands) connected to a gas chromatograph (GC1300, Thermo Fisher Scientific) equipped with a Methanizer and a Flame Ionization Detector (FID) to detect CH_4 and CO_2, an electron capture detector (ECD) for detection of N_2O and two sets of a pair Rt-Q-Bond capillary columns (L; 15 m and 30 m, ID; 0.53 mm, Restek, Interscience, Breda, The Netherlands). Helium was used as a carrier gas, and oven temperature was set at 80°C. Five different concentrations of CH_4 (0.1, 0.2, 0.6, 1.2, 2 ppm), CO_2 (100, 200, 600, 1,200, 2,000 ppm), and N_2O (0.05, 0.1, 0.3, 0.6, 1.0 ppm) from a gas mixture (2 ppm CH_4, 2,000 ppm CO_2, 1 ppm N_2O) (Linde AG, Velsen-Noord, The Netherlands) were used as a standard. If higher concentrations of CO_2 and N_2O were measured, additional single gas calibration gases (Linde AG) of the respective gases (CO_2: 4,000 and 10,000 ppm; N_2O: 10 and 100 ppm) were used. Chromeleon™ Chromatography Data System 7.1 (CDS, Thermo Fisher Scientific) Software was used to analyse the obtained gas chromatograms from the GC and was used to calculate the standard curves. The gas flux rates were determined by linear regression from the three time points. All fluxes with a $R^2 < 0.70$ were discarded.

Measuring Methane Oxidation and Organic Matter

To determine near atmospheric soil methane emission or uptake under influence of the different amendments after 7, 14, 21, and 28 d, the bottles were closed for 6 days and ∼10 ppm CH_4 was added to the headspace. CH_4 decrease was measured every day in duplicates from each bottle using an Ultra GC gas chromatograph (Interscience, Breda, The Netherlands) equipped with a Flame Ionization Detector (FID) and a Rt-Q-Bond (L; 30 m, ID; 0.32 mm, Restek, Interscience) capillary column. Helium was used as a carrier gas, and oven temperature was set at 80°C. Chromeleon™ Chromatography Data System 7.1 (CDS, Thermo Fisher Scientific) Software was used to analyse the obtained gas chromatograms from the GC.

Sample Storage and Soil Organic Matter Measurements

After finishing the incubation ∼10 g of soil samples were stored at −20°C for later DNA extractions. Another ∼50 g of soil was dried at 30°C and stored for soil nutrient determination. To measure the soil organic matter content after incubation, 10–15 g of soil was dried in a porcelain cup at 105°C for 1 day. Afterwards, the dried sample was burned in an oven at 430°C for another day, both times the sample was weighed. To calculate the organic matter content per g 100 g^{-1} dry soil the following formula was used: 100* (g dry soil—g ashed soil)/g dry soil.

DNA Extraction and qPCR Assays

DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Venlo, The Netherlands) according to manufacturer's instruction. We performed qPCR assays targeting *amoA* for ammonium oxidizing Achaea (AOA) and bacteria (AOB), *nifH* (N-fixers), *nosZ* clade I/II (denitrifiers), *mcrA* (methanogens), *pmoA* (methane oxidizers), 16S rRNA gene for Archaea and Bacteria as well as the 18S rRNA gene of fungi. Each assay was performed in duplicate for each DNA extract with primers, primer concentration, and PCR profiles as shown in **Supplementary Table 1**. Briefly, each qPCR (total volume 20 μl) for all assays consisted of 10 μl 2× SensiFAST SYBR (BIOLINE, Alphen aan den Rijn, The Netherlands), 1 μl of forward and reverse primers each (10 pmol μl^{-1}; Sigma-Alderich, Zwijndrecht, The Netherlands), 1 μl bovine serum

TABLE 1 | Amendment description, total C and N contents of amendment and soil.

Soil/residues	Total C (μg C mg dw sample^{-1})	Total N (μg C mg dw sample^{-1})	C:N	Description (source/location)
Soil	16.44 ± 0.34	1.12 ± 0.07	14.76	Clay soil from an agricultural field with onions as the last crop (Lelystad, The Netherlands)
Sewage Sludge	202.74 ± 12.82	41.81 ± 1.80	4.85	Sampled from an anaerobic digester after sludge thickening (Vallei Veluwe, The Netherlands)
Digestate	290.07 ± 14.14	24.59 ± 1.64	11.82	Residue product of biogas formation from manure (ACRRES, The Netherlands)
Compost1	145.68 ± 39.07	11.08 ± 2.19	13.04	Mature compost derived from organic materials e.g., plant clippings and grass (Attero, The Netherlands)
Compost2	118.40 ± 13.77	6.25 ± 0.65	18.96	Van Iersel fungal dominant humic compost. Basic ingredient is wood shreds (Soiltech, The Netherlands)
CC residue mixture	347.02 ± 15.78	15.50 ± 1.78	22.39	Consist of *Brassica carinata*, *Trifolium incarnatum*, *Secale cereal* collected from a field in November 2016 (Joordens, The Netherlands)

albumin (5 μg μl^{-1}; Invitrogen, Breda, The Netherlands), 4.5 μl DNase- and RNase-free water and 2.5 μl diluted template DNA. The qPCR for the EUBAC(bacterial 16S rRNA gene) assay (total volume 15 μl) consisted of 7.5 μl 2× SensiFAST SYBR (BIOLINE), 0.75 μl of forward and reverse primers each (5 pmol μl^{-1}; Sigma-Alderich), 1.5 μl bovine serum albumin (5 μg μl^{-1}; Invitrogen), 1.5 μl DNase- and RNase-free water, and 3 μl diluted template DNA. Standard curves were obtained using serial 10-fold dilutions of a known amount of plasmid DNA from different pure cultures representing the target gene fragment (10^8–10^1 gene copies) containing the respective gene fragment. The qPCR was performed with an iCycler IQ5 (Applied Biosystem, Carlsbad, CA, USA). Negative controls were always run with water instead of template DNA. PCR reactions were done with 1:20 and 1:60 diluted DNA extracts. Amplification efficiencies for all assays were between 79 and 98% with R^2 values between 0.969 and 0.995. Amplicon specificity was inferred from the melt curve.

Statistical Analyses of Collected Data

All statistical analyses were done using R version 3.0.1 (R Development Core Team, 2013). The mean total GHG fluxes, the GWP, the organic matter loss and abundance of the different functional marker genes were tested for normality by Kolmogorov–Smirnov test and for homogeneity of variance by Levene's test. If necessary, normal distribution was achieved by log-transformation of the data. Treatment effects and differences between means were assessed using one-way ANOVA followed by Tukey's *post-hoc* test. All levels of significance were defined at $P < 0.05$.

RESULTS

GHG Flux Measurements

The fluxes of the three major GHG (CH_4, CO_2, N_2O) from the soils amended with the organic amendments were measured continuously through the experiment at different level of SM and different applied concentrations of organic amendments. An overview about values of the different GHG as well as the calculated GWP_{100yr} for the different samples is shown in **Table 2**.

CH_4

The CH_4 flux measurements under 65% SM (**Supplementary Figures 1A,B**) showed variation over time considering uptake or emission of CH_4. Both amounts of organic amendments applied (5 and 20 t/ha) led to similar fluxes during the incubation without fluctuation. However, total CH_4 fluxes (**Figures 1A,B**) varied between treatments, mostly releasing CH_4 over time irrespective of the amount of organic amendment used. Only three amendments (digestate, D + C2, S + C1 at 20 t/ha) led to increased methane uptake. Under 40% SM, minor fluctuations in CH_4 fluxes over time were detected with both organic amendment amounts (**Supplementary Figures 1C,D**). Calculated mean cumulative CH_4 fluxes (**Figures 1C,D**) demonstrated that all samples emitted CH_4 during the incubation.

CO_2

Measured CO_2 fluxes under 65% SM (**Supplementary Figures 2A,B**) showed the same trends, irrespective of the amounts of organic amendment applied. Highest CO_2 fluxes were observed for cut and powdered cover crop residues, respectively, followed by digestate and the sewage sludge + compost 2 combination. Independent of the amount applied, cut as well as powdered CC residues continuously released CO_2 over the complete incubation. Both types of compost led to the lowest CO_2 fluxes among the organic amendments used and were comparable or lower than the CO_2 fluxes of the un-amended soil. The mean cumulative CO_2 fluxes (**Figures 2A,B**) reflect the dynamics of the CO_2 fluxes over time and treatments (**Supplementary Figures 2A,B**). Highest CO_2 emissions were observed for cut CC residue material, followed by powdered CC residue, digestate, and

TABLE 2 | Overview of mean total CH_4. CO_2, N_2O, and calculated GWP_{100yr} values of the different organic amendments, amounts, and soil moisture concentration that were used.

OA	Amount [t/ha]	Mean total CH_4-C [μg kg soil^{-1}]		Mean total CO_2-C [mg kg soil^{-1}]		Mean total N_2O-N [mg kg soil^{-1}]		GWP_{100yr} [mg CO_2-C kg soil^{-1}]	
		40%SM	65%SM	40%SM	65%SM	40%SM	65%SM	40%SM	65%SM
Un-amended	None	75 ± 50.2	83 ± 18.0	958 ± 71.3	1,068 ± 132.6	0.015 ± 0.008	0.063 ± 0.034	959 ± 71.2	1,072 ± 134.8
C1	20	100 ± 28.6	70 ± 53.7	1830 ± 117.5	2,357 ± 256.4	0.057 ± 0.008	0.290 ± 0.052	1834.4 ± 117.1	2375.9 ± 257.7
	5	142 ± 123.4	125 ± 23.8	818 ± 464.6	1,717 ± 73.3	0.002 ± 0.015	0.0414 ± 0.014	819 ± 464.8	1,744 ± 79.9
C2	20	91 ± 27.1	88 ± 42.7	1,058 ± 27.3	1,586 ± 14.3	0.013 ± 0.001	0.042 ± 0.014	1,060 ± 27.1	1,589 ± 13.7
	5	91 ± 24.1	88 ± 32.5	777 ± 35.2	1,426 ± 75.7	0.009 ± 0.005	0.425 ± 0.457	778 ± 34.9	1,453 ± 13.6
Cut CC	*20*	134 ± 52.2	70 ± 37.4	32,372 ± 2762.6	46,157 ± 1289.2	13.651 ± 1.879	16.877 ± 2.182	33,218 ± 2670.6	47,201 ± 1381.7
	5	70 ± 46.5	84 ± 22.8	6,303 ± 1057.9	11,689 ± 1220.0	0.107 ± 0.033	5.482 ± 2.244	6,310 ± 1059.5	12,028 ± 1358.8
Powder CC	20	118 ± 27.9	70 ± 30.5	20,098 ± 1538.7	26,177 ± 1006.6	19.345 ± 3.967	6.397 ± 2.67	21,295 ± 1422.1	26,688 ± 1031.1
	5	82 ± 19.1	109 ± 47.2	5,286 ± 1205.6	7,996 ± 1429.6	0.031 ± 0.013	4.666 ± 5.425	5,289 ± 1205.9	8,236 ± 1143.8
Digestate	20	80 ± 40.3	−77 ± 21.3	4,554 ± 780.5	6,583 ± 316.5	0.280 ± 0.335	6.204 ± 2.207	4,572 ± 800.9	6,966 ± 183.7
	5	104 ± 53.8	107 ± 94.0	2,322 ± 277.4	2,750 ± 490.8	0.032 ± 0.049	2.029 ± 0.457	2,325 ± 279.4	2,877 ± 129.9
D+C1	20	48 ± 32.1	30 ± 25.7	2,734 ± 177.8	3,807 ± 348.0	0.105 ± 0.060	2.259 ± 0.189	2,741 ± 180.8	3,947 ± 359.0
	5	46 ± 28.8	18 ± 55.1	1,560 ± 383.7	2,070 ± 95.6	−0.033 ± 0.093	1.059 ± 0.30	1,558 ± 389.5	2,135 ± 84.4
D+C2	20	75 ± 74.9	−70 ± 61.1	2,135 ± 34.1	3,848 ± 1239.4	0.056 ± 0.049	8.183 ± 10.67	2,139 ± 31.9	4,354 ± 1895.3
	5	57 ± 15.4	12 ± 29.6	1,118 ± 208.5	1,711 ± 103.1	0.028 ± 0.011	0.586 ± 1.211	1,120 ± 208.1	1,747 ± 70.0
S+C1	20	53 ± 23.9	−40 ± 44.5	4,884 ± 362.0	6,057 ± 2144.8	1.485 ± 0.271	28.589 ± 15.345	4,976 ± 345.2	7,825 ± 3042.5
	5	85 ± 40.8	9 ± 38.9	1,853 ± 137.3	2,527 ± 63.0	0.263 ± 0.190	9.306 ± 4.354	1,870 ± 130.0	3,102 ± 289.9
S+C2	20	107 ± 71.2	16 ± 23.8	4,561 ± 336.3	6,170 ± 209.3	0.707 ± 0.047	32.501 ± 3.094	4,605 ± 336.1	8,178 ± 399.2
	5	88 ± 24.1	62 ± 64.5	1,648 ± 203.7	2,266 ± 186.1	−0.016 ± 0.091	8.756 ± 2.053	1,647 ± 208.7	2,808 ± 313.5

*GWP_{100yr} calculations derived from the cumulative CH_4 (**Supplementary Figure 1**), CO_2 (**Supplementary Figure 2**), and N_2O (**Supplementary Figure 3**) fluxes. OA, organic amendments; un-amended, soil without organic amendment; C1, compost1; C2, compost2; cut CC, cut cover crop residue mixture; powder CC, powder cover crop residue mixture mix; D+C1, digestate + compost1; D+C2, digestate + compost2; S+C1, sewage sludge + compost1; S+C2, sewage sludge + compost2.*

the sewage sludge amendments. This was true for both tested amounts. Highest CO_2 fluxes under 40% SM were always observed for cut CC residue material followed by powdered CC residues, digestate and the two sewage sludge treatments (**Supplementary Figures 2C,D**). While high amounts of CC residues showed emission of CO_2 over the whole incubation period, no emissions were detected after 21 d with low amounts. Similarly, cumulative CO_2 fluxes (**Figures 2C,D**) were always lower with lower amounts of organic amendments, the extent of which differed between the type of organic amendment. While both cover crop residue treatments were 4- to 5-fold higher, all other organic amendments were only 1.4- to 2.7-fold higher when 20t/ha was applied.

Lower SM always lead to lower CO_2 fluxes when same amounts organic amendments were applied.

N_2O

Both sewage sludge combinations showed the highest N_2O flux rates at 65% SM, regardless of the applied amounts of organic amendments, followed by digestate and cut CC residue material (**Supplementary Figures 3A,B**). Both composts, as well as the un-amended soil, showed almost no N_2O fluxes. In general, 20 t/ha led to higher overall measurable N_2O fluxes. These findings are also underlined by the cumulative N_2O fluxes (**Figure 3**). The N_2O fluxes of both sewage sludge combination, digestate, digestate + compost 1, and both CC residue mixtures were 2- to 4-fold lower with 5 t/ha. The digestate + compost 2 amendment showed a 13-fold reduction, while the un-amended and both single compost applications did not lead to any N_2O emission at all. After 14 d of incubation both combinations of digestate with compost at an application rate of 5 t/ha resulted in lower N_2O emissions.

Only low N_2O emissions were detected at 40% SM (**Supplementary Figures 3C,D**). All organic amendments applied at a rate of 5 t/ha showed no N_2O emissions during the complete incubation period while at 20 t/ha only small amounts of N_2O were released in the first 14 d of incubation. After 14 d both CC residue amendments (cut and powdered) showed a rapid increase in N_2O emissions, which peaked at day 21. After 28 d the cut CC residues still released N_2O from the soil, while the powdered CC residue enabled soil N_2O uptake from this point onward.

GWP Analyses

We derived the GWP in mg CO_2 equivalent per kg soil by combining the cumulative CH_4, CO_2, and N_2O flux (**Supplementary Figures 1**–**3**). In these calculations, the GWP value for CH_4 and N_2O are considered to be 28 and 265, respectively over a hundred- year time frame, while the GWP value for CO_2 is considered to be 1 (IPCC, 2014).

The GWP values showed similar trends as the cumulative CO_2 fluxes, irrespective of the SM and amount of organic amendment

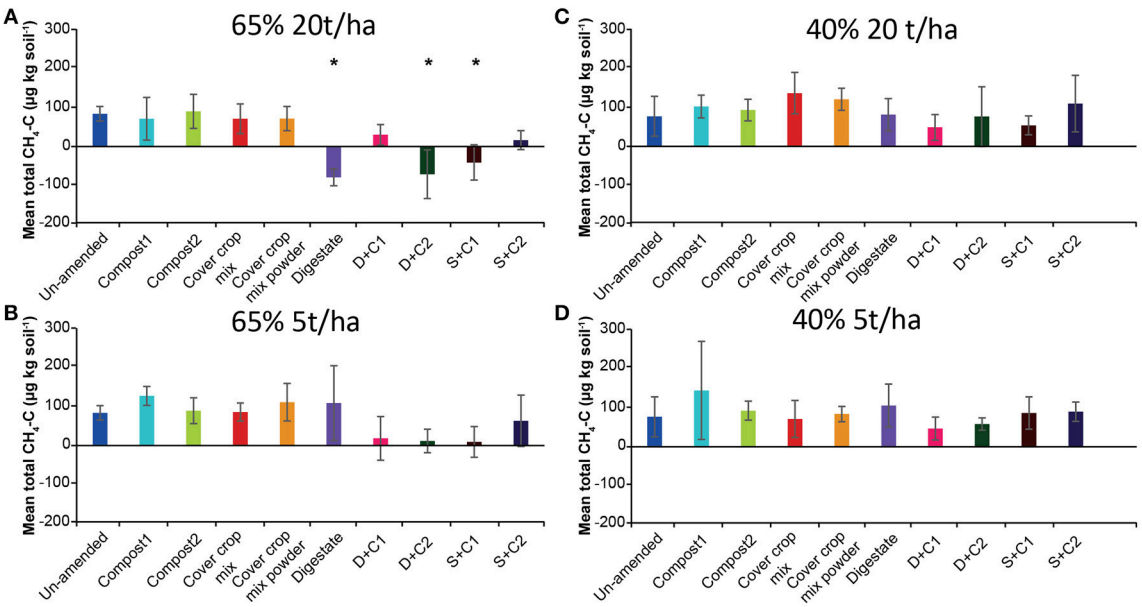

FIGURE 1 | Mean total CH_4 emitted or consumed over the period of 28 d in un-amended clay soil and after amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1, and sewage sludge+compost2 (mean ± SD; $n = 3$) at **(A)** high amount (20 t/ha) and high water content (65%), **(B)** low amount (5 t/ha) and high water content, **(C)** high amount and low water content (40%), and **(D)** low amount and low water content, derived from the cumulative CH_4 (**Supplementary Figure 1**) fluxes. Asterisk (*) indicate significant differences in the mean total CH_4 fluxes between the soils with organic amendments and the un-amended soil within the four separate superordinate treatments (ANOVA: $P < 0.05$).

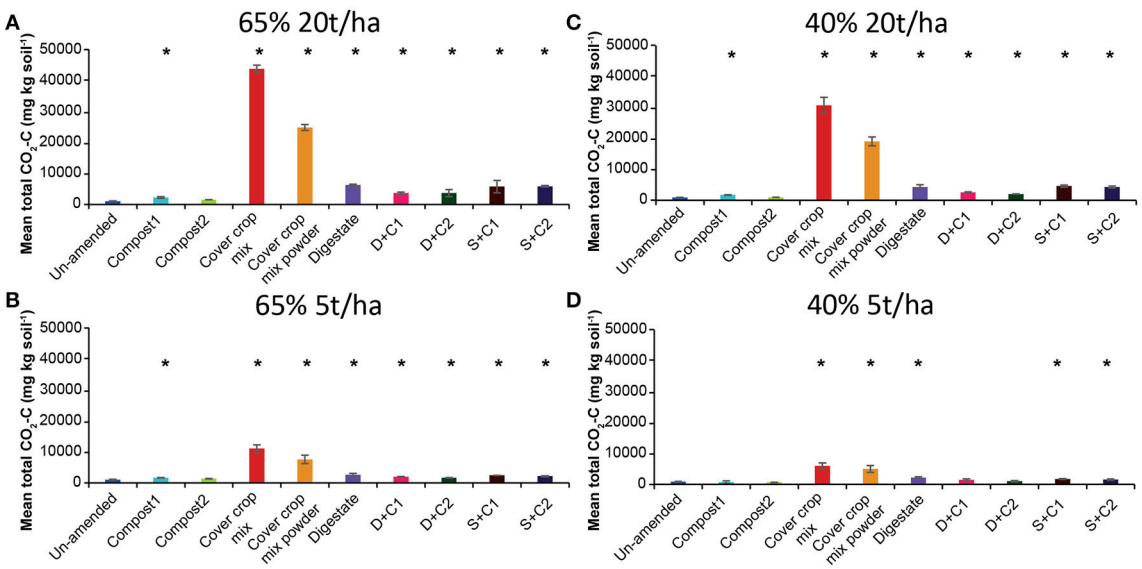

FIGURE 2 | Mean total CO_2 emitted over the period of 28 d in un-amended clay soil and after amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1, and sewage sludge+compost2 (mean ± SD; $n = 3$) at **(A)** high amount (20 t/ha) and high water content (65%), **(B)** low amount (5 t/ha) and high water content, **(C)** high amount and low water content (40%), and **(D)** low amount and low water content, derived from the cumulative CO_2 (**Supplementary Figure 2**) fluxes. Asterisk (*) indicate significant differences in the mean total CO_2 fluxes between the soils with organic amendments and the un-amended soil within the four separate superordinate treatments (ANOVA: $P < 0.05$).

(**Figure 4**). Notably, compost1 and 2 treatments led to lower GWP as compared to un-amended soil with low amounts applied under 40% SM (**Figure 4**).

CH_4 Fluxes After Addition of 10 ppm CH_4

CH_4 fluxes after the addition of 10 ppm CH_4 at multiple times, did not differ significantly between the four major

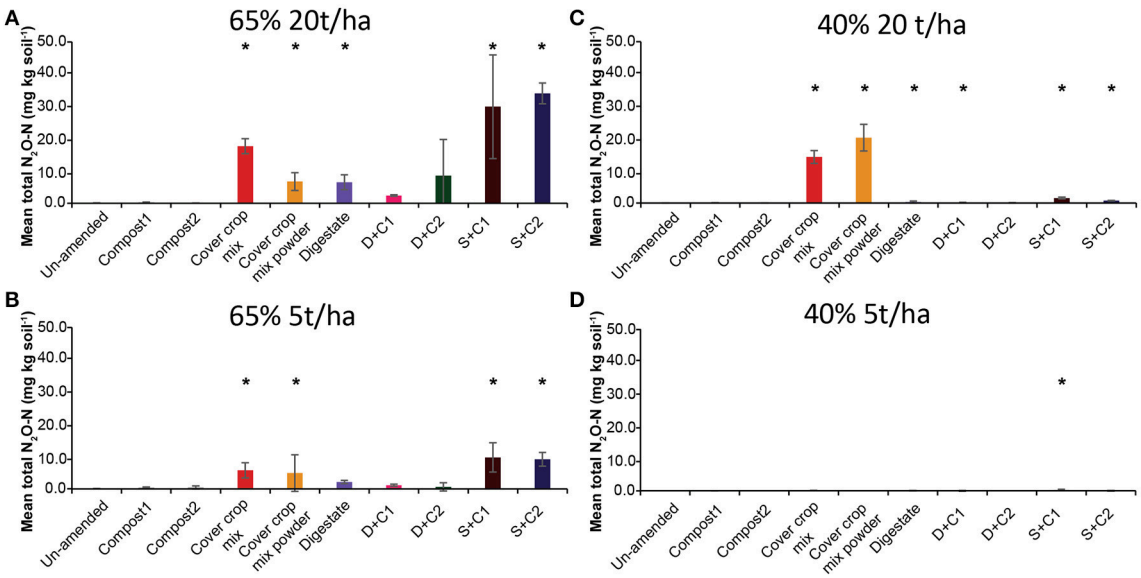

FIGURE 3 | Mean total N_2O emitted over the period of 28 d in un-amended clay soil and after amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1, and sewage sludge+compost2 (mean ± SD; $n = 3$) at **(A)** high amount (20 t/ha) and high water content (65%), **(B)** low amount (5 t/ha) and high water content, **(C)** high amount and low water content (40%), and **(D)** low amount and low water content, derived from the cumulative N_2O (**Supplementary Figure 3**) fluxes. Asterisk (*) indicate significant differences in the mean total N_2O fluxes between the soils with organic amendments and the un-amended soil within the four separate superordinate treatments (ANOVA: $P < 0.05$).

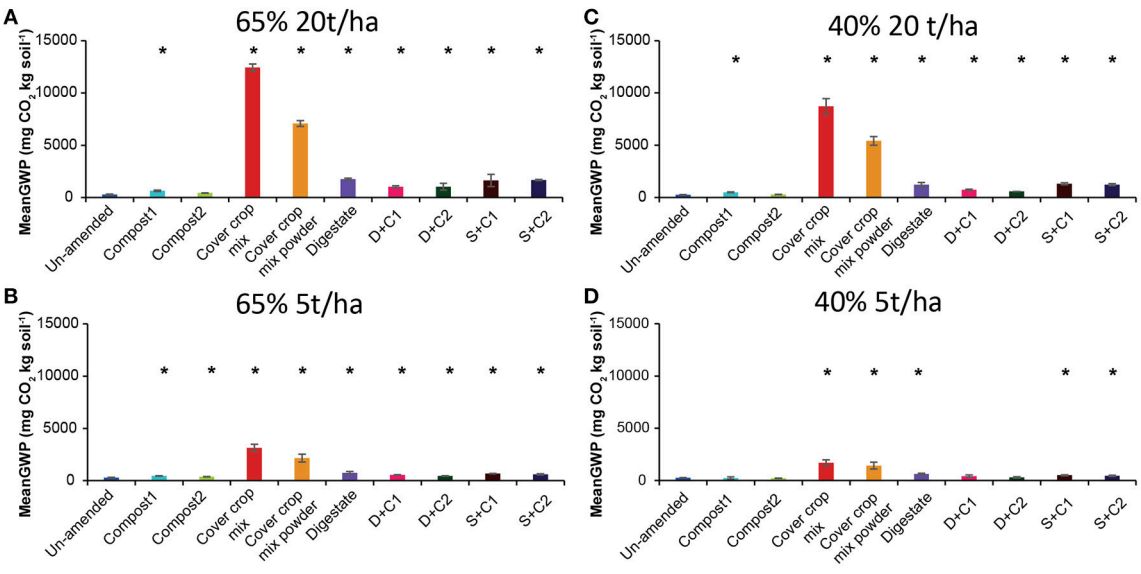

FIGURE 4 | Mean global warming potential (GWP) over the period of 28 d in un-amended clay soil and after amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1, and sewage sludge+compost2 (mean ± SD; $n = 3$) at **(A)** high amount (20 t/ha) and high water content (65%), **(B)** low amount (5 t/ha) and high water content, **(C)** high amount and low water content (40%), and **(D)** low amount and low water content, derived from the cumulative CH_4 (**Supplementary Figure 1**), CO_2 (**Supplementary Figure 2**), and N_2O (**Supplementary Figure 3**) fluxes. Asterisk (*) indicate significant differences in the GWP between the soils with organic amendments and the un-amended soil within the four separate superordinate treatments (ANOVA: $P < 0.05$).

treatments irrespective of SM and organic amendment rate applied (**Supplementary Figure 4**). The fluxes in most cases vary between 0 and −0.003 μmol m^{-2} min^{-1}, which can be referred to as CH_4 uptake. At the last sampling point the amendment with compost2 at 40% SM and 5 t/ha increased to an uptake of −0.008 μmol m^{-2} min^{-1}, which was the highest uptake measured.

However, most organic amendments improve their CH_4 uptake over time.

Organic Matter

When low amounts of organic amendment are applied at 65% SM, the organic matter loss is constant through all treatments ranging from −0.4 to −0.6% loss of the original OM content which was around 2.5–3% (**Figure 5**). At high concentration of organic amendments the loss of OM is lower being around −0.4% with exception of the cut CC residue amendment, resulting in 1.4% loss in organic matter. In general incubations at 40% SM lost more organic matter than their counterpart at 65% SM (**Figure 5**). The lowest losses were observed for digestate, compost1, and D+C1 with a loss of ∼-0.55%. These organic amendments are followed by compost2, D+C2, S+C1, and S+C2 with a loss of −0.8 to −1.0% organic matter content. The highest loss could be observed for cut and powdered CC residue mixture with −1.2 and −1.4%, respectively.

Abundance Analyses of Microbial Groups

To assess changes in the abundance of the microbial communities, the ratio was calculated between gene copy numbers of the analyzed genes in the initial soil and at the end of the incubation. The individual gene copy numbers of all samples analyzed can be found in **Supplementary Tables 2, 3**.

The overall bacterial abundance stayed either stable or increased over time (**Figure 6A**), with high amounts of CC residues leading to the highest stimulation in abundance (4- to 7-fold). All other organic amendments at high application rate led to at least to a doubling of bacterial numbers, while numbers in the un-amended remained constant. When applying low amounts of organic amendments, microbial abundances did not change in any of the treatments.

In contrast to the bacterial abundance, archaea communities either remained stable or decreased over the time (**Figure 6B**). Typically, all digestate combinations, both composts and sewage sludge combinations at high application rate did not lead to change in archaeal abundance, while it decreased in all other treatments.

Overall, fungal abundance was rather constant during the incubation (**Figure 6C**). However, the cut CC residue mixture led to a 15- and 5-fold increase in fungal abundance at high and low organic amendment application rate, respectively while the 20 t/ha powdered CC residue treatment increased around 3-fold. Compost 2 at low application led a 10-fold in increase. All other treatments at high application rate did not lead to change in fungal abundance.

For most of the functional marker genes there was no change in the un-amended soil, except for a decrease of AOAs and a doubling of *nosZ* clade II (**Figure 7**).

Both *nosZ* clades showed an increase in abundance, in all organic amendment-treatments, irrespective of the application rate (**Figures 7A,B**). While the two clades with low amendments increased mainly between 1.2- and 2.5-fold, a 2- to 7-fold increase was observed with 20 t/ha. The highest increase occurred in the incubation with cut CC residue material with 28-fold in *nosZ* clade I. In general, the *nosZ* clade II was 10- to 100-fold more abundant than *nosZ* clade I (**Supplementary Table 2**).

At low application rates organic amendments had no effect on the bacterial *amoA* abundance (**Figure 7C**). At high concentrations, the cut CC residue, both sewage sludge combinations and all treatments with digestate lead to an increase in bacterial *amoA* of 2- to 8-fold (**Figure 7C**).

In contrast to the abundance of the bacterial *amoA*, archaeal *amoA* abundance decreased in all organic amendment-treatments (**Figure 7D**). The strongest decrease was observed for the digestate and sewage sludge combinations with both composts, which decreased 3- to 4-fold in both applied concentrations. In all compost, CC residue and digestate amendments AOA gene copy numbers were 2- to 10-fold higher than for AOBs. This is contrast with the sewage sludge treatments, which at low amendment led to higher numbers of AOA, whereas AOBs showed a 2- to 4-fold higher abundance at high organic amendment (**Supplementary Table 2**).

The abundance of N-fixers in the cut and powdered CC residue mixture increased in the application with 20 t/ha by 3- and 6-fold, respectively (**Figure 7E**). The only other treatment with a positive effect on the abundance of *nifH* was the sewage sludge + compost 2 amendment, which showed an increase of ∼3-fold.

The methanogenic abundance did not changed for both cover crop treatments, but increased 3-fold for compost1, 5-fold for sewage sludge+compost1 and between 10- and 14-fold for the remaining organic amendments at high rates of application while at low rates *mcrA* gene abundance stayed stable (**Figure 7F**).

Gene copy number of methanotrophs (*pmoA*) increased for all samples with 20 t/ha, except in the digestate amendment, in which no differences to un-amended soil were reported (**Figure 7G**). The compost2 amendment and the combination with compost2 showed the strongest effect on the copy numbers with a 4- to 6-fold increase. Low organic amendment application rates only showed minor positive effects on the abundance of methanotrophs.

The abundance of the two CC residue amendments at low SM and high organic amendment application behaved very similar for all analyzed genes (**Supplementary Table 4**). The abundance of the archaeal 16S rRNA gene and archaeal *amoA* dropped by 2-fold, while it stayed stable for *nifH*, *mcrA,* and *pmoA*. A 5-fold increase was observed for the fungal 18S rRNA gene and *nosZ* clade I for the cut CC residues, while the powder led to a 3- and 2-fold increase, respectively. *nosZ* clade II numbers increase for both CC residue materials around 3-fold. While the cut CC residue material resulted in a 2-fold increase for the bacterial 16S rRNA gene and the bacterial *amoA*, the powdered CC residue material did not show a change for these two genes.

DISCUSSION

In this study, we investigated the influence of combinations of organic amendments on the GHG balance and the CH_4 uptake as well as on dynamics of different soil microbial groups that are involved in producing or reducing GHGs in

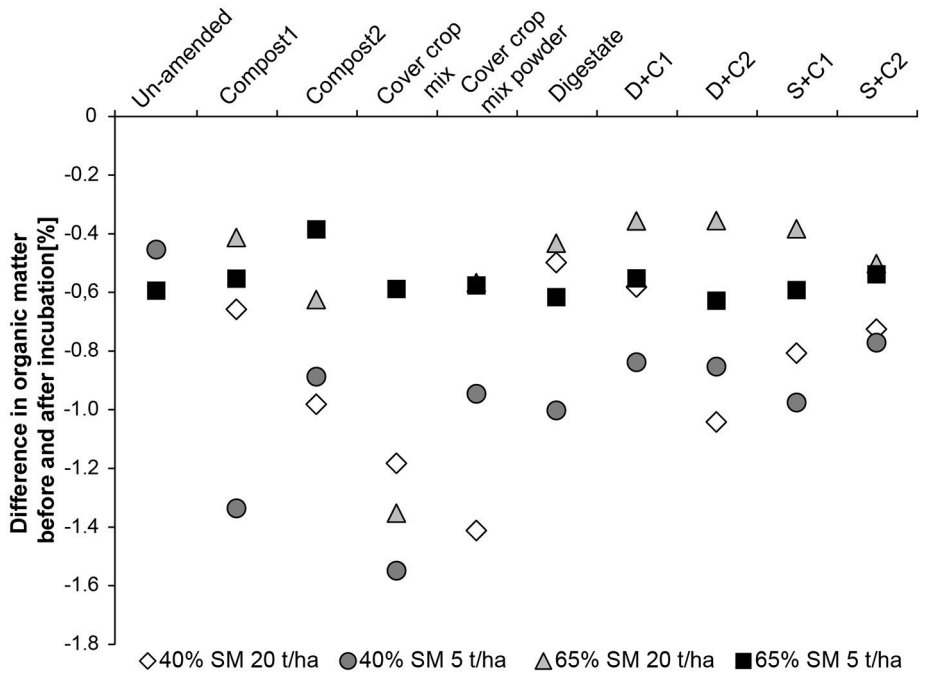

FIGURE 5 | Loss in organic matter content during the incubation period of 28 d in un-amended clay soil and during amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1, and sewage sludge+compost2 (mean ± SD; $n = 3$) at (△) high amount (20 t/ha) and high water content (65%), (■) low amount (5 t/ha) and high water content, (◇) high amount and low water content (40%), and (●) low amount and low water content.

agricultural soil. Several combinations of amendments led to reductions of CO_2, CH_4, and/or N_2O emissions compared to un-amended soil. Most optimal GHG balance was obtained by compost amendments, which resulted in similar overall GHG balance as compared to the un-amended soil. Additionally, we could observe a strong increase in microbial communities involved in GHG consumption in all amendments, with the strongest increase associated with cover crop residue mixtures.

GHG Dynamics and GWP in Relation to Different Organic Amendments and Manipulation of Soil Moisture

CH_4

We did not observe significant uptake of CH_4 in any of our samples except for digestate (D), D+C2, S+C1 at high SM and high application rate, which led to CH_4 uptake over the complete incubation period (**Figure 1**). However, the *in vitro* methane uptake capacity at near atmospheric (i.e., 10 ppm) methane concentrations increased in all samples over time. As proposed by Ho et al. (2015), it seems that the methanotrophic community needs elevated methane to gear up the enzyme machinery. A similar result was found in rice soils where high methane concentration spikes were necessary to induce atmospheric methane uptake (Cai et al., 2016). Especially the 5 t/ha compost2 treatment under 40% SM showed a very strong improvement in CH_4 uptake at the end of the incubation. Potentially, the release of rare earth metals (e.g., La, Ce, Nd), which are stored in the compost (La ∼2.2 μg g^{-1}; Ce ∼3.8 μg g^{-1}; Nd ∼2.2 μg g^{-1}; El-Ramady, 2011) stimulated the CH_4 uptake (Keltjens et al., 2014). Recent studies found that for some methanotrophs rare earth metals are essential as cofactors in the active center of an alternative methanol dehydrogenase (Keltjens et al., 2014; Pol et al., 2014; Shiller et al., 2017). Furthermore, it was shown that the La-dependent methanol dehydrogenase can also be more efficient hydrolytic catalysts because they are stronger Lewis acids (electrophilic electron acceptor) than the Ca dependent one (Lim and Franklin, 2004). This La-dependent methanol dehydrogenase which can also be found in the newly isolated atmospheric CH_4 oxidizer belonging to the USCα cluster (Pratscher et al., 2018). However, all studies with rare earth metals and their effect on CH_4 oxidation were performed in liquid cultures. Another possible explanation for the increase in CH_4 oxidation rates at the end of the incubation in compost2 incubations, could be its relatively low C- and N-content in comparison to the other organic amendments. This could lead to higher amounts of essential substrates (O_2) or lower amounts of inhibiting compounds (e.g., NH_4^+) for methane oxidation (Conrad and Rothfuss, 1991; Bender and Conrad, 1992; Malyan et al., 2016). In contrast, the higher amount of C- and N-compounds in the other organic amendments could result in a reduced or delayed start of CH_4 oxidation. Furthermore, it is known that compost could lead to an increase in the soil's cation binding capacity (Epstein et al., 1976), leading to lowering of

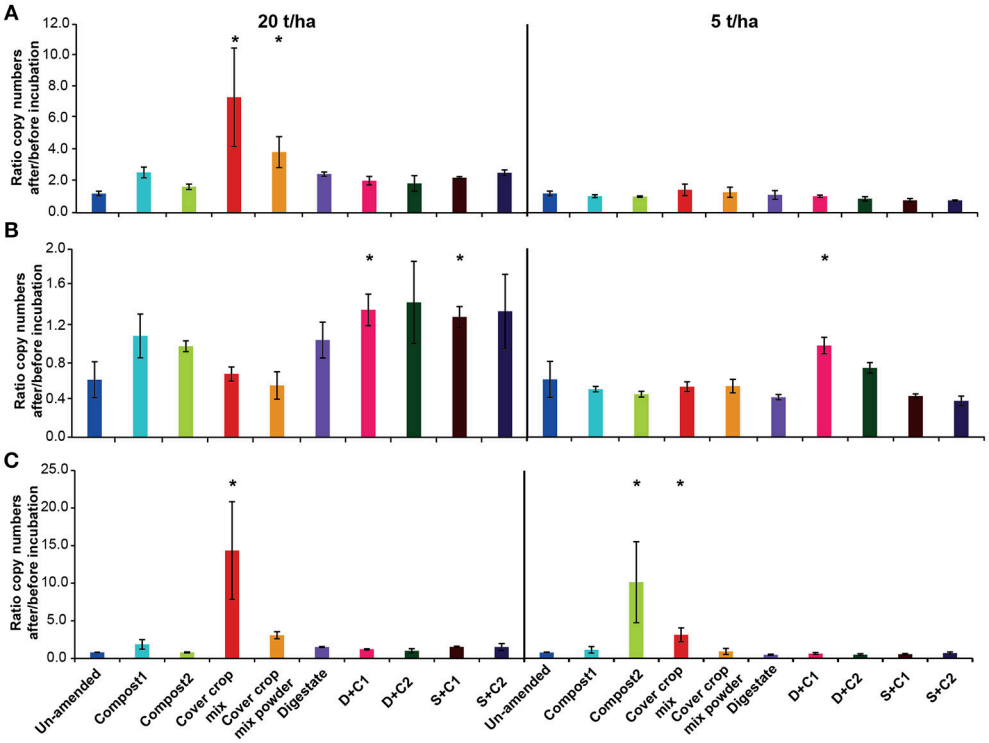

FIGURE 6 | Ratio of the copy numbers of **(A)** bacterial 16S rRNA gene, **(B)** archaeal 16S rRNA gene, and **(C)** fungal 18S rRNA gene after and before an incubation of un-amended clay soil and during amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1 and sewage sludge+compost2 (mean ± SD; $n = 3$) for 28 d. Asterisk (*) indicate significant differences in the ratio of the individual genes in the soils with organic amendments and the un-amended soil within the four separate superordinate treatments (ANOVA: $P < 0.05$).

the availability of ammonium ions, potentially inhibiting the particulate methane monooxygenase (Singh and Seneviratne, 2017).

CO_2

The first addition of water induced a direct emission of CO_2 from the soil in samples with organic amendments. The extend of these CO_2 emissions is strongly dependent on the amendment used. The lowest CO_2 emissions were obtained with both compost amendments, showing similar values as the un-amended soil, where the fungal based compost emitted less CO_2 than the compost from green cut materials (**Table 2**). The reason for this could be a low total C concentration together with not easily degradable C-compounds (Ryals et al., 2015). Based on this it seems that the different material of the compost can contribute better or worse to reducing GHG, which would need further analyses.

The decrease respiration in organic matter added through the experiment did not correlate with most of the CO_2 fluxes. Only the CO_2 fluxes under moist conditions ($R^2 = 0.633$) and high amount of organic amendment ($R^2 = 0.783$) correlated with the decrease of organic matter. This is in accordance with our previous study (Ho et al., 2017), demonstrating that C:N alone is not a good predictor of amendment effects on GHG fluxes. In this study the organic amendment with the highest C:N ratio was the fungal based compost which showed the lowest measurable CO_2 fluxes of all organic amendments. However, the highest measured CO_2 fluxes were emitted by both CC residue mixtures which indeed have the second highest C:N ratio. We observed a correlation between the total C concentration measured in the organic amendments and the CO_2 fluxes. The quality and composition of the amendments, seem to be more important for influencing the CO_2 fluxes. For example the sewage sludge+compost2 amendment has the same total C-content as compost1, but emitted 4-fold higher CO_2 fluxes. In accordance with this, digestate has a lower total C concentration compared to CC residue material, but emitted 15-fold less CO_2. One explanation is that the digestate is not as easily degradable as the plant material for the microorganisms, since its origin is already anaerobically digestaed manure. It was already shown that CO_2 respiration from digestate is highly dependent on the initial source from which the digestate is produced, which led to a broad range of CO_2 respiration rates (Alburquerque et al., 2012). According to our results, this statement can be extended to a variety of organic amendments.

Surprisingly, we saw a second peak of increased CO_2 emission after 21 d in almost all treatments. This may be explained by the fact after 14 d substrates which are more difficult to degrade are reduced to a more accessible form of shorter chain molecules. Succession in microbial community composition may

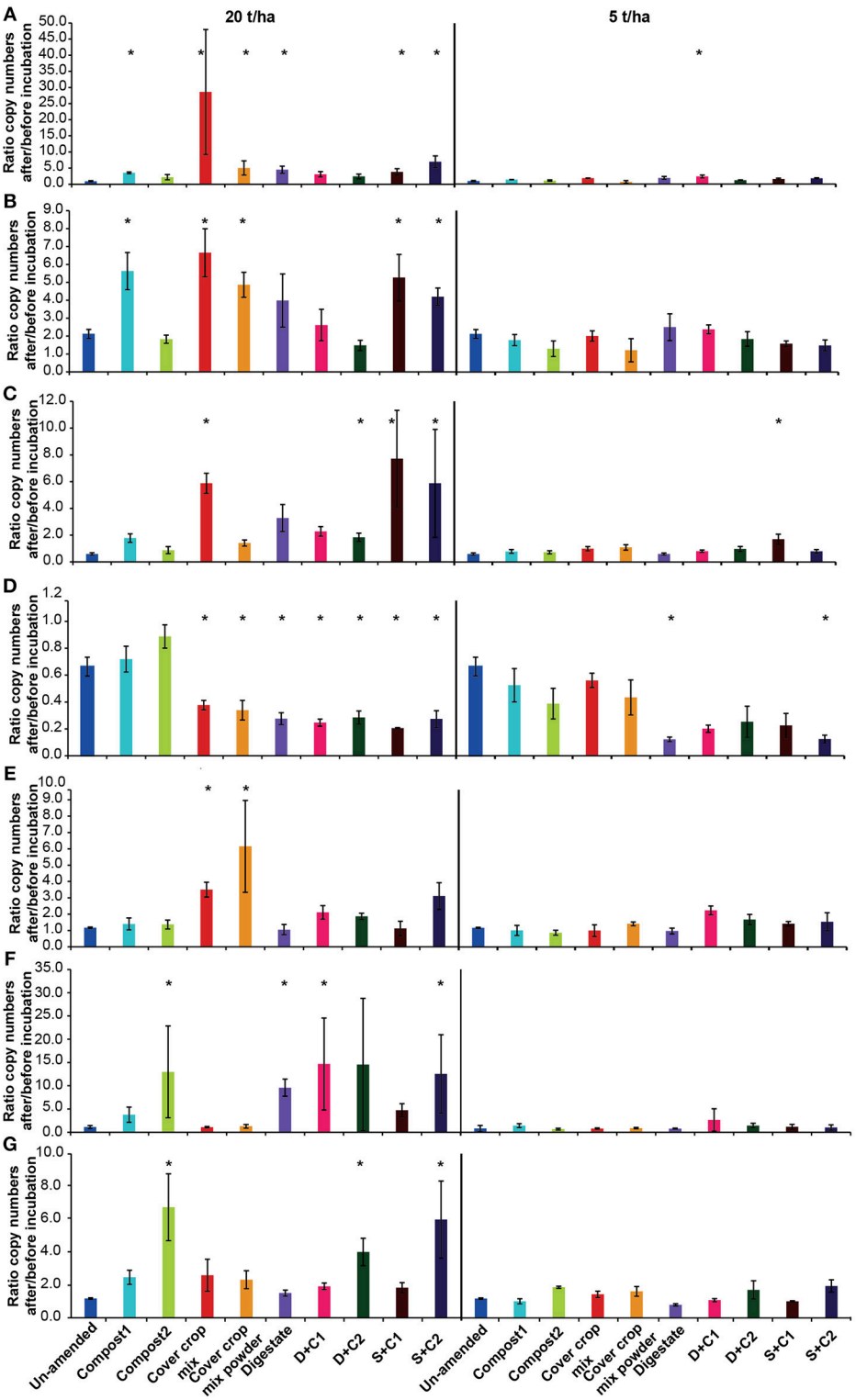

FIGURE 7 | Ratio of the copy numbers of the functional marker genes **(A)** *nosZ* clade I, **(B)** *nosZ* clade II, **(C)** bacterial *amoA*, **(D)** archaeal *amoA*, **(E)** *nifH*, **(F)** *mcrA*, and **(G)** *pmoA* after and before an incubation of un-amended clay soil and during amendments with compost1, compost2, CC residues mixtures, digestate, digestate+compost1, digestate+compost2, sewage sludge+compost1, and sewage sludge+compost2 (mean ± SD; $n = 3$) for 28 d. Asterisk (*) indicate significant differences in the ratio of the individual genes in the soils with organic amendments and the un-amended soil within the four separate superordinate treatments (ANOVA: $P < 0.05$).

be involved which can take place in just a short period of time (13–15 d) following amendment with organic residues as shown by Ho et al. (2017). Additionally, changes in soil parameters (e.g., O_2 availability, N availability) may cause a second phase of CO_2 respiration due to alleviation of initial limitations.

N_2O

Surprisingly, the highest N_2O fluxes were not observed from the N richest organic amendment (digestate), but from the combinations of sewage sludge with compost, followed by the CC residue mixtures (**Table 2**). Hence, the N_2O emission is not only depending on the N-content of the organic amendments, but also in which form the N-source is provided to the microorganisms. These observations are similar to our findings of the relatively weak correlation between C-content and CH_4/CO_2 fluxes. Additionally, we could not find any correlation of C/N or C-content to N_2O fluxes (data not shown). Contrary to a recent study we also did not observe a linear relation between N fertilization and N_2O emission (Shcherbak et al., 2014) in a study where all soil and environmental parameters were kept stable.

Only in case of the high organic amendment application we observed a second N_2O flux peak after 21 d of incubation. In these incubations, the existing input of fresh N through the organic amendments was probably already processed and either turned into gaseous N, microbial N, or remains in refractory form. The microbial biomass or refractory N may release ammonium by mineralization, but this may take more time explaining the temporal pattern observed. Another explanation maybe that the soil parameters changed and stimulated the production of N_2O again (e.g., through more anoxic zones). The results of the abundance analyses from these samples (**Supplementary Table 3**) revealed a strong increase of fungi in these samples, which could be causing the observed N_2O production in our incubations. Fungi are known for possessing denitrification genes to produce N_2O, but as yet have not been demonstrated to harbor N_2O-reductase gene (Takaya, 2002; Shoun et al., 2012). It was also shown that denitrifying fungi already prefer drier conditions than denitrifying Bacteria (Chen et al., 2015). Additionally, since a SM of 40% normally does not favor denitrification processes (Skiba et al., 2002; Bateman and Baggs, 2005), changes in soil structure or chemistry (e.g., pH, O_2 availability, aggregate composition) could have occurred leading to "hotspots" of N_2O production as proposed to be responsible for local, temporary high denitrification activity (Groffman et al., 2009).

The water content has a more pronounced influence on the N_2O emission than on the CH_4 and CO_2 fluxes. At low SM almost no N_2O emission was detected. Since high SM reduces O_2 availability and gas diffusivity and therefore will favor denitrification (Skiba et al., 2002), it can be assumed that in our incubation denitrification processes are the main source of N_2O production. It was already observed in other studies that an increasing SM led to an increase of N_2O production by denitrification (peak above 65% water-filled pore space), since the optimal SM concentration for nitrification peaks at around 55–65% water-filled pore space (Bateman and Baggs, 2005; Vargas et al., 2014; Sanz-Cobena et al., 2016). Contrary to this, the high amount of CC residue mixtures showed a strong increase in N_2O emission at a low SM (**Figure 4**) just after 15 d. Even more surprising was the uptake of N_2O after 28 d for the powdered CC residue mixture. This can either be caused by the high concentrations of N_2O stimulating N_2O reducers, or by a change in the soil characteristics (e.g., pH, O_2 availability). Growth of fungi, which occurred in the CC residue bottles after some days of incubation, could also increase production of N_2O activating the N_2O-reducing community in the soil. It was shown recently that through application of plant residues, hotspots of N_2O emission can occur, by enhanced water absorption from the plant residues which will lead to reduced O_2 concentrations in the surrounding (Kravchenko et al., 2017). Combined with mineralized N and fungal growth this could explain the N_2O peak caused by CC residues. To our knowledge this is the first time that such a behavior of N_2O emission/consumption was observed after applying crop residues to the soil. More studies that confirm these results need to be conducted in the future.

Abundance of Microorganisms in Relation to GHG Fluxes and Organic Amendment Application

Microbial dynamics following application of organic amendments clearly offers scope for modulating functional groups involved in consumption of GHGs. In this light, the CC residues materials showed the best results, by increasing the abundance of the denitrifiers (*nosZ)*, methanotrophs (*pmoA),* and nitrogen fixers (*nifH)* genes, while only moderately increasing the nitrifiers (AOB) and methanogens (*mcrA)*. This could be either through the introduction of microbes already present in the organic amendments or stimulation of growth from indigenous microorganisms harboring these genes. Here, the effect is highly related to the amount of organic amendment applied to the soil. Small amounts of organic amendments have only a minor effect on the different microbial groups, which is also in accordance with the distinct lower GHG flux measurements from these incubations. On the opposite site, organic amendments cannot only increase the gene copy numbers, but can also lead to a decrease of microbial groups (AOA) in comparison to an un-amended soil.

The overall bacteria and fungi abundance correlate quite well with the CO_2 respiration rates ($R^2 = 0.942/R^2 = 0.858$, respectively). The strong increase, especially in the CC residue application in bacterial and fungal abundance, could mainly occur due to the high application rate of the CC residue in our experiment. Normally, around 4- to 6-fold lower amounts of CC residues are plowed under in the field after the winter (Marinari et al., 2015; Coombs et al., 2017). However, we observe also an increase in the fungal abundance at the low amount of applied CC residues, which is comparable to recent studies (Maul et al., 2014).

The differences in abundance of the different groups are highly influenced by the different organic amendments that are used. For example, the application with the fungi based compost has a great effect (7-fold increase) on the abundance of the methanotrophs, compared to the green cut compost

material which (like the other organic amendments) had only a doubling effect on the abundance of methanotrophs. Like mentioned before, a stimulation of rare-earth metal-dependent methanotrophs, which harbor the *XOXF* dependent methanol dehydrogenase gene, in these samples could be a possible explanation (Gu and Semrau, 2017; Krause et al., 2017). However, in a previous study (Ho et al., 2015) USCα *pmoA* sequences, which are known to poses the XOXF enzyme and is capable of atmospheric CH_4 oxidation, was not detected in soil samples from the same location. This would rather support the hypotheses that the increase in *pmoA* copies is due to the introduction of methanotrophs by the organic amendment.

In contrast to the methanotrophic community, we observe more distinct differences of the effect of organic amendments on the methanogenic abundance. Especially organic amendments (compost and digestate) that undergo a treatment in which anoxic habitats are formed to provide a perfect environment for methanogens (Hellmann et al., 1997; Alburquerque et al., 2012). Especially, CC residue amendment increased the ratio of methanotrophs to methanogens, which can harbor a positive effect on the ratio of CH_4 consumption to CH_4 production (Conrad, 2007).

In our soil the newly found *nosZ* clade II (Jones et al., 2013) is 10- to 100-fold more abundant than *nosZ* clade I. While clade I is mainly associated with soil type (clay), nutrient status, total organic carbon, organic matter or C:N ratio, it is unclear what the drivers for the abundance of clade II in soils are (Highton et al., 2016; Hallin et al., 2017). Our soil is a clay soil, which would be expected to show a higher correlation to *nosZ* clade I bacteria, but instead we see a clear preference of N_2O-reducers with a *nosZ* clade II gene. We think that the differentiation between the two clades cannot be broken down to just one or two single soil characteristics. More knowledge about the ecology of *nosZ* clade II bacteria, which seem to be the major drivers for soil N_2O sink capacity (Jones et al., 2014; Domeignoz-Horta et al., 2016a), is necessary. This knowledge may be used to design strategies to enrich agricultural soils either directly with *nosZ* clade II microorganisms or using amendments that are rich in these denitrifiers. In our study almost all organic amendments had a stimulating effect on the two *nosZ* clades. The rise in N_2O production may have stimulated the N_2O-reducers during the incubation (Hallin et al., 2017).

The archaeal 16s rRNA gene and archaeal *amoA* are the only two genes that are decreasing during the incubation. For archaea and especially the AOA inside the archaea kingdom it was already shown that they are more affected by rewetting stress compared to bacteria and AOB (Conrad et al., 2014; Thion and Prosser, 2014). The decrease in the archaeal *amoA* seem to be higher with the addition of either CC residues, digestate or sewage sludge to the soil (**Figure 7**). Potentially, the high N-content in these organic amendments, along with the high water level is known to favor denitrification processes (Skiba et al., 2002). Furthermore, it is believed that the addition of fertilizer normally lead to an increase in the AOB/AOA ratio (Wertz et al., 2012; Hartmann et al., 2013; Kastl et al., 2015), since it was shown that AOB grow faster after the addition of fertilizer, this may also true for our study. Even though a recent study showed that this effect is not occurring in every occasion by showing that AOA and AOB had changed in the same way during an incubation (Orellana et al., 2018).

It is not surprising that the treatments with CC residues harbored the highest abundance of N-fixing bacteria, since 1/3 of the CC residues mixtures we added were legumes (Sprent et al., 2017). N-fixers cannot directly be linked to a GHG production or consumption, but can have an indirect effect on N_2O production by converting N_2 to NH_4 which then can be consumed by nitrifiers in the soil (Galloway et al., 1995).

CONCLUSION

In our study we analyzed different organic amendments and their influence on the GWP as well as functional microbial groups which are involved in GHG transformations in an agricultural soil. Our results indicate that compost amendments perform best with respect to the soil GWP calculated from the three major GHGs (CH_4, CO_2, N_2O) and have a similar GWP as the un-amended soil (**Table 2**). Combinations of sewage sludge and digestate with both composts have also moderate effects on the soil GWP and will provide higher nutrients supply for plants. Although CC residues had the least favorable GWP, it still harbors a great long-term benefit to reduce GHG emissions from agricultural soils in manipulating the microbial communities. The CC residue amendment increased microbial groups that are involved in the reduction of GHGs (N_2O-reducers, methanotrophs) or keeping the producing microbial community stable (methanogens, nitrifiers) compared to other organic amendments and the un-amended soil. This could provide a better GWP in the long-term. The next step would be to study the effect of plants on the GWP and have a deeper investigation of the associated microbial communities that are involved in GHG consumption and perform a longer running long-term incubation experiment to verify the short-term results. Further well-aerated agricultural soils need to be investigated in their potential as a sink for CH_4, especially in combination with organic fertilizers and the potential of rare earth metals in these organic amendments. Understanding the underlying mechanisms of how organic fertilizers influence and possibly decrease GHG would allow us to develop a strategy to reduce GHG emission from agricultural soils without affecting the plant yield.

AUTHOR CONTRIBUTIONS

KB designed the study, performed the laboratory experiment, performed all lab work (flux measurements, nucleic-acid extractions, qPCR analysis, analytical analyses), performed statistical analysis, evaluated the data, and wrote the manuscript. SD helped with the set-up of the laboratory experiment, evaluated the data, and wrote the manuscript. GK helped with collecting the organic amendments and wrote the manuscript. PB designed the study, evaluated the data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.03035/full#supplementary-material

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Catch Crop Residues Stimulate N_2O Emissions During Spring, Without Affecting the Genetic Potential for Nitrite and N_2O Reduction

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Agricultural soils are a significant source of anthropogenic nitrous oxide (N_2O) emissions, because of fertilizer application and decomposition of crop residues. We studied interactions between nitrogen (N) amendments and soil conditions in a 2-year field experiment with or without catch crop incorporation before seeding of spring barley, and with or without application of N in the form of digested liquid manure or mineral N fertilizer. Weather conditions, soil inorganic N dynamics, and N_2O emissions were monitored during spring, and soil samples were analyzed for abundances of nitrite reduction (*nirK* and *nirS*) and N_2O reduction genes (*nosZ* clade I and II), and structure of nitrite- and N_2O-reducing communities. Fertilization significantly enhanced soil mineral N accumulation compared to treatments with catch crop residues as the only N source. Nitrous oxide emissions, in contrast, were stimulated in rotations with catch crop residue incorporation, probably as a result of concurrent net N mineralization, and O_2 depletion associated with residue degradation in organic hotspots. Emissions of N_2O from digested manure were low in both years, while emissions from mineral N fertilizer were nearly absent in the first year, but comparable to emissions from catch crop residues in the second year with higher precipitation and delayed plant N uptake. Higher gene abundances, as well as shifts in community structure, were also observed in the second year, which were significantly correlated to NO_3^- availability. Both the size and structure of the nitrite- and N_2O-reducing communities correlated to the difference in N_2O emissions between years, while there were no consistent effects of management as represented by catch crops or fertilization. It is concluded that N_2O emissions were constrained by environmental, rather than the genetic potential for nitrite and N_2O reduction.

Keywords: catch crop, fertilization, nitrous oxide emissions, denitrifier genes, N_2O-reduction genes

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INTRODUCTION

Global anthropogenic emissions of nitrous oxide (N_2O), a potent greenhouse gas and ozone-depleting substance, constitute 6.7 Tg nitrogen (N) annually according to Ravishankara et al. (2009). Agriculture is the single largest source of these emissions, contributing an estimated 5.8 Tg N and 4.2–7.0 Tg N using bottom-up and top-down approaches, respectively

(Del Grosso et al., 2008). These N_2O emissions are, directly or indirectly, related to the application of mineral fertilizers and manure for crop production, and decomposition of crop residues (Davidson, 2009). Soil N losses outside the main growing season reduce the overall N use efficiency of a cropping system, and winter cover crops (catch crops) are increasingly used as a measure against N leaching (Aronsson et al., 2016). When catch crop residues decompose following spring incorporation, N_2O emissions can be triggered (Flessa et al., 2002). Some studies indicate that the main source of N_2O is heterotrophic denitrification (Chen et al., 2013; Li et al., 2016; Parkin et al., 2016), although at low oxygen tensions the contribution from nitrifier-denitrification may also be significant (Poth and Focht, 1985; Zhu et al., 2013). Understanding the mechanisms, by which catch crop residues enhance N_2O emissions, may help to develop new management practices in order to prevent indirect N_2O emissions from N leaching during winter from being replaced by direct emissions during spring.

Denitrification is mediated through a sequence of enzyme-catalyzed reactions, in which nitrate (NO_3^-) is reduced via nitrite (NO_2^-) and nitric oxide (NO) to N_2O or N_2 under anoxic conditions by a diverse group of microorganisms. The denitrification pathway is modular, meaning that all steps in the pathway are not necessarily performed by the same organisms (Graf et al., 2014). Therefore, the abundances of *nir* genes, encoding enzymes that convert nitrite (NO_2^-) to nitric oxide (NO), and *nos* genes, encoding enzymes responsible for N_2O reduction to N_2, inform about the balance between genetic potential for N_2O production and consumption within a denitrifying community. A causal relationship between denitrification gene abundance and N_2O emissions has been shown in experiments, where the relative abundance of organisms with or without *nosZ* genes was manipulated (Philippot et al., 2011; Domeignoz-Horta et al., 2016). Under field conditions, denitrification gene abundances and N_2O emissions are sometimes, but not always, correlated (Hallin et al., 2009; Morales et al., 2010; Wang et al., 2017). Gene pools may not always reflect rates of N_2O emissions due to subsequent controls over gene transcription and enzyme activities (Philippot and Hallin, 2005; Wallenstein et al., 2006; Röling, 2007). Thus, gene abundances may reflect the genetic potential within the cropping system, rather than short-term response to dynamic environmental conditions.

Emissions of N_2O associated with incorporation of (catch) crop residues can vary due to differences in plant C:N ratio or decomposability. Li et al. (2016) reported that leguminous catch crop residues resulted in net N mineralization and significant N_2O emissions even at 40% water-filled pore space (WFPS), while ryegrass caused net N immobilization and much lower N_2O emissions. While residue N availability is important for denitrifier activity and N_2O emissions, especially if soil NO_3^- availability is low, residue C decomposability is also critical by constituting a sink for oxygen (O_2). Thus, residue decomposition may interact with soil water content in determining soil O_2 status around organic hotspots. For example, Li et al. (2013) found that crop residues consistently increased N_2O emissions at 30 and 60% WFPS, while at 90% WFPS the emissions were reduced by residue amendment, presumably because there was a shift in the N_2O:N_2 product ratio of denitrification due to more reducing conditions. Finally, when catch crop residue incorporation in spring is followed by N fertilization, there is a potential for interactions between the external N source and the decomposing residues, which may enhance denitrification (Frimpong and Baggs, 2010) and N_2O emissions (Duan et al., 2017).

Our aim was to better understand the complex interactions between soil conditions, crop residues and N amendments during spring, and the response of nitrite- and N_2O-reducing communities, since this understanding is a precondition for effective strategies to mitigate N_2O emissions. For this purpose, we performed a 2-year field study in which N_2O emissions were monitored during spring in a factorial experiment that involved rotations with or without catch crops, and with or without application of N as digested liquid manure or mineral fertilizer. By the end of each monitoring period in June, the soil was sampled to analyze the abundance of nitrite and N_2O reduction genes, and the structure of the communities harboring these genes. We hypothesized (1) that N-rich fertilizer and catch crop residues would interact positively on N_2O emissions; (2) that N_2O emissions derived from mineral N would depend more on soil O_2 status, and hence rainfall, than emissions derived from catch crop residues; and (3) that the abundance and composition of denitrifying communities would reflect the long-term effects of cropping system on metabolizable C and N availability.

MATERIALS AND METHODS

Long-Term Crop Rotation Field Experiment

The study made use of a long-term crop rotation experiment, established in 1996, that is located at 56°30′N, 9°34′E in Western Denmark (Olesen et al., 2000). The sandy loam is classified as a Typic Hapludult and has 77.9% sand, 13.3% silt and 8.8% clay in the plow layer (0–25 cm soil depth). This depth interval further contains 23 g kg^{-1} soil organic carbon (SOC) and 1.8 g kg^{-1} total N, and it has a pH_{CaCl2} of 6.5, a cation exchange capacity of 12.3 meq 100 g^{-1}, and an average bulk density of 1.35 g cm^{-3}. Mean annual rainfall is 704 mm and mean annual air temperature 7.3°C.

Five different cropping systems were compared, representing systems with or without catch crops, and with or without N fertilization (**Table 1**). All systems had rotations with spring barley (*Hordeum vulgare)*, hemp (*Cannabis sativa*), pea (*Pisum sativum*)/barley, spring wheat (*Triticum aestivum)* and potato (*Solanum tuberosum*). All crops were represented each year in two fully randomized blocks. Where a catch crop was present before spring barley (+*CC*), this was a mixture of rye (*Secale cereale*), hairy vetch (*Vicia villosa*) and rapeseed (*Brassica napus*). Four of the five rotations were under organic management (*O4*), and the last rotation under conventional management (*C4*), where the identifiers *O4* and *C4* are used in accordance with previous studies from this long-term crop ration experiment (e.g., Chirinda et al., 2010; Brozyna et al., 2013).

Field plots within each rotation were selected in which the main crop in the previous year was potato, and the main

TABLE 1 | Cumulative N_2O emissions during spring, N_2O emission factors (EFs), and yield-scaled EFs of spring barley in five crop rotations. For calculation of EFs, the N_2O emissions were corrected for background emissions in treatment *O4-CC-N* with no external N input. Significant differences between rotations within a year are indicated by lower-case letters, and differences between years within a rotation by capital letters.

System[#]	Catch crop	N fertilizer[§]	Nitrous oxide flux, kg N ha^{-1}				Emission factor		Yield-scaled EF, kg N_2O-N kg^{-1} N in plant uptake	
			2011		2012		2011	2012	2011	2012
Conventional	*−CC*	*+N*	0.27	bA	0.96	cB	0.000	0.007	0.000	0.005
Organic	*+CC*	*+N*	0.79	cA	1.39	dB	0.004	0.007	0.006	0.015
Organic	*+CC*	*−N*	0.8	cA	0.91	cA	0.017	0.023	0.006	0.011
Organic	*−CC*	*+N*	0.2	aA	0.28	bB	−0.001	0.001	−0.001	0.004
Organic	*–CC*	*–N*	0.25	abA	0.18	aA	NA	NA	NA	NA

NA, Not applicable.

[#]Crop sequence (all rotations): spring barley (Hordeum vulgare); hemp (Cannabis sativa); pea (Pisum sativum)/barley; spring wheat (Triticum aestivum); potato (Solanum tuberosum). In +CC rotations, a catch crop consisting of a rye (Secale cereale), hairy vetch (Vicia villosa) and rapeseed (Brassica napus) mixture was established after all crops in the rotation except hemp.

[§]In the conventional system, the N source was NPK fertilizer, while in the organic rotations the N source was anaerobically digested liquid manure (see text for details).

crop in the experimental years (2011 and 2012) was spring barley. A rotation with neither catch crop nor N fertilization was not represented in the basic design, and instead manure application was excluded from a 1.5 m strip of *O4-CC+N* plots, which represented the treatment *O4-CC-N*. In *O4* rotations, the N fertilizer was anaerobically digested liquid manure, which contained 3.6% dry matter (DM), 6.5 kg Mg^{-1} total N and 3.9 kg Mg^{-1} total ammonia-N (TAN) in 2011, and 2.6% DM, 8.2 kg Mg^{-1} total N and 5.4 kg Mg^{-1} TAN in 2012. The two organic rotations with manure application received 99.4 kg ha^{-1} TAN in 2011, and 132 kg ha^{-1} TAN in 2012. The conventional rotation received 120 kg ha^{-1} N in NPK 23-3-6 (%, by weight) fertilizer, with similar amounts of ammonium (NH_4^+) and NO_3^- in both years.

Field Operations

The amount of N returned to the soil through incorporation of above-ground catch crop biomass was estimated by cuts to 1 cm height in mid-November of 2010 and 2011, respectively. Total DM and N percentage of cuts were determined. In 2011, rotovation and plowing (with incorporation of catch crops where present) took place on 6 April, N fertilization on 12 April, and seeding on 19 April. In 2012, the rotovation and plowing took place on 4 April, N fertilization on 10 April, and seeding on 11 April. There were no further field operations during the N_2O monitoring period. In early August of both years, the above-ground biomass (including spring barley and weeds) was cut to determine DM production and N uptake in harvested biomass.

Nitrous Oxide Measurements

The dimensions of field plots were 12 × 15 m, with a 6 × 15 m harvest plot in the middle, and to each side sampling plots with dedicated 1 × 1 m microplots for experimental purposes. For the present study, two available microplots per field plot were randomly selected for monitoring of N_2O emissions. Two-part static chambers were used with permanently installed stainless steel collars covering a 0.75 × 0.75 m area. The chambers (height 20 cm) of 4 mm white expanded PVC were vented and further equipped with a battery-powered fan for mixing of the chamber headspace during deployment. When chambers were deployed for flux measurements, gas samples (10 mL) were collected through a septum using a polypropylene syringe and hypodermic needle, and stored in evacuated 6 mL exetainer vials (Labco, Ceredigion, UK) for later analysis. Five gas samples were taken over the course of *c.* 2 h starting around 9:30, the first sample at the time of deployment.

In 2011, the N_2O monitoring started immediately after tillage, and two N_2O-flux measurement campaigns were conducted in the week between tillage and fertilization; then collars were temporarily removed for manure application and incorporation, and seeding. Since 2011 showed no significant N_2O emissions prior to fertilization, the first N_2O flux measurement campaign in 2012 took place on the day of seeding. Three N_2O flux measurement campaigns were then carried out during the first week, followed by weekly campaigns until mid-June.

Nitrous oxide concentrations in the gas samples were determined using an Agilent 7890 GC system with a CTC CombiPal autosampler (Agilent, Nærum, Denmark). The gas chromatograph had a 2-m back-flushed pre-column with Hayesep P, and a 2-m main column with Porapak Q connected to an electron capture detector. The carrier gas was N_2 at a flow rate of 45 mL min^{-1}, and Ar-CH_4 (95/5%) at a flow rate of 40 mL min^{-1} was used as make-up gas. Temperatures of injection port, column and detector were 80, 80, and 325°C, respectively.

Soil Sampling

From the time of N fertilization, and then weekly until the end of N_2O monitoring, soil samples were collected adjacent to micro-plots used for N_2O flux measurements. Ten subsamples (20 mm diameter, 0–20 cm depth) were taken from each field plot and pooled. Subsamples (10 g) were extracted in 1 M KCl and filtered extracts frozen at −20°C until analyzed for NH_4^+ and NO_3^- concentrations by standard colorimetric methods (Keeney and Nelson, 1982). Gravimetric soil water content was determined by drying 10 g of soil for 24 h at 105°C. For each sampling day, soil WFPS and relative gas diffusivity were calculated using treatment

specific measurements of dry bulk density (Chirinda et al., 2010). Relative gas diffusivity was calculated as (Moldrup et al., 2005):

$$\frac{D_p}{D_0} = \Phi^2 \left(\frac{\varepsilon}{\Phi}\right)^{2+[\log(\varepsilon_{100}{}^{0.25})/\log(\varepsilon_{100}/\Phi)]},$$

where D_p and D_0 are gas diffusivity in soil and air, respectively ($m^2\ s^{-1}$), Φ is total porosity ($m^3\ m^{-3}$ soil), ε is volumetric air content ($m^3\ m^{-3}$ soil), and ε_{100} is volumetric air content at -100 cm H_2O.

After the final N_2O emission measurement campaign in June of each year, two 250 cm^3 soil samples were collected from 0 to 10 cm depth for molecular analyses within each of the permanently installed collars used for N_2O monitoring. These samples were sieved and mixed separately, and subsamples frozen at $-20°C$ until DNA isolation.

DNA Isolation

Microbial genomic DNA was isolated from soil samples using Genomic Spin Kit (A&A Biotechnology, Gdynia, Poland) following a modified protocol. A 500-mg soil sample was added to a tube containing small glass beads, followed by 1 mL extraction buffer (A&A Biotechnology). Cells in the soil were lysed using a FastPrep instrument (MP Biomedicals, Solon, OH, USA) for 30 s at a speed of 5.5, followed by centrifugation at 14,000 × *g* for 1 min, and then the supernatant was transferred to a sterile 1.5-mL Eppendorf tube. Ammonium acetate (5 M) was added to the tube to a final concentration of 2 M, and the tube was incubated on ice for 5 min after vortexing. Then, the tube was centrifuged at 16,000 × *g* for 10 min at 4°C, and the supernatant was transferred to a 9-mL plastic tube. Two mL guanidine HCl (7 M) was added to the tube and mixed by vortexing, and then 900 μL of the mixture was transferred to a spin column and centrifuged at 14,000 × *g* for 15 s. After centrifugation, the catch tube was emptied, and the process was repeated with another 900 μL liquid until the entire sample had run through the spin column. Finally, the spin column was washed, and the DNA was eluted according to the manufacturer's instructions.

The extracts were analyzed by 1% (w/v) agarose gel electrophoresis, and the bands containing genomic DNA were cut out for DNA recovery using SpinPrep Gel DNA Kit (Millipore, Hellerup, Denmark). The quantities of extracted DNA were determined using Qubit dsDNA BR assays (Invitrogen, Carlsbad, CA, USA). After quantification, the DNA were diluted to 10 ng μL^{-1} and kept at $-20°C$ until used for downstream analysis.

Quantification of *nirK, nirS*, and *nosZ* Genes

Quantitative real-time PCR (qPCR) was performed using a Bio-Rad CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Prior to gene quantification, the presence of potential PCR inhibitors in each soil DNA extract was tested by quantifying a known amount of the pGEM-T plasmid (Promega, USA) using plasmid specific T7 and SP6 primers in the presence of extracted DNA or water. The 15 μL mixture for inhibition test contained 1 × DyNAmo Flash SYBR Green qPCR Master Mix (Thermo Scientific, Waltham, MA, USA), 1 μg bovine serum albumin (BSA; New England BioLabs, MA, USA), 0.25 μM of each primer, 1×10^5 copies of the plasmid, and 2 μL of either soil DNA (20 ng) or water. No inhibition was observed with the amount of DNA used.

Standards ranging from 1×10^2 to 10^8 gene copies μL^{-1} were prepared from linearized pGEM plasmids with insertions of fragments of the target genes (*nirK*, *nirS*, *nosZ*-I, or *nosZ*-II). The genes *nirK* and *nirS* were amplified with primers F1aCu/R3Cu (Hallin and Lindgren, 1999) and Cd3aF/R3cd (Throbäck et al., 2004), respectively; and *nosZ* clades I and II were amplified using primers 1840F/2090R (Henry et al., 2006) and *nosZ*-II-F/*nosZ*-II-R, respectively (Jones et al., 2013). The 15 μL qPCR mixture consisted of 1 × DyNAmo Flash SYBR Green qPCR Master Mix, 1 μg bovine serum albumin, 0.25 μM (for *nirK*) or 0.8 μM (for *nirS* and *nosZ*) of each primer, and 2 μL (20 ng) of template. Primers and thermal cycling conditions are detailed in **Table S1** in Supplementary Material. Each gene was amplified twice on two separate plates. Dissociation curve analysis and agarose gel electrophoresis of amplicons were performed at the end of each run to confirm the specificity of amplification. Amplification efficiencies were 90, 94, 98, and 85% for *nirK*, *nirS*, *nosZ*-I, and *nosZ*-II, respectively. Results were processed using Bio-Rad CFX Manager software version 3.1 with default settings.

Terminal Restriction Fragment Length (T-RFLP) Analysis

PCR for T-RFLP analysis was performed on a Bio-Rad C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The same primers as those for qPCR were used for amplification, with the modification that the 5′ ends of the forward primers were labeled with the fluorescent dye hexachlorofluorescein (HEX). The 40-μL PCR mixture contained 20 μL DreamTaq Green PCR Master Mix (Thermo Scientific), 4 μg bovine serum albumin, 0.25 μM (for *nirK*) or 0.8 μM (for *nirS* and *nosZ*) of each primer, and 20 ng of soil DNA. The thermal cycling conditions were identical to those used for qPCR, with the modification of exclusion of the data acquisition step and the melting curve analyses. Amplicons were analyzed by agarose gel electrophoresis to confirm successful amplification and correctness of fragment sizes. Amplicons of each gene were digested by two different restriction endonucleases separately to produce terminal restriction fragments (T-RFs): *nirK* amplicons were treated by HaeIII and HpyCH4IV, *nirS* by HaeIII and HhaI, *nosZ*-I by BstUI and Sau96I, and *nosZ*-II by HpyCH4IV and NlaIII (all restriction enzymes were from New England BioLabs, Ipswich, MA, USA). Enzyme digestions were performed according to manufacturer's instructions. T-RFLP profiling was performed using a 3,730xl DNA Analyzer (Applied Biosystems, Waltham, MA, USA) at Uppsala Genome Center, Uppsala University, Sweden, and data on peak positions and sizes were extracted using the Peak Scanner software (Applied Biosystems).

T-RFs from different soil samples were aligned using an in-house R package (see **Supplementary Material** for R source code and analysis parameters), which uses tables of peak size, area, and height exported from Peak Scanner as input and aligns

profiles in a series of steps. First, each profile was processed using a Gaussian smoothing function, which eliminated double or shoulder peaks by a peak merging algorithm. The peaks were then relativized by dividing peak areas and heights by the sum of each within the same profile. Next, peaks across all samples were differentiated into "noise" and "signal" peaks using the iterative approach described by Abdo et al. (2006), where noise peaks were defined as having relative areas or heights <3 standard deviations from a theoretical baseline of 0 relative fluorescence units (RFUs) across all samples. After removal of noise peaks, signal peaks were aligned across all profiles using the iterative dynamic programming algorithm described by Vähämaa et al. (2007). Briefly, two de-noised T-RFLP profiles were selected at random and aligned in a pair-wise manner using dynamic programming, where dissimilarities between peaks in each profile account for differences in peak size as well as area and height. New profiles were then added to the alignment in random order using a modified version of the dynamic programming algorithm, where the set of aligned T-RFLP profiles were converted into single profiles of average peak size, area, and heights. Once all samples were aligned, an overall alignment score was calculated based on the sum of peak dissimilarities. Then followed an iterative process, where a sample is chosen at random and removed from the alignment, then realigned to the remaining samples. Once this was done for all samples, the overall alignment score was recalculated and, if the score was improved from the previous alignment, another iteration was carried out using the improved alignment. Following the best possible alignment the algorithm was terminated, producing a table of aligned peak sizes, areas, and heights across all samples. Following automated alignment, plots of electropherograms and false gel images can be generated to allow for visual inspection and, if necessary, manual correction of fragment binning prior to downstream analysis. After peak alignment, the T-RFLP profiles of each gene derived from the two different enzyme digestions were combined prior to statistical analysis.

Statistical Analyses

Nitrous oxide fluxes were estimated using HMR (Pedersen et al., 2010), which is available as an add-on package in R (R Core Team, 2015). HMR calculates trace gas flux based on linear or non-linear concentration-time data series as required; linear or non-linear regression was selected manually based on scatter plots of concentration change over time.

The cumulative N_2O emissions for each of the combinations of year, crop rotation, catch crop and N input used in the experiment were estimated by integrating the N_2O emissions over the period of observation. To do so, a gamma linear mixed model was adjusted to the N_2O emissions observed on sampling days in each of the 20 sampling positions represented each year. The model contained a fixed effect representing the combination of year, crop rotation, catch crop, fertilization method and sampling date, and a random component designed to account for the correlations generated by repeated measurements. The integrals over time, representing the cumulative N_2O emissions in each field plot, were approximated by contrasts (i.e., linear combinations of the model parameters) with coefficients coinciding with the weights of the trapezoidal approximation of the respective integrals, as described in Duan et al. (2017) (**Supplementary Material)**. The analyses were performed with the software R (R Core Team, 2015) using the packages *lme4* for adjusting generalized linear mixed models, and *pairwiseComparisons* (http://home.math.au.dk/astatlab/software/pairwisecomparisons) for making inferences on the contrasts and *post-hoc* analyses. The *p*-values implicitly used in the *post-hoc* analyses were adjusted for multiple comparisons using the false discovery rate (FDR) (Benjamini and Yekutieli, 2001).

Effects of rotations, catch crop, fertilization, and year on gene copy numbers were evaluated by multivariate analysis of variance using the *manova* function in R. Pairwise differences at $\alpha = 0.05$ were identified by package *lsmeans* with Tukey's multiple comparison test. Bray-Curtis dissimilarities in the T-RFLP profiles were visualized by ordination analysis (non-metric multidimensional scaling, NMDS) using the *vegan* package. The abundances of T-RFs were presented as relative peak areas, and then transformed using Wisconsin double standardization before being supplied to the *metaMDS* function. The ordination was performed using a random start for 100 runs, with 100 iterations in each run. The number of dimensions from one to six was tested, and three dimensions were selected for final analysis with the assistance of scree plots. Following ordination, a test was conducted to find whether there was a correlation between T-RFLP profiles and soil properties.

Soil properties, including NH_4^+ and NO_3^- concentrations, soil water content, D_p/D_0 values, and cumulative N_2O emissions, were averaged using the trapezoidal rule. A matrix containing these soil properties was fit to the ordination using the *envfit* function with 1,000 permutation tests. Based on the *p*-values of the results, gradients of soil properties that had a significant effect ($p < 0.05$) were shown in the ordination plots using the *ordisurf* function. Ordination and fitting of environmental vectors were performed with T-RFLP profiles of denitrifier genes (*nirK* and *nirS*), N_2O reduction genes (*nosZ*-I and *nosZ*-II), as well as with a combined profile of all four denitrification genes (*nirK*, *nirS*, *nosZ*-I, and *nosZ*-II).

RESULTS

Weather Conditions in 2011 and 2012

The weather in 2011 was generally warmer than in 2012 during the monitoring period, with average temperatures of 11.7°C in 2011 and 9.9°C in 2012 (**Figure 1**). In particular, there was a cold spell in early April of 2012, with air temperature declining to 1°C. The 2 experimental years also differed with respect to precipitation. The spring of 2011 was drier than that of 2012, with little precipitation before mid-May. In contrast, 2012 had several periods with significant rainfall between early April and mid-May. Average daily precipitation during the monitoring period was 1.4 mm in 2011, and 2.1 mm in 2012.

Soil WFPS varied between 35 and 48% in 2011, and between 29 and 58% in 2012 (**Figure S1**). Relative gas diffusivity varied between 0.050 and 0.084 in 2011, and between 0.030 and 0.116 in 2012 (**Figure S1**). Temporal dynamics reflected the distribution

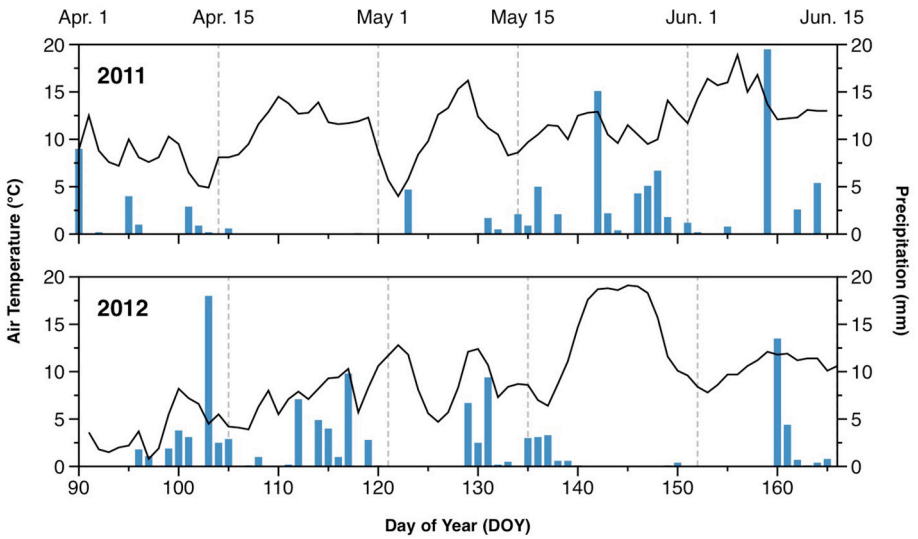

FIGURE 1 | Air temperature (lines) and daily rainfall (bars) during the monitored periods in 2011 and 2012.

of rainfall, with dry periods during April (DOY105–122) in 2011, and in late May and early June (DOY139–159) in 2012. During early spring (April and May), the WFPS was consistently higher, and D_p/D_0 lower, in treatments with catch crops (*O4+CC+N* and *O4+CC-N*) in 2012 compared to 2011, whereas WFPS and D_p/D_0 were similar in 2011 and 2012 in the two organic rotations without a catch crop (*O4-CC+N* and *O4-CC-N*; **Figure S1**). Higher wetness in 2012 was also indicated for the conventional rotation, *C4-CC+N*.

Nitrogen Dynamics

The input of N in the form of mineral fertilizer in the conventional system or as digested manure in the organic systems (*+N*), as well as from catch crop residues (*+CC*), was reflected in soil concentrations of NH_4^+ and NO_3^- (**Figure 2**). The background levels of both NH_4^+ and NO_3^- in early spring were low, as seen in the treatment *O4-CC-N*, and in all treatments before N fertilization in 2011 (**Figure 2**). All treatments showed a similar pattern of mineral N dynamics after fertilization, with NH_4^+ disappearing within 2–4 weeks, and a transient accumulation of NO_3^-. When compared to 2011, the extent of soil NO_3^- accumulation in 2012 was higher in treatments *C4-CC+N* and *O4+CC+N*, and depletion of soil NO_3^- occurred later in all treatments.

The accumulation of mineral N was higher in treatments receiving mineral fertilizer (*C4-CC+N*) or manure (*O4-CC+N*, *O4+CC+N*) compared to those with crop residues only (*O4+CC-N*). This does not directly reflect the differences in N availability, since the retention time in soil before plant N uptake would have been shorter with a more gradual release of N from catch crop residues. In accordance with this, the N uptake with catch crop residues only (*O4+CC-N*) was greater than the uptake with digested manure only (*O4-CC+N*) in both years (**Table 2**); there was little added effect of combining catch crop residues with digested manure (*O4+CC+N*). The conventional system with NPK fertilizer (*C4-CC+N*) had higher plant N uptake than all four organic rotations.

N_2O Emissions

The N_2O emissions during spring showed several notable trends (**Figure 3**). Firstly, emissions of N_2O were higher in both years in rotations with a catch crop (*O4+CC+N* and *O4+CC-N*). In contrast, organic rotations without catch crop incorporation in spring (*O4-CC+N* and *O4-CC-N*) had low N_2O footprints in both years, irrespective of fertilization with digested manure. The conventional rotation without catch crop (*C4-CC+N*) showed different patterns in the 2 years, with little or no N_2O emission in 2011, but substantial emissions in 2012. In both years, the N_2O emissions in all treatments had returned to the background level by the time of the last sampling. The temporary decline in N_2O emission rates around DOY125 in 2011, and DOY130 in 2012, coincided with transient cold spells (**Figure 3**).

Cumulative N_2O emissions were significantly higher in 2012 than in 2011 for treatments with a catch crop (*O4+CC+N* and *O4+CC-N)*, and for the conventional rotation (*C4-CC+N*) (**Table 1**). In 2011, the cumulative emissions of N_2O from rotations with catch crop residue incorporation were significantly higher than from rotations receiving N fertilizer only. In 2012, the treatment receiving N in both catch crop residues and digested manure had the highest N_2O emissions, but the emissions from treatment *C4-CC+N* were also significant and similar to those from treatment *O4+CC-N*. In 2012, the N_2O emissions from the organic rotation receiving digested manure only (*O4-CC+N*) were again low and only marginally higher than from the unamended reference (**Figure 3**).

Area-based N_2O emission factors (EFs) were calculated with reference to N input in catch crop residues and N fertilization; emissions were corrected for background emissions, assumed to

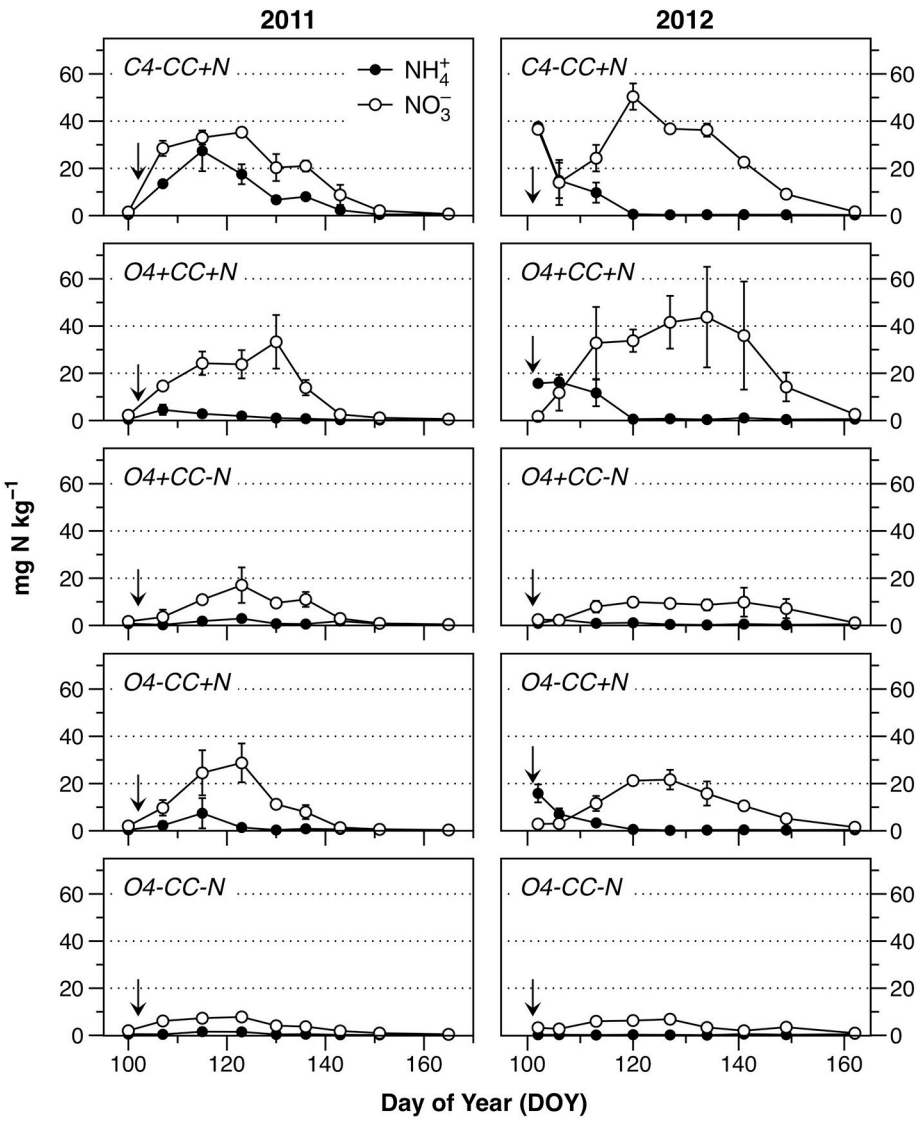

FIGURE 2 | Content of NH_4^+ (filled circles) and NO_3^- (open circles) in the soil during the monitored periods in 2011 and 2012. Fertilization took place on April 12 (DOY102) in 2011 and April 10 (DOY101) in 2012 (indicated by the arrows). The data represent means of four observations in two replicate plots, while error bars represent standard error ($n = 2$).

be represented by treatment *O4-CC-N*. For treatment *O4+CC-N* with catch crop residues as only N input, the area-based N_2O EF was high in both years (1.7–2.3%) compared to the rotation with both catch crop residue incorporation and digested manure (*O4+CC+N*) at 0.4–0.7%. The EF for treatment *O4-CC+N* receiving digested manure was consistently low. In contrast, the N_2O EFs for treatment *C4-CC+N* receiving mineral fertilizer differed in the 2 years, with no increase in N_2O emissions in 2011 and 0.7% in 2012. Yield-scaled EFs were calculated with reference to the N content in plant biomass harvested in each of the experimental treatments in August 2011 and August 2012, respectively (**Table 2**). Yield-scaled EFs were higher in 2012 compared to 2011 (**Table 1**).

Abundances of Denitrifier Genes

The abundances of *nirK* genes were 1.38–1.56 $\times$ 10^8 and 1.79–2.54 $\times$ 10^8 copies g^{-1} dry soil in 2011 and 2012, respectively, and around three times higher than the copy numbers of *nirS* genes (**Figure 4**). The *nosZ*-I genes were significantly more abundant than *nosZ*-II genes, with copy numbers ranging from 8.82–11.1 $\times$ 10^7 copies g^{-1} soil in 2011 to 1.18–1.95 $\times$ 10^8 copies g^{-1} soil in 2012, which was three to four times the abundance of *nosZ*-II genes. Within each treatment, the abundances of all four genes increased significantly from 2011 to 2012, except for *nosZ*-II genes in *O4+CC-N* and *O4-CC+N*. In contrast, there were no significant effects of the experimental variables (rotation, catch crop, N addition or interactions) with respect to gene abundances within each year, as determined by multivariate analyses of

TABLE 2 | Nitrogen input (kg N ha^{-1}) in catch crop residues and fertilizers, and N content in above-ground plant biomass 2 weeks prior to harvest in late August.

	Rotation	Catch crop N[#]	Fertilizer N[#]	Plant N uptake
2011	*C4−CC+N*	–	120	138.6a
	O4−CC+N	–	100	78.1b
	O4+CC−N	32.3a	0	88.8b
	O4+CC+N	40.7a	100	92.0b
2012	*C4−CC+N*	–	120	148.0a
	O4−CC+N	–	132	75.1b
	O4+CC−N	32.2a	0	85.8b
	O4+CC+N	38.0b	132	92.9b

Data represent means and standard error (n = 2); letters indicate significant differences in plant N uptake ($p < 0.05$). [#]The conventional treatment (C4-CC+N) received NPK mineral fertilizer, while the treatments in the organic system (O4+CC+N and O4-CC+N) received digested manure.

variance. The average ratios of *nir* to *nos* gene copy numbers (*nir*/*nos* ratios) for all treatments were approximately 1.56 in both years, and there were no significant difference ($p > 0.05$) across treatments and/or years.

Denitrifier Community Structure

The ordination of the combined T-RFLP profiles of *nirK*, *nirS*, and *nosZ* clade I and II genes show two distinct clusters, representing samples from 2011 and 2012, which reveals a shift in community structure between years (**Figure 5A**). These changes correlated strongly to NO_3^- concentrations ($p = 0.042$), as well as cumulative N_2O emissions ($p = 0.035$). Gradients of D_p/D_0 also partly described this inter-annual variation; however, the correlation was not significant ($p = 0.114$). Samples were more scattered in 2011 compared to 2012, suggesting less overall heterogeneity in 2012. Separate ordination analyses of T-RFLP profiles for nitrite reduction genes (*nirK* and *nirS*; **Figure 5B**) and N_2O reduction genes (*nosZ* clade I and II; **Figure 5C**) show that the changes in community structure between years were associated with denitrifiers carrying *nir* genes rather than *nos*-harboring N_2O-reducers. A significant shift along the gradient of NO_3^- concentrations was also observed for the *nir* communities (**Figure 5B**; $p = 0.032$). In contrast, no correlation between community structure and environmental variables was found for N_2O-reducing communities, and there was no effect of management on the structure of any of the communities in either year.

DISCUSSION

Oxygen Supply and Demand

WFPS is often used as a proxy for soil O_2 status (Chen et al., 2008), and approximately 60% WFPS has been considered to be an upper limit for well-aerated soil conditions (Linn and Doran, 1984). Some studies, however, suggest that relative gas diffusivity, D_p/D_0, is a better predictor of N_2O emissions from intact (Petersen et al., 2008) as well as repacked soil (Balaine et al., 2013), and in the present study both metrics of soil O_2 status were therefore calculated (**Figure S1**). Both WFPS and D_p/D_0 indicated that soil O_2 availability was lower during April and May of 2012 (WFPS ∼55%, D_p/D_0 ∼0.04) compared to 2011 (WFPS ∼45%, D_p/D_0 ∼0.06) in the conventional rotation, and in the two organic rotations with catch crops. In accordance with this, the N_2O emissions were also significantly higher in 2012 in treatments *C4-CC+N* and *O4+CC+N*, whereas the difference was not significant in *O4+CC-N* (**Table 1**). Generally, emissions of N_2O occurred at bulk soil conditions that should not support N_2O emissions, i.e., <60% WFPS (Linn and Doran, 1984; Balaine et al., 2013). In a related study, Chirinda et al. (2010) found evidence for soil compaction at 0–5 cm depth in *C4-CC+N*, which may have increased soil water-retention and restricted the O_2 supply in this treatment, but does not explain N_2O emissions in the two organic rotations where, instead, organic hotspots may have been the main source. Parkin (1987) demonstrated that nearly all denitrification activity in a soil core was associated with a single decaying leaf. The depletion of O_2 around residues was demonstrated by Højberg et al. (1994) using an O_2 microsensor, and by mapping of O_2 distribution with planar optodes (Kravchenko et al., 2017). Kravchenko et al. (2017) further showed that plant residues absorbed water in order to equilibrate with the soil water potential, thereby attaining 4–10 times more water by volume than the surrounding soil. Parkin (1987) calculated that a 160-μm water film would be sufficient to develop anaerobic conditions at surfaces of decomposing plant material, and hence water absorption may represent a barrier for O_2 supply allowing denitrification and N_2O emissions to occur even in well-aerated soil. In accordance with this, Li et al. (2016) found consistent N_2O emissions from leguminous catch crop residues incubated at 40, 50, and 60% WFPS, and denitrification was shown to be the main source of N_2O at all three soil water levels. This was also the case in a manipulation experiment with intact soil from organic crop rotations incubated at water potentials of −10, −30, and −100 hPa (Petersen et al., 2013a). Thus, in soil environments with organic hotspots, denitrification can occur over a wide range of soil moisture conditions—what matters is the balance between O_2 supply and O_2 demand, which could also account for much of the variation in N_2O emissions observed in the present study.

Nitrogen Availability

Degradable organic carbon and O_2 limitation are only two of the three requirements for denitrification, the third being the electron acceptors NO_3^- or NO_2^-. Background levels of NO_3^- in the soil were low (**Figure 2**), but increased instantaneously with NPK fertilization, and more gradually with digested manure and catch crop residues as N source. A phase of net N immobilization may occur when crop residues and liquid manure are applied to soil. For example, Trinsoutrot et al. (2000) found that rapeseed incorporation resulted in net N immobilization for *c.* 2 weeks, and Sung et al. (2010) reported little N immobilization from rye, but substantial net N mineralization from hairy vetch. This implies that N_2O emissions during the initial stage of decomposition will depend on soil NO_3^- availability for several days, as reported by Petersen et al. (1996) in a study of cattle manure hotspots. The supply of NO_3^- from the soil will rapidly decline as a result of decreasing concentration gradients, and

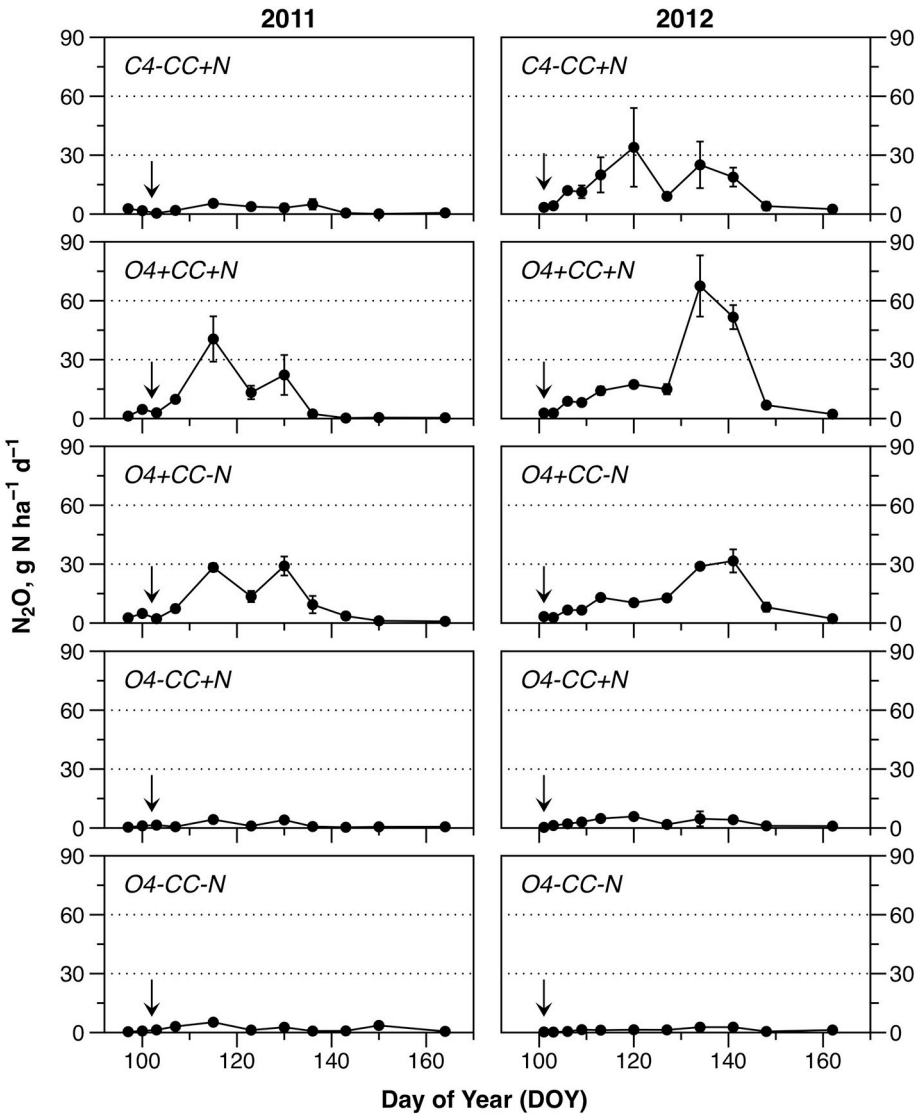

FIGURE 3 | Nitrous oxide emissions during the monitored periods in 2011 and 2012. Fertilization took place on April 12 (DOY102) in 2011 and April 10 (DOY101) in 2012 (indicated by arrows). The data represent means of four observations in two replicate plots, while error bars represent standard error ($n = 2$).

sustained N_2O emissions therefore depend on mineralization and nitrification of N input via manure or crop residues. The digested manure used in this study contained only 2.6–3.6% DM, and thus most of the liquid phase would have infiltrated the bulk soil, along with dissolved C and N. The intimate contact between soil and manure probably enhanced microbial N immobilization (Sørensen and Jensen, 1995), thereby reducing N availability for nitrification and denitrification, resulting in lower N_2O emissions. Kong et al. (2017) incubated ^{15}N-labeled residues of white clover in soil mesocosms and found that the enrichment of N_2O increased gradually during a 2-week period (though much less so when residues had been treated with a nitrification inhibitor to prevent nitrification of mineralized N). Generally, residue quality will determine the extent of net N mineralization from decomposing residues (Li et al., 2016).

Soil mineral N dynamics indicated that plant N uptake was delayed in 2012 compared to 2011. This was probably related to a difference in soil temperature, since by DOY140 (mid-May) the sum of plant-growing degree days, calculated according to Léon (1992), were 219 and 131 in 2011 and 2012, respectively (**Figure S2**). The longer residence time for mineral N in the soil probably increased the potential for N_2O emissions by increasing the average soil NO_3^- availability.

Positive effects of catch crops on yields are normally seen when access to mineral N in fertilizers or manure is suboptimal (Li et al., 2015; Marcillo and Miguez, 2017). However, the relationship between N input and plant N uptake in this study was weak and suggested that plant availability of the N supplied in digested manure and residues was relatively low. An earlier study from the same long-term crop rotation experiment found

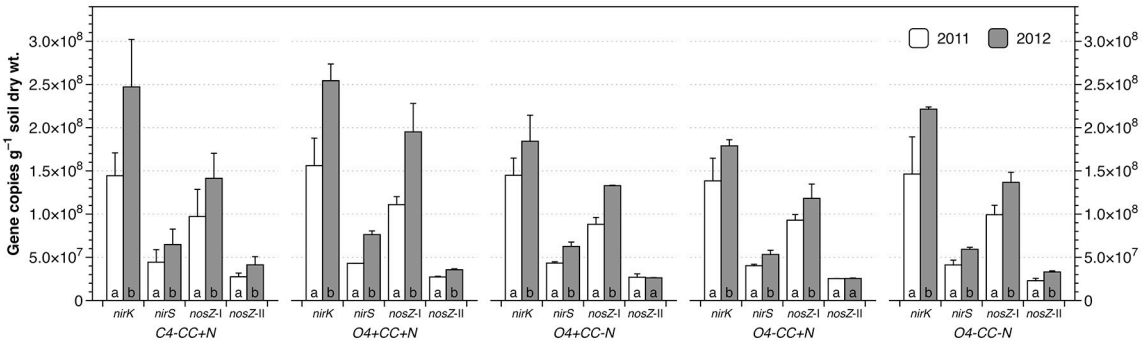

FIGURE 4 | Abundance of *nirK*, *nirS*, *nosZ*-I, and *nosZ*-II genes in different cropping systems in 2011 and 2012. The bars represent the average in each treatment, and error bars show standard error ($n = 2$). Within each treatment, genes marked with the same letters are not significantly different at $\alpha = 0.05$ between 2011 and 2012.

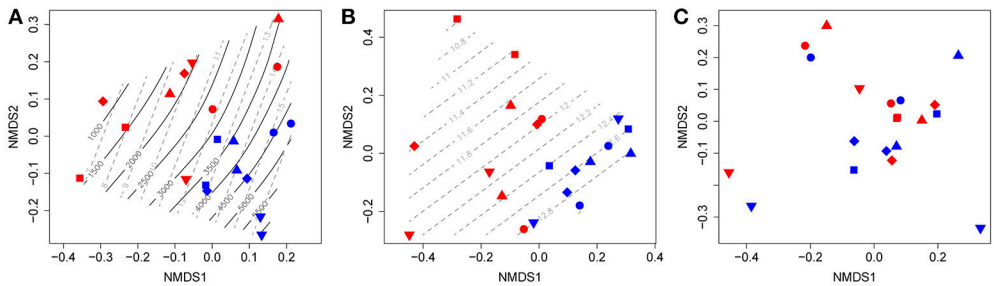

FIGURE 5 | Non-metric multi-dimensional scaling (NMDS) ordination of T-RFLP profiles for **(A)** collective denitrification genes (*nirK, nirS, nosZ*-I, and *nosZ*-II); **(B)** nitrite-reduction genes (*nirK* and *nirS*); and **(C)** N_2O-reduction genes (*nosZ*-I and *nosZ*-II). Each point represents the T-RFLP profile of one field plot. Environmental vectors that were significantly correlated to shifts in T-RFLP profiles ($p < 0.05$) were fitted to the ordination and presented as gradients: black solid lines represent cumulative N_2O emissions (g N_2O-N ha^{-1}), and gray dashed lines represent average soil NO_3^- content (mg N kg^{-1} soil). ●: *C4-CC+N*; ■: *O4-CC-N*; ♦: *O4-CC+N*; ▲: *O4+CC-N*; ▼: *O4+CC+N*. Red points represent samples from 2011, and blue points those from 2012.

that differences in yield of winter wheat could not be explained by labile N pools (potentially mineralisable N, microbial biomass N) alone (Petersen et al., 2013b). Instead, a multiple regression analysis showed that total N and depth of the A horizon, and cumulative N input during the previous 12 years, all contributed significantly to N availability. It implies that available N, and N_2O emissions, are not exclusively derived from the most recent input of manure and residues, and that long-term effects of management influence N_2O emissions *via* net N mineralization, nitrification and denitrification.

Denitrifier Community Dynamics

Genetic potential for net N_2O emissions was indicated by *nir*/*nos* gene copy ratios > 1 across all treatments and both years. The increase in N_2O emissions in 2012 was corroborated by a significant increase in the abundances of *nirK* and *nirS* genes, suggesting that the size of the community matters (Hallin et al., 2009). However, there was no difference in *nir*/*nos* ratios between the 2 years, and no correlation was found between *nir*/*nos* ratios and N_2O emissions. This lack of correlation indicates a more complex regulation of the N_2O balance than mere gene copy numbers, and that subsequent regulations of gene transcription and enzymatic activities are important in the shorter term (Röling, 2007). Expression of *nosZ* may be impaired by low pH (Liu et al., 2014), and in most cases N_2O reductase loses activity if exposed to O_2 (Thomson et al., 2012). However, Højberg et al. (1994) did not find evidence for a decrease in pH around a decaying clover leaf, and O_2 supply was probably lower, not higher, in 2012 compared to 2011 (cf. **Figure S1**). In contrast, the accumulation of NO_3^- around decomposing residues or manure-saturated soil volumes would have been greater in 2012, resulting in lower ratios of metabolizable C vs. NO_3^-, which is known to increase the N_2O:N_2 product ratio (Benckiser et al., 2015). The increase in N_2O emissions from 2011 to 2012 was also associated with changes in the collective communities carrying *nir* and *nos* genes (**Figure 5**). Although net N_2O emissions were the result of a balance between N_2O production and consumption, the inter-annual shift observed for the collective denitrifier communities was only found for communities carrying *nirS*, but not *nirK* nor *nosZ*, genes (**Figure 5**; **Figure S3**). This suggests that *nirS*-type denitrifiers accounted for the higher N_2O emissions in 2012. Different responses of *nirS*- and *nirK*-type denitrifiers is consistent with the concept that the two variants respond differentially to environmental factors (Hallin et al., 2009; Jones and Hallin, 2010; Braker and Conrad, 2011).

Organic or mineral N fertilizers, and catch crop residue decomposition, have the potential to modify denitrifier communities through effects on soil NO_3^- and O_2 availability,

and metabolizable carbon (Hallin et al., 2009; Enwall et al., 2010; Tatti et al., 2015). In the present study, however, a statistically supported response in denitrifier gene abundances to nitrogen management was not observed. Furthermore, management appeared to have limited effect on the composition of denitrifier communities, even after more than a decade with the same crop rotation (**Figure 5**). The main difference was instead between year 2011 and 2012, which was associated with a difference in NO_3^- and, to some extent, O_2 availability. Higher NO_3^- availability in general in 2012 could be explained by delayed plant uptake, as discussed above, and the O_2 supply was reduced because of higher precipitation. Under such conditions, with more anoxic periods and fluctuating soil O_2 status, the denitrifiers have an advantage compared to obligate aerobic microorganisms. The higher NO_3^- availability combined with lower O_2 availability in the first month after tillage and fertilization, and the availability of metabolizable C, probably together stimulated the activity and growth of *nirS* denitrifiers in 2012 compared to 2011, leading to the inter-annual shift in community composition and elevated N_2O emissions. Hence, the pressure caused by the year-to-year differences in abiotic parameters was stronger than selective pressure from management for these functional groups. This suggests that climatic factors rather than management could impact future N_2O emissions from denitrification and climate feedbacks.

PERSPECTIVES

Both area-based and yield-scaled N_2O emission factors increased in all treatments between 2011 and 2012, although treatments and cropping histories were identical. The annual application of 100 kg N ha^{-1} or more in digested manure resulted in no or barely measurable emissions of N_2O in both 2011 and 2012, whereas N_2O emissions in treatments with catch crop residue incorporation were high in both years despite lower N input (cf. **Tables 1**, **2**). The NPK treatment, in contrast, showed low and high emissions in 2011 and 2012, respectively. These observations challenge the methodology of the Intergovernmental Panel on Climate Change (IPCC, 2006), in which emissions are estimated from N input only. The IPCC methodology is a statistical approach and acknowledges the diversity of soil conditions by defining a large uncertainty range (0.003–0.03) for the default N_2O emission factor of 0.01. However, the patterns of N_2O emissions and soil characteristics observed here across five experimental rotations and 2 years suggest that there may be scope for better predictions of N_2O emissions by taking site-specific conditions into account. This should include soil physical properties and precipitation, but also the amount and quality of organic C input as a potential driver for denitrification in organic hotspots. Given that catch crop residues, by the inclusion of above-ground parts, will often have a higher degradability and lower C:N ratio compared to roots and stubble of harvested crops (Trinsoutrot et al., 2000), and that incorporation takes place in spring where soil water content is often higher than at harvest, there is an urgent need to consider catch crop residues as a driver for N_2O emissions, and search for mitigation options.

CONCLUSIONS

Rotations with a catch crop during winter had significantly higher N_2O emissions after spring incorporation than rotations without catch crop, and stimulated N_2O emissions more consistently than addition of N, either as mineral fertilizer or digested manure. Contrary to our original hypothesis, there was limited evidence for a positive interaction between crop residues and N fertilizer application, whereas the importance of rainfall for N_2O emissions from mineral N fertilizer was confirmed. This indicates an important role of crop residues in regulating N_2O emissions from sandy soils, where transformations of residue-derived N probably took place in organic hotspots with O_2 limitation caused by intense turnover of degradable residue carbon. The abundance of denitrifier genes increased from 2011 to 2012, and the inter-annual shift in community composition was associated with gradients in NO_3^- availability. The changes in both the community size and structure were correlated to higher N_2O emissions in 2012 compared to 2011. However, management differences between the five rotations had limited effect on the abundance and structure of nitrite- and N_2O-reducers. Together these results suggest that rotations with catch crops significantly stimulated N_2O emissions from agricultural soil, but had limited effect on the genetic potential for denitrification and N_2O reduction.

AUTHOR CONTRIBUTIONS

SP designed the study and organized the field experiment. Y-FD performed molecular analyses in collaboration with SH and AP. CJ developed the R package used for T-RFLP alignment. RL provided consultation on statistical analysis of N_2O emission data. Y-FD and SP wrote the first draft of the manuscript. All authors contributed to the development of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02629/full#supplementary-material

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Nitrification Rates Are Affected by Biogenic Nitrate and Volatile Organic Compounds in Agricultural Soils

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The processes regulating nitrification in soils are not entirely understood. Here we provide evidence that nitrification rates in soil may be affected by complexed nitrate molecules and microbial volatile organic compounds (mVOCs) produced during nitrification. Experiments were carried out to elucidate the overall nature of mVOCs and biogenic nitrates produced by nitrifiers, and their effects on nitrification and redox metabolism. Soils were incubated at three levels of biogenic nitrate. Soils containing biogenic nitrate were compared with soils containing inorganic fertilizer nitrate (KNO_3) in terms of redox metabolism potential. Repeated NH_4–N addition increased nitrification rates (mM $NO_3{}^{1-}$ produced g^{-1} soil d^{-1}) from 0.49 to 0.65. Soils with higher nitrification rates stimulated ($p < 0.01$) abundances of 16S rRNA genes by about eight times, *amoA* genes of nitrifying bacteria by about 25 times, and *amoA* genes of nitrifying archaea by about 15 times. Soils with biogenic nitrate and KNO_3 were incubated under anoxic conditions to undergo anaerobic respiration. The maximum rates of different redox metabolisms (mM electron acceptors reduced g^{-1} soil d^{-1}) in soil containing biogenic nitrate followed as: $NO_3{}^{1-}$ reduction 4.01 ± 0.22, Fe^{3+} reduction 5.37 ± 0.12, $SO_4{}^{2-}$ reduction 9.56 ± 0.16, and CH_4 production (μg g^{-1} soil) 0.46 ± 0.05. Biogenic nitrate inhibited denitrificaton 1.4 times more strongly compared to mineral KNO_3. Raman spectra indicated that aliphatic hydrocarbons increased in soil during nitrification, and these compounds probably bind to NO_3 to form biogenic nitrate. The mVOCs produced by nitrifiers enhanced ($p < 0.05$) nitrification rates and abundances of nitrifying bacteria. Experiments suggest that biogenic nitrate and mVOCs affect nitrification and redox metabolism in soil.

Keywords: nitrification, biogenic nitrate, redox metabolism, mVOCs, 16S rRNA, *amoA*

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INTRODUCTION

Nitrification is a key biogeochemical process for the global nitrogen cycle (Nelson et al., 2016). Therefore, in-depth knowledge on nitrification is essential for agricultural, environmental, and economic reasons. Nitrification of ammonia to nitrate is a two-step process usually performed by two distinct groups of chemolitho-autotrophic microbes (Alfreider et al., 2017), one step oxidizes $NH_4{}^+$ to $NO_2{}^{1-}$, while the other oxidizes $NO_2{}^{1-}$ to $NO_3{}^{1-}$ (Li Y. et al., 2018). In the first step, most of the $NH_4{}^+$ is converted to $NO_2{}^{1-}$, but a small portion of the N is emitted as N_2O (Liimatainen et al., 2018). This is produced as a byproduct when the intermediate HNO is produced during the oxidation of NH_2OH to $NO_2{}^{1-}$. HNO is further oxidized to $NO_2{}^{1-}$ and finally to

NO_3^{1-} (Weber et al., 2015). Complete ammonia oxidation (comammox) is energetically feasible and bacteria (*Nitrospira* sp.) capable of performing both steps have been identified (Daims et al., 2015). These bacteria encode all enzymes necessary for ammonia oxidation via nitrite to nitrate in their genomes (van Kessel et al., 2015).

Most ammonia oxidizing bacteria (AOB) belong to the *Betaproteobacteria* (β-AOB) (Pan et al., 2018). There are two distinct phylogenetic clusters within the β-AOB, the *Nitrosomonas* cluster and the *Nitrosospira* cluster (Zhao et al., 2015). The *Nitrosomonas* cluster comprises members of the genus *Nitrosomonas*. The *Nitrosospira* cluster comprises the genera *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio*. Nitrite (NO_2^{1-}) oxidizing bacteria have been described in four genera; *Nitrobacter*, *Nitrococcus*, *Nitrospina*, and *Nitrospira* (Han et al., 2017). Our understanding of the nitrogen cycle has been revised in the past few years by the discovery of ammonia oxidizing archaea (AOA) (Leininger et al., 2006). AOA are members of the proposed archaeal phylum *Thaumarchaea* (Gribaldo et al., 2010). However, AOA are difficult to cultivate, so some aspects of their physiology and contribution to biogeochemical pathways are still speculative. AOA are found in almost all environments. Crenarchaeotal 16S rRNA gene sequences have been recovered from different environments including Pacific and Atlantic oceans (Flood et al., 2015), lake sediments (Lliros et al., 2014), the guts of animals (Radax et al., 2012), agricultural soils (Tourna et al., 2011), and forest soils (Isobe et al., 2012). Typically AOA greatly outnumber AOB. In soil samples, the copy number of crenarchaeotal *amoA* is one to three orders of magnitude higher than bacterial *amoA* (Wuchter et al., 2006).

Nitrification is carried out by the microbial membrane-bound enzymes. The ammonia monooxygenase (AMO) is responsible for the conversion of NH_3 to hydroxylamine (Bock and Wagner, 2013). The end product of nitrification, NO_3^{1-}, may binds to cationic molecules present in soil or extracellular microbial molecules. Thus, the NO_3^{1-} produced by nitrifiers can be different in nature than inorganic NO_3^{1-}. The nitrates produced from nitrification may bind to extracellular complex organic compounds to form "biogenic nitrate." Contrastingly, inorganic forms of NO_3 ($NaNO_3$, KNO_3, NH_4NO_3, etc.) are in the form of salts. The bonding between NO_3^{1-} and cations (Na, K, NH_4, etc) in inorganic NO_3 fertilizer is stronger than the bonding between NO_3^{1-} and cellular organic cations in biogenic nitrate. Therefore, nitrate in the inorganic nitrate fertilizer preferably does not bind to cellular organic cations unlike the nitrate produced through nitrification. It is also reported that nitrifiers produce soluble microbial products (SMPs) which serve as supplementary organic substrates for heterotrophic bacteria (Dolinšek et al., 2013). The SMPs are mainly constituted of proteins and humics (Li J. et al., 2018). There is a possibility that after nitrification the product (NO_3^{1-}) binds to SMPs forming "biogenic nitrate." Like inorganic nitrate, the biogenic nitrate has two main biological functions. Either it is assimilated by plants and microbes (under aerobic condition) (Rubio-Asensio et al., 2014) or it is denitrified when anoxic conditions prevail. Nitrate reduction or denitrification is carried out by dissimilatory nitrate reducing bacteria (Castro-Barros et al., 2017). However, due to its complexation with SMPs, the availability and fate of biogenic nitrate can be different from inorganic fertilizer nitrate (KNO_3).

Like other microorganisms, nitrifiers can produce volatile organic compounds (VOCs). However, information on the VOCs emitted by nitrifiers is scarce. Microbial VOCs (mVOCs) act as signal molecules for different microorganisms (Insam and Seewald, 2010). The mVOCs can modulate activities of the producing species, or of different microbial species. However, it is unclear how the volatiles produced by nitrifiers influence the activity of nitrifiers and denitrifiers. The manuscript aims to define how the NO_3^{1-} derived from nitrification is different from that in chemical inorganic nitrate fertilizers.

MATERIALS AND METHODS

Soil Sampling and Characterization

Experiments were carried out using soil samples collected during September 2016 from the experimental fields of the Indian Institute of Soil Science, Bhopal, Madhya Pradesh, India (23.30 N, 77.40 E, 485 m above mean sea level). The soil is a heavy clayey Vertisol (typic Haplustert, WRB code VR), characterized by: 5.7 g kg^{-1} organic C, 225 mg kg^{-1} available N, 2.6 mg kg^{-1} available P, and 230 mg kg^{-1} available K. The textural composition of soil was: sand 15.2%, silt 30.3%, clay 54.5%, electrical conductivity (EC) 0.43 dS m^{-1}, and pH 7.5. The soil had 863.24 μM NO_3^{1-}, 0.01 μM Fe^{2+}, and 101.02 μM SO_4^{2-}. Concentration of these ions was estimated by wet chemical method as given below (chemical analysis). After collection, the soil was hand-processed after breaking the clods and removing roots and stones. Soil was then passed through 2-mm mesh sieve and was used within 2 days of collection.

Nitrification and Biosynthesis of Biogenic Nitrate

Biogenic nitrate is defined here as the nitrate produced via nitrification. To biosynthesize biogenic nitrate, microcosms were prepared where nitrification was carried out three times (**Figure 1**). The choice of having three repeated NH_4 additions was based on the fact that in agriculture, N fertilizer is often applied in split doses, and for most crops, three split doses of N are recommended (Arregui and Quemada, 2008). Repeated nitrification resulted in different levels of nitrate (biogenic nitrate). Experiments were carried out in six 1000-ml bottles (**Figure 1**). Three bottles served as controls (AC1–AC3) and the other three were used for biosynthesis of biogenic nitrate and estimation of nitrification (labeled as BC1–BC3). To each bottle 200 g soil was added and sterilized double distilled water was added to maintain soil at 60% moisture holding capacity (MHC). There was no ammonium amendment to "AC" bottles, while 2 ml of 1 M NH_4–N (NH_4Cl) was added the "BC" bottles, giving a final concentration of 10 mM. Soils were mixed thoroughly using a glass rod and bottles were closed with butyl rubber caps. All the bottles were incubated at 30 ± 2°C. At different times, bottles BC1–BC3 were opened and 1-g soil subsamples were taken out to determine NO_3^{1-} concentrations. Control bottles

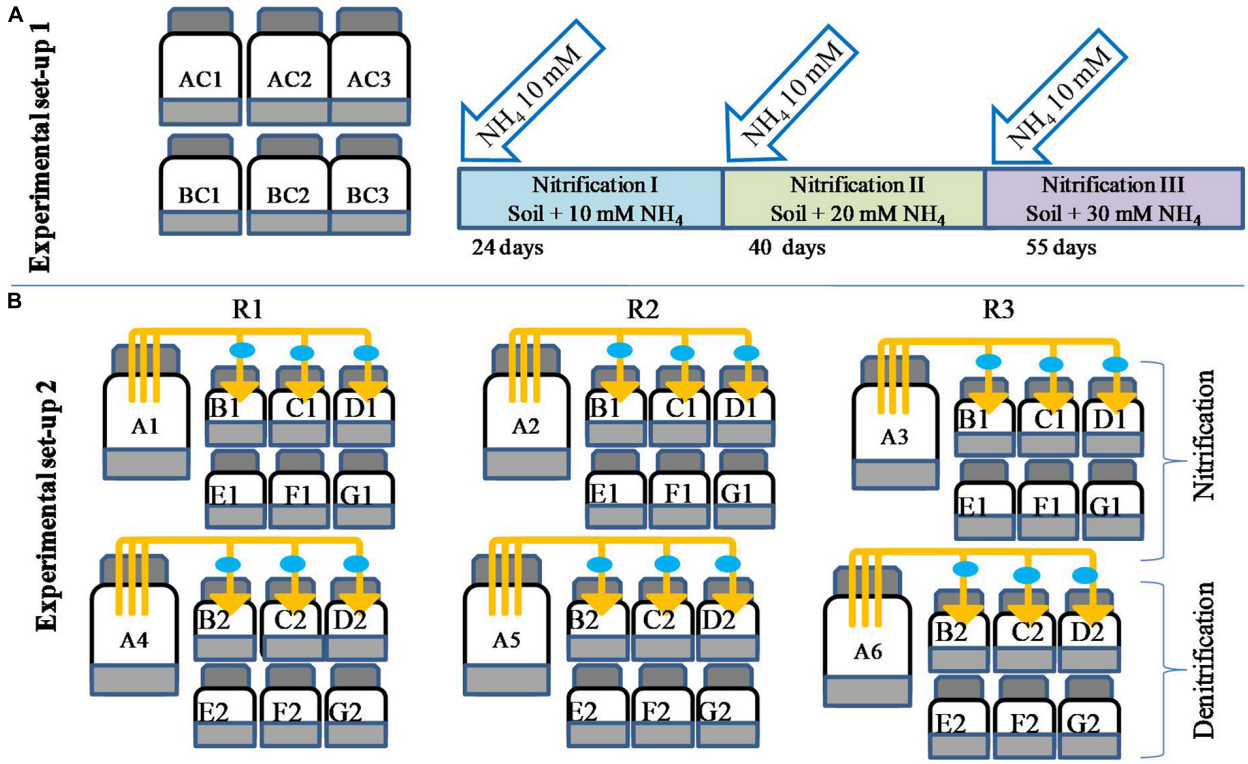

FIGURE 1 | Microcosm design for biosynthesis of biogenic nitrate and estimation of nitrification potential of soil under repeated NH_4–N amendment (setup 1, **A**). Microcosm setup to evaluate the effect of microbial volatiles (mVOCs) on nitrification and denitrification (setup 2, **B**). Bottles of 250 ml volume contained 50 g soil and were un-amended (AC1–AC3) or amended with 10 mM NH_4 (BC1–BC3). After complete nitrification of 10 mM NH_4 (24 days) a second dose of NH_4 was added (BC bottles) and after complete nitrification (40 days) a third dose of 10 mM NH_4 was added (BC bottles). The third nitrification stage was completed in 55 days of incubation. The three complete nitrification phases were designated as nitrification I, nitrification II, and nitrification III. The second setup **(B)** was designed to evaluate the effect of mVOCs (A1–A6) on nitrification and denitrification. All experimental treatments included three replicates (R1, R2, R3). The six source bottles (A1–A6) were connected to 18 sink bottles (130 ml volume containing 20 g of soil), shown as B1, C1, D1, B2, C2, and D2. The control bottles (130 ml bottle containing 20 g soil) were without exposure to mVOCs (E1, F1, G1 and E2, F2, G2). The connectors fitted with 0.2-μm filters (filled circle) were used to connect source and sink bottles. After each nitrification stage, one sink bottle and one control bottle were further incubated to determine nitrification and denitrification rates as mentioned the methodology.

were also opened for analysis to mimic the treated ones. Nitrate measurement continued till the NO_3^{1-} concentrations in BC bottles reached a plateau. Nitrification of the first dose of NH_4–N (10 mM) was referred as "nitrification I." After nitrification I, 10 mM of NH_4–N was again added to BC bottles and the same incubation and measurement protocol applied until the NO_3^{1-} was again stabilized. This second nitrification stage was referred as "nitrification II." Subsequently, the bottles were again opened and amended with a third dose of 10 mM NH_4–N in BC bottles. The third nitrification stage was referred as "nitrification III." The three nitrification stages (nitrification I, II, and III) produced three levels of biogenic nitrate. After completion of each nitrification stage, 20-g soil was taken from the bottles (AC and BC) and incubated to evaluate redox metabolism as described below.

The Effect of Biogenic Nitrate on Redox Metabolism

To evaluate the effect of biogenic nitrate on redox metabolism, experiments were carried out as described above (AC1–AC3 and BC1–BC3). In addition, 18 130-ml vials were also used for this analysis. Nine vials were kept for evaluating redox metabolism using soil mixed with equivalent amount of inorganic fertilizer nitrate (KNO_3) as observed in nitrification vials (labeled as A). Another nine vials were used for evaluating redox metabolism using the soil in which biogenic nitrate was produced (labeled as B). Each set of nine vials was represented as three nitrification phases and three replicates. Soil (20 g) from AC1–AC3 and BC1–BC3 bottles (collected after nitrification I, II, and III) were placed into 130-ml serum vials. Soils were mixed with 10 mM of CH_3COONa, and 50 ml of sterile distilled water. Acetate served as carbon source for anaerobic microbial metabolism. After mixing the contents, bottles were closed with rubber septa and sealed using aluminum crimp seals. Bottles were incubated at 30 ± 2°C with shaking at 100 revolutions per minute (rpm) on an orbital shaker for 30 days. To determine the temporal variation in the reduction of the terminal electron acceptors, 3 ml of slurry from each vial was withdrawn using a syringe (Dispovan, India). Before sampling, the syringes were first flushed with pure N_2 to maintain anoxic conditions. Slurry samples

were processed following standard methods to estimate NO_3^{1-}, Fe^{3+}, SO_4^{2-} (see below). Changes in the concentrations of all electron acceptors (NO_3^{1-}, Fe^{3+}, SO_4^{2-}) were measured at each sampling time to estimate the rates of redox metabolism. Headspace gas samples of the vials were analyzed via gas chromatography (see below) to quantify CH_4 production at the end of the incubation period (30 days).

Effects of N_2O and Microbial Volatiles on Nitrification

To test the effect of N_2O on nitrification, experiments were carried out by placing 20 g soil into six 130-ml sterilized serum bottles. Soils were moistened with sterilized double distilled water to maintain 60% MHC and NH_4–N was added to a final concentration of 10 mM. After mixing the contents, bottles were closed with rubber septa and sealed with aluminum crimp seals. Three bottles were kept as controls and three were injected with pure N_2O (Inox Pvt. Ltd., Bhopal, India) to a final mixing ratio of 10 ppmv. Control vials were injected with pure helium (99%) instead of N_2O. All bottles were incubated at 30 ± 2°C for 30 days. At different incubation, periods bottles were opened and 1 g amounts of soil taken to measure NO_3^{1-}. After each NO_3^{1-} measurement, bottles were re-incubated with 10 ppmv N_2O.

To evaluate the effect of mVOCs on nitrification and denitrification, an experiment was set up as shown in **Figure 1**; 50 g amounts of soil were placed into 250-ml bottles, and sterile double distilled water added to maintain soil at 60% MHC. To each bottle, 10 mM NH_4–N was added. Bottles were closed with rubber stoppers and tightened with screw caps. Three bottles were controls and six "source bottles" were the source of mVOCs originating from nitrification. Another set of 36 "sink bottles" were 130 ml serum bottles each containing 20 g of soil at 60% MHC. The headspace of one source bottle was connected with three sink bottles using silicon tubes (45 cm long × 0.5 cm internal diameter), each fitted with a needle (1.20 mm × 38 mm) at one end and a 0.2 μm syringe filter (25 mm) and needle (1.20 mm × 38 mm) at the other end. The syringe filters were used to restrict any microbial cross contamination between source bottles and sink bottles. The needles of both ends of the silicon tubes were pierced into the rubber caps of source and sink bottles. Gas phases of source and sink bottles were mixed via repeated (10 times) flushing (withdrawing and injecting) of the headspace of the sink bottles using a 50 ml syringe. A total of 18 sink bottles were connected with six source bottles, and another 18 sink bottles were not connected and served as "mVOCs control." All bottles were kept in an incubator maintained at 30 ± 2°C in the dark. Headspace gas samples of all sink bottles were analyzed for N_2O. Nitrification was measured only in the bottles labeled as "controls."

Nitrification of 10 mM NH_4–N in the source bottles was repeated three times as described earlier. The three nitrification stages were referred to as nitrification I, nitrification II, and nitrification III. At the completion of each nitrification phase, three sink bottles and three control bottles were removed and used for evaluating nitrification and denitrification rates. To measure nitrification in these bottles, 10 mM NH_4–N was added and the accumulation of NO_3^{1-} was determined. Denitrification was measured by adding 10 mM NO_3^{1-} (KNO_3) and 50 ml of sterile distilled water. Decline in NO_3^{1-} concentrations was measured to determine denitrification.

Chemical Analyses

Soil nitrate content was estimated after extraction with $CaSO_4$ and reaction by the phenol disulfonic acid method (Jackson, 1958). Reduced Fe^{2+} was determined by extracting slurries with 0.5 N HCl and ferrozine assay (Stookey, 1970). Sulfate (SO_4^{2-}) content was estimated by extracting slurries with $Ca(H_2PO_4)_2$ and turbidometric analysis (Searle, 1979). The slopes of regression lines relating the changes in NO_3–N concentrations with the incubation time were used to determine the potential rates of nitrification or denitritrification (nitrification: μg NO_3^{1-} produced g^{-1} soil d^{-1}; denitrification: μg NO_3^{1-} consumed g^{-1} soil d^{-1}) (Schmidt and Belser, 1982). Potential iron (Fe^{3+}) reduction rates were estimated from the increase of Fe^{2+} in slurries over time, and potential sulfate reduction rates were determined from declining SO_4^{2-} concentrations.

Gas samples of 0.1 ml were withdrawn from the headspaces of the vials using a gas-tight syringe. After each sampling, the headspace was replaced with an equivalent amount of high purity (>99%) helium (He) to maintain atmospheric pressure. Gas analysis was carried out using a gas chromatograph (GC 2010, CIC, India) fitted with flame ionization detector (FID) and electron capture detector (ECD). Gas samples were introduced through the port of an on-column injector. The GC was calibrated before and after each set of measurements using different mixtures of gasses (CO_2 or CH_4 or N_2O) in N_2 (Inox Gas, Bhopal, India) as primary standards. Primary standards were CO_2 (500, 1000 ppmv), CH_4 (10 and 100 ppmv), and N_2O (1 and 10 ppmv).

To quantify CO_2 and CH_4, a Porapak Q column (2 m length, internal diameter 3.175 mm, 80/100 mesh, stainless steel column) was used in combination with the FID. The CO_2 was quantified after its conversion to CH_4using a attached methanizer module at 350°C. The injector, column, and detector (FID) were maintained at 120, 60, and 330°C, respectively. N_2O was estimated using a stainless steel column (2 ft; diameter, 1/8 in) filled with chromosorb 101 (60–80 mesh) coupled to the ECD. The oven temp was 30°C, the injector and detector (ECD) temp were 120 and 330°C, respectively.

Raman Spectroscopic Analysis of Soil

To test the hypothesis that NO_3^{1-} derived through nitrification is a complex mixture of NO_3^{1-} and cellular derived bio-molecules, and to reveal any compositional changes of soil due to nitrification, soil samples were analyzed by Raman spectrophotometry (Guizani et al., 2017). Soil samples of un-nitrified control and after third nitrification (nitrification III) were dried at room temperature. The dried soil samples were ground using a mortar and pestle and passed through a 0.1-mm sieve. Samples were scanned in a high-resolution Raman spectrometer (RamanStation^{TM} 400F, Perkin-Elmer®, Beaconsfield, Buckingham-shire, United Kingdom) fitted with

Czerny-Turner type achromatic spectrograph. The spectral resolution was 0.4 $cm^{-1}pixel^{-1}$ at the spectral range of 200–1050 nm and the source of excitation was a 632.8 nm, air cooled He–Ne laser. Nomenclature of wavelengths and the representing functional groups was based on the earlier publications (Socrates, 2004; Colthup, 2012). Data obtained from the instrument were normalized. Wavelengths representing each functional group were considered for analysis. Intensities of the peaks were added and the average of three replicates was calculated.

DNA Extraction

DNA was extracted from 0.5 g field soil samples using the ultraclean DNA extraction kit (MoBio, United States) according to the manufacturer's instructions. The DNA concentrations were determined in a biophotometer (Eppendorf, Germany) by measuring absorbance at 260 nm (A260), assuming that 1 A260 unit corresponds to 50 ng of DNA per μl. DNA extraction was further confirmed by electrophoresis on a 1% agarose gel. The extracted DNA was dissolved in 50 μl TE buffer and stored at −20°C until further analysis.

Real-Time PCR Quantification of Total Bacteria, Ammonia Oxidizing Bacteria, and Ammonia Oxidizing Archaea

Microbial abundance was estimated from two experimental setups: first with soil samples of un-incubated control, nitrification I, II, and III soils, and second with soil samples exposed to microbial volatiles (mVOCs) of nitrification III and un-exposed controls. The microbial groups estimated were total bacteria, AOB, and AOA. Real-time PCR was performed on a Step one plus real-time PCR (ABI, United States). Reaction mixtures contained 2 μl of DNA template, 10 μl of 2X SYBR green master mix (Affymetrix, United States), and 200 nM of each primer (GCC Biotech, New Delhi). The final volume of PCR reaction mixture was made to 20 μl with PCR grade water (MP Bio, United States). Primers targeting bacterial 16S rRNA genes, bacterial *amoA* genes, and archaeal *amoA* gene were used to quantify the respective microbial abundance. The primers (5'–3') for bacteria were 1F (CCT ACG GGA GGC AGC AG) and 518R (ATT ACC GCG GCT GCT GG) (Baek et al., 2010); nitrifying bacteria 1F (GGG GTT TCT ACT GGT GGT) and amoA 2R1 (CCC CTC TGG AAA GCC TTC TTC) (Okano et al., 2004); nitrifying archaea arc-Amo-F (STA ATG GTC TGG CTT AGA CG; S = G or C); and arc-amoa-R (GCG GCC ATC CAT CTG TAT GT) (Mutlu and Guven, 2011). Thermal cycling was carried out by an initial denaturizing step at 94°C for 4 min, 40 cycles of 94°C for 1 min, the assay-dependent annealing temperature for 30 s, 72°C for 45 s; and a final extension at 72°C for 5 min. The annealing temperature for 16S rRNA genes was 52°C, and for *amoA* genes of bacteria and archaea were 50 and 52°C, respectively. Fluorescence was measured during the elongation step. Data analysis was carried out with Step one plus software (ABI, United States) as described in user's manual. The cycle at which the fluorescence of target molecule number exceeded the background fluorescence (threshold cycle [C_T]) was determined from dilution series of target amplicons with defined target molecule amounts. C_T was proportional to the logarithm of the target molecule number. The quality of PCR amplification products was determined by melting curve analysis with temperature increase of 0.3°C per cycle. Standard for bacteria prepared by using *Escherichia coli* strain JM 109 (Promega Inc., United States). For preparing standard for amoA genes of nitrifying bacteria and nitrifying archaea, the PCR products of bacterial amoA and archaeal amoA genes were separately cloned to TOPO TA cloning vector (Invitrogen, United States). Constructed plasmids were transformed into competent cells (One Shot Top 10, Invitrogen, United States). Transformed cells (white colonies) were multiplied in LB broth for 24 h at 37°C and their concentration was estimated using a Biophotometer (Eppendorf, Germany). Plasmids from the *E. coli* or transformed cells were extracted using a plasmid extraction kit (Axygen, United States). Concentration of plasmids was quantified and expressed as ng μL^{-1}. Serial dilution for each plasmid was prepared and real-time PCR carried out. Standard curve for each gene was prepared by plotting plasmid concentration (representing cell number or gene copies) versus C_T values (**Supplementary Table S1**).

Statistical Analysis

All statistical analyses were carried out using the "agricolae" packages of the statistical software R (2.15.1) (Ihaka and Gentleman, 1996). Data obtained were presented as arithmetic mean of three replicated observations. Effect of factors (NH_4 amendment) on the parameters (nitrification, denitrification, Fe^{3+} reduction, SO_4^{2-} reduction, CH_4 production, and microbial abundance) was tested by analysis of variance (ANOVA). Low *P*-value and high F statistics indicated significant impact of the factors on the variables. To define the significant difference among the treatments, Tukeys honestly significant difference (HSD) test was performed.

RESULTS

Nitrification Activity of Soil

Variation of NO_3^{1-} concentrations during repeated stages of nitrification is shown in **Figure 2**. Nitrification increased steadily after 5 days of incubation. Nitrification of the first dose of 10 mM NH_4–N occurred within 24 days. Subsequent amendment of NH_4–N stimulated nitrification. The second dose of 10 mM NH_4–N was nitrified by 40 days while the third dose of 10 mM NH_4–N was nitrified by 55 days. The added NH_4–N was nitrified by about 84% in nitrification I, 92% in nitrification II, and 87% in nitrification III stages. Potential nitrification rates (PNRs) increased with repeated nitrification (**Table 1**). PNR of fresh soil was 0.49 mMg^{-1} soil d^{-1} while the PNR of nitrification III soil was highest of 0.65 mMg^{-1} soil d^{-1}.

Effect of Nitrification on Microbial Abundance

Abundances of total bacteria, nitrifying bacteria, and nitrifying archaea all increased after repeated nitrification (**Table 1**). The

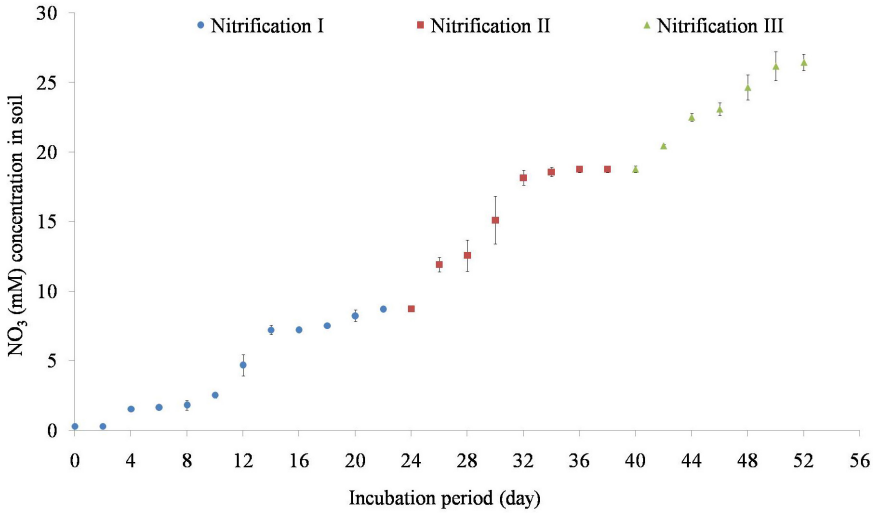

FIGURE 2 | Temporal variation of nitrification in response to 10 mM NH_4–N amendment. Nitrification was estimated as the increase in NO_3^{1-} concentration afterNH_4–N amendment. After complete nitrification, soils were again amended with 10 mM NH_4 for a second and a third time to complete three nitrification stages (nitrification I, nitrification II, and nitrification III). Each data point represents an arithmetic mean with standard deviation of three replicates.

TABLE 1 | Nitrification and microbial abundance in soil after nitrification of three successive 10 mM NH_4–N amendments.

Nitrification	Nitrification rate (mM NO_3^{1-} produced g^{-1} soil d^{-1})	Bacteria ($\times 10^6$ 16S rRNA gene copies g^{-1} soil)	Nitrifying bacteria ($\times 10^4$ bacterial amoA gene copies g^{-1} soil)	Nitrifying archaea ($\times 10^4$ archaeal amoA gene copies g^{-1} soil)
Unincubated control		5.00 ± 1.00	4.00 ± 1.46	6.00 ± 1.00
Nitrification I	0.49 ± 0.01	16.67 ± 5.69	32.33 ± 6.43	58.00 ± 8.19
Nitrification II	0.56 ± 0.09	29.00 ± 7.81	66.00 ± 11.53	71.00 ± 16.52
Nitrification III	0.65 ± 034	43.67 ± 4.51	102.33 ± 8.50	94.33 ± 7.77

The three nitrification stages were referred as nitrification I, nitrification II, and nitrification III. Microbial abundance was estimated after complete >80% oxidation of the added ammonium. Soil without added ammonium served as a control. Values represent arithmetic means and standard deviation of three replicates.

bacterial population varied from 5 to 43.67 ($\times 10^6$ cells g^{-1} soil). The nitrifying bacterial population ranged from 4 to 102 ($\times 10^4$ cells g^{-1} soil) and the nitrifying archaeal population varied from 6 to 94.33 ($\times 10^4$ cells g^{-1} soil). The lowest abundances were measured in control treatments and the highest in the nitrification III soil samples.

Effect of Nitrification on Redox Metabolism

Redox metabolism followed the classical trend of sequential reduction of terminal electron acceptors (**Figure 3**), starting with NO_3^{1-} reduction followed by Fe^{3+} reduction, SO_4^{2-} reduction, and CH_4 production. Soil amended with inorganic KNO_3 exhibited detectable nitrate reduction after 2 days and complete denitrification within 5 days. Iron reduction peaked at 5 days and SO_4^{2-} reduction after 2 weeks. Potential redox metabolic rates are presented in **Table 2**. Denitrification rates increased with NO_3^{1-} concentration originating from either nitrification phases or KNO_3. However, the denitrification rate was lower in the soil that had undergone nitrification than compared to the KNO_3 treated soil. Denitrification may have been inhibited by biogenic nitrate. The reduction rate of Fe^{3+} was also lower in the nitrification soil. Similarly, the reduction of SO_4^{2-} was also low in the nitrification soil. Production of CH_4 was estimated after the end of incubation. CH_4 production was low in nitrification soil compared to non-nitrification soil (**Table 2**).

Statistical Analyses

Analysis of variance indicated that NH_4–N addition significantly and positively influenced nitrification ($p < 0.0001$) (**Table 3**). It also significantly influenced NO_3^{1-} reduction ($p < 0.0001$), and Fe^{3+} reduction ($p < 0.01$) compared to or inorganic nitrate amendment. However, SO_4^{2-} reduction and CH_4 production were not significantly affected. Abundances of 16S rRNA genes, *amoA* genes of nitrifying bacteria, and *amoA* genes of nitrifying archaea were significantly ($p < 0.0001$) influenced by the NH_4 amendment.

Raman Spectra of Soil in Response to Nitrification

Soil samples were scanned by a Raman spectrometer to examine how soil organic carbon changed due to the metabolism of nitrifiers (**Figure 4**). Nitrified soil (nitrification III) had high absorbance for the wavelengths (wavenumbers cm^{-1}) between 500–1000 and 1500–2000. Absorbance intensity was

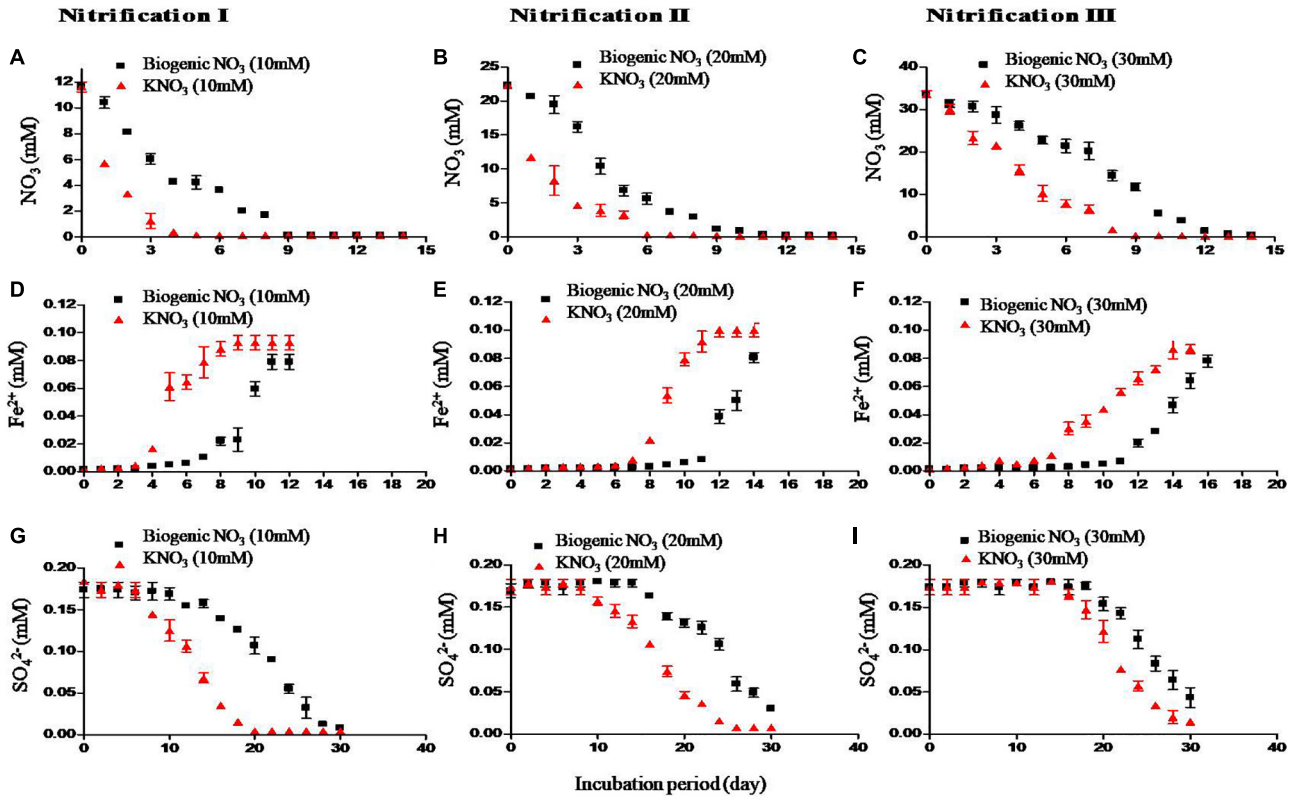

FIGURE 3 | Effect of nitrification on redox metabolism. Soil samples after three nitrification stages were incubated to undergo redox metabolism. Inorganic NO_3^{1-} (KNO_3) was used to compare with biogenic NO_3^{1-} (i.e., nitrate produced from nitrification). First row – denitrification (NO_3^{1-} reduction) **(A–C)**, second row – iron (Fe^{3+}) reduction **(D–F)** measured as increase of Fe^{2+} concentration, and third row – SO_4^{2-} reduction **(G–I)**. The three nitrifications stages were nitrification I (left), nitrification II (center), and nitrification III (right). Each data point represents an arithmetic mean and standard deviation of three replicates.

low for the wavelengths ranging from 1200–1600. Raman intensity for the above wavelengths was plotted for both the samples (**Figure 4**). Nitrification increased the concentration of C–C, C–S, C–O–C molecules and decreased C–NO_2, and CH_2 molecules.

Production of N_2O and CO_2 From Soil During Nitrification

Headspace N_2O and CO_2 production were measured from control soil (no added nitrogen) and soil after the three nitrification stages (**Table 4**). N_2O production rates varied from 4.06 to 19.39 $\mu g\ g^{-1}$ soil d^{-1}. The lowest rate was in control soil and the highest was in nitrification III soil. The amount of headspace CO_2 varied from 465 $\mu g\ g^{-1}$ soil d^{-1} in control soil to 649 $\mu g\ g^{-1}$ soil d^{-1} in nitrification III soil. The values of N_2O varied significantly among the treatments.

Effect of N_2O and Nitrifying Microbial Volatiles on Nitrification and Denitrification

The effect of N_2O on the nitrification and denitrification was evaluated by exposing soil to 0 or 10 ppm of N_2O. Production of NO_3^{1-} was measured during nitrification, while the decline of NO_3^{1-} was measured during denitrification. Nitrification of 10 mM NH_4 was completed in 3 weeks whereas the denitrificaion of NO_3^{1-} (∼10 mM) was completed within 8 days. Added N_2O had no significant effect on nitrification and denitrification (**Figure 5**).

The effect of volatiles originating from nitrification was tested on nitrification and denitrification (**Figure 5**). The composition of nitrifier-derived mVOCs was not evaluated in this study because the primary aim was to reveal the influence of mVOCs on nitrification and denitrification. Soils were exposed to microbial volatiles of three repeated nitrification (nitrification I, II, and III) phases. The mVOCs originating from nitrifiers significantly stimulated nitrification (**Figure 5**). Time required for complete nitrification of the added NH_4 was significantly reduced due to the volatiles. Nitrification rates (mM NO_3^{1-} produced g^{-1} soil d^{-1}) varied from 0.425 in control soil to 0.844 in nitrification III soil. Nitrification and denitrification values of the controls remained unchanged over the three nitrification phases. However, mVOCs of nitrifiers did not influence denitrification. Potential denitrification rates (mM NO_3^{1-} reduced g^{-1} soil d^{-1}) varied from 1.37 to 1.38 with no statistical difference among the treatments (**Table 4**).

TABLE 2 | Influence of biogenic NO_3^{1-} and inorganic fertilizer KNO_3 on soil denitrification rate, iron reduction rate, sulfate reduction rate, and methane production rate.

Source of NO_3^{1-}	Nitrification phases	Denitrification rate (mM NO_3^{1-} reduced g^{-1} soil d^{-1})	Iron reduction rate (μM Fe^{3+} reduced g^{-1} soil d^{-1})	Sulfate reduction rate (μM SO_4^{2-} reduced g^{-1} soil d^{-1})	CH_4 production (μg CH_4 produced g^{-1} soil)
Biogenic NO_3^{1-}	Nitrification I	1.22 ± 0.04	5.29 ± 0.26	9.41 ± 0.14	0.54 ± 0.04
	Nitrification II	2.03 ± 0.02	2.95 ± 0.10	9.35 ± 0.03	0.41 ± 0.08
	Nitrification III	2.80 ± 0.04	2.89 ± 0.10	9.19 ± 0.06	0.38 ± 0.04
Inorganic fertilizer NO_3^{1-}	Nitrification I	1.63 ± 0.17	8.55 ± 0.58	10.89 ± 0.17	0.63 ± 0.11
(KNO_3)	Nitrification II	2.84 ± 0.18	5.84 ± 0.10	10.45 ± 0.19	0.57 ± 0.10
	Nitrification III	4.01 ± 0.22	5.37 ± 0.12	9.56 ± 0.16	0.46 ± 0.05

The three nitrification stages were referred as nitrification I, nitrification II, and nitrification III. Soil sub-samples collected at the end of the three nitrification phases were incubated for redox metabolism. Values represent arithmetic means and standard deviations of three replicates.

Microbial Abundance in Response to Microbial Volatiles

The effect of nitrifying mVOCs on the soil microbial abundance was estimated by quantifying the 16S rRNA genes of eubacteria, *amoA* genes of nitrifying bacteria, and *amoA* genes of nitrifying archaea. Exposure of soils to mVOCs of nitrification (nitrification III) did not increased microbial abundance in soils (**Table 5**). This indicated that the mVOCs were not a substantial substrate for growth of soil microorganisms. However, prior exposure of soils to mVOCs and subsequent incubation for nitrification resulted in a significant increase in the growth of nitrifying bacteria. Probably, the mVOCs may have activated the nitrifiers in some way resulting high microbial abundance.

Raman Spectra of Soil Exposed to Nitrifying Microbial Volatiles

Soils after exposure to the nitrification III and control (unexposed) treatments were analyzed by Raman spectra (**Figure 5**). The Raman intensity across the total wavelengths of the two samples was mostly equivalent with no apparent change.

DISCUSSION

NO_3^{1-} influences (mostly negatively) reduction of Fe^{3+} (Ionescu et al., 2015), SO_4^{2-} (Ontiveros-Valencia et al., 2013), and methanogenesis (Rissanen et al., 2017). Denitrification is thermodynamically more favorable than the reduction of other electron acceptors (Fe^{3+}, SO_4^{2-}, CO_2). This influence of inorganic nitrate on redox metabolism is well understood. However, the role of biogenic NO_3^{1-} on redox metabolism is not known. Therefore, the interaction between nitrification (which produces biogenic nitrate) and redox metabolism was explored.

Soils were amended with 10 mM NH_4–N and the progress of nitrification was monitored. The PNRs measured were similar to those observed in other soils (Fierer et al., 2001). The nitrification was repeated three times to generate three levels of NO_3^{1-} (biogenic nitrate). Nitrification rates increased over repeated NH_4–N amendments, as did the abundance of both nitrifying bacteria and archaea. After each nitrification stage, the soils were evaluated for redox metabolism. Soil samples were incubated under flooded moisture regime to test the effect of the biogenic nitrate versus inorganic nitrate (control) on redox metabolism. Biogenic nitrate inhibited reduction of electron acceptors compared to inorganic NO_3^{1-}.This is reasonable as any compound or processes that inhibited denitrification will ultimately affect the reduction of subsequent terminal electron acceptors (Fe^{3+}, SO_4^{2-}, CO_2).

The production of biogenic nitrate via nitrification significantly ($p < 0.05$) inhibited redox metabolism compared to the addition of inorganic NO_3^{1-}. Several soil factors may have been affected by the nitrification phase. One possibility is that nitrifiers produced biomolecules which inhibited the redox metabolism. To identify those biomolecules, soils of non-nitrified control soil and soil from the nitrification III stage were analyzed by Raman spectrometer. Soils of nitrification III were selected for Raman spectra analysis because these soils had undergone maximum nitrification. Raman spectra differentiated soil of control (with an equivalent amount of KNO_3) from soils of nitrification III. Nitrification increased the abundance of functional groups including C–C, C–S, C–O–C. Spectra also indicated that nitrification decreased the amount of functional groups including C–NO_2, CH_2/CH_3, C–NO_2, C–N, esters, and alkynes. Therefore, the

TABLE 3 | Analysis of variance (ANOVA) to determine the effect of NH_4 amendment on nitrification, denitrification, sulfate reduction, CH_4 production, abundance of bacterial 16S rRNA genes, *amoA* of nitrifying bacteria, and *amoA* of nitrifying archaea.

Parameters	F statistics	*P*-value
Nitrification	80.37	< 0.0001
NO_3^{1-} reduction	2997	< 0.0001
Fe^{3+} reduction	21	< 0.01
SO_4^{2-} reduction	1.349	0.285
CH_4 production	3.955	0.187
16S rRNA genes of eubacteria	52.73	< 0.0001
amoA genes of nitrifying bacteria	103.5	< 0.0001
amoA genes of nitrifying archaea	16.21	< 0.01

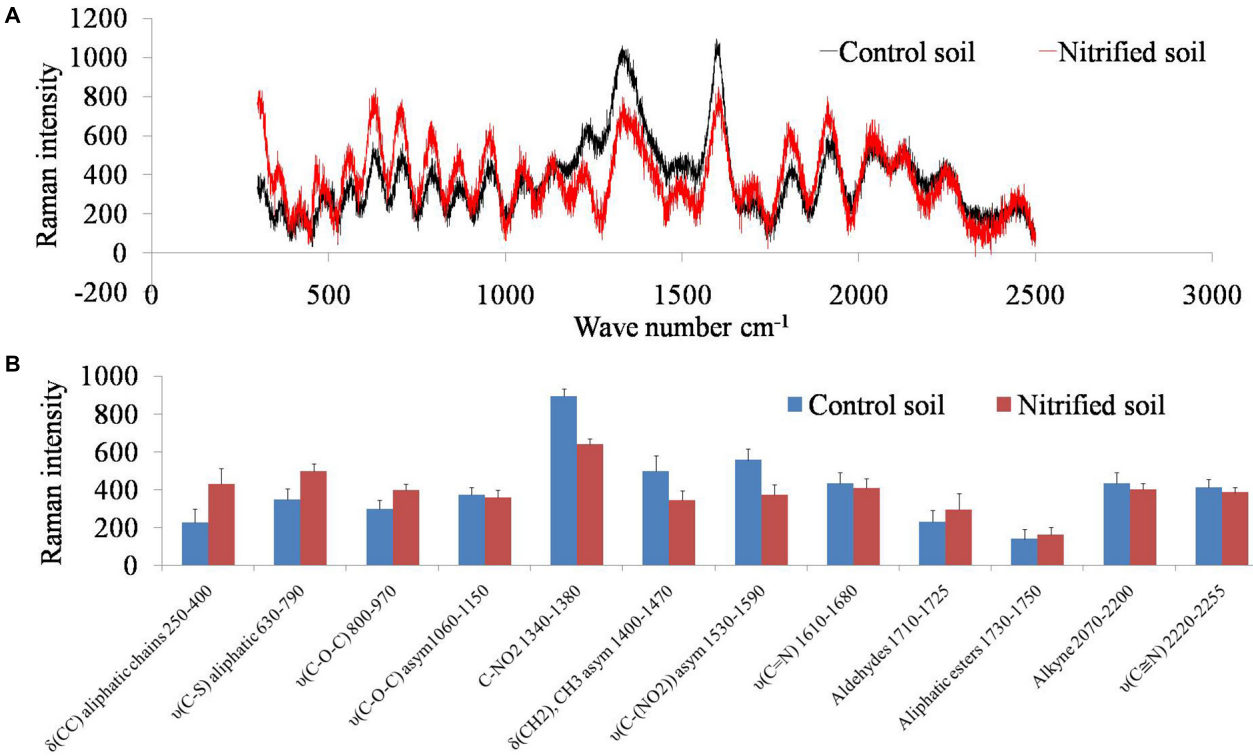

FIGURE 4 | Raman spectra of the non-nitrified (control) and nitrified (after nitrification III) soil **(A)**. The *x*-axis represents wavenumber cm^{-1} and the *y*-axis represents Raman intensity. Raman intensity of different functional groups (wavenumber cm^{-1}) of nitrified (nitrification III) and control soils **(B)**. The *x*-axis represents functional groups and the *y*-axis represents Raman intensity. Data points are arithmetic means and standard deviations of three replicates.

TABLE 4 | Production rates of N_2O, CO_2, potential rates of nitrification and denitrification in soil in response to repeated ammonium additions.

Nitrification	N_2O production (μg produced g^{-1} soil d^{-1})	CO_2 production (μg produced g^{-1} soil d^{-1})	Potential nitrification rate (mM NO_3^{1-} produced g^{-1} soil d^{-1})	Potential denitrification rate (mM NO_3^{1-} reduced g^{-1} soil d^{-1})
Control	4.06 ± 0.06	465 ± 50.16	0.42 ± 0.01	1.37 ± 0.05
Nitrification I	11.97 ± 0.84	575 ± 94.85	0.47 ± 0.02	1.38 ± 0.06
Nitrification II	15.87 ± 1.80	605 ± 36.02	0.57 ± 0.01	1.38 ± 0.01
Nitrification III	19.39 ± 2.61	649 ± 39.02	0.84 ± 0.06	1.38 ± 0.04

The three successive nitrification stages are referred as nitrification I, nitrification II, and nitrification III. Soil without added ammonium served as control. Values represent arithmetic means and standard deviations of three replicates.

inhibition of redox metabolism by nitrification may have been due to the presence and/or absence of these functional groups. Under anaerobic conditions, denitrifiers oxidize aliphatic bonds (C–C and C–O–C) to CO_2 through NO_3^{1-} dependent oxidation (Zedelius et al., 2011). Theoretically, the occurrence of aliphatics would stimulate the redox metabolism by acting as substrates for the anaerobic microorganisms. However, in the current experiment, they were correlated with reduced redox metabolism. Probably, the biogenic nitrate was less reactive (denitrifying) than the inorganic NO_3^{1-} as mentioned above. This could be due to the complex interaction or bonding between NO_3^{1-} and the extracellular aliphatics. In control (non-nitrified soil), the C–NO_2 functional groups were high. Spectral data indicated occurrence of biogenic nitrate in soil that has undergone nitrification. Biogenic nitrate is a complex form of nitrate containing organic molecules. The organic molecules can be short- or long-chain aliphatics. The complex structure and bonding between aliphatics and NO_2^{1-} /NO_3^{1-} makes it less reactive to undergo denitrification (**Figure 6**). It has been found that organic compounds may inhibit denitrification (Gilbert et al., 1997). Probably, the biogenic NO_3^{1-} was denitrified after separation of NO_3^{1-} and aliphatics, which might have been carried out by anaerobic microorganisms (Rabus et al., 2016). The processes of decomposition of the biogenic nitrate by microorganisms probably delayed the availability of NO_3^{1-} for denitrification. Thus, due to the delayed denitrification, there was delay in the reduction of subsequent electron acceptors comprising Fe^{3+}, SO_4^{2-}, and CH_3COO^{1-} (CH_4 production).

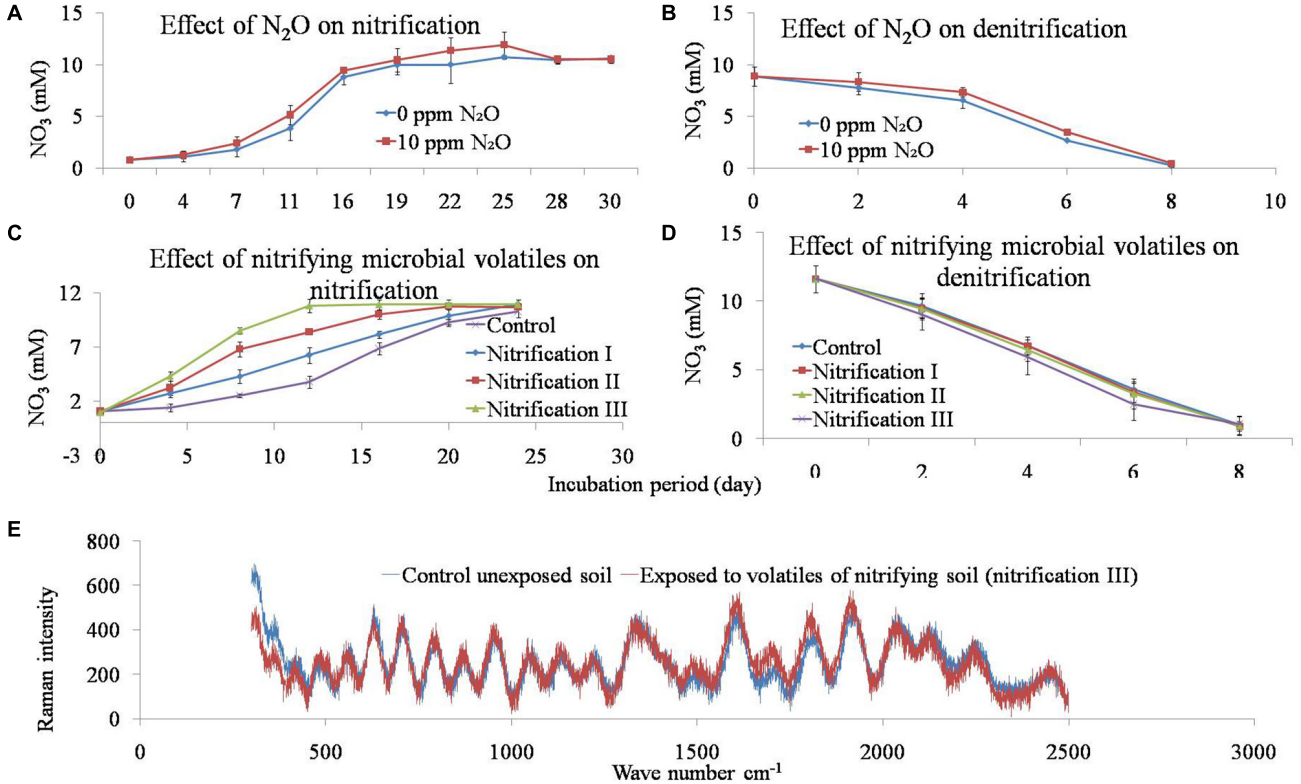

FIGURE 5 | Effect of N_2O and microbial volatile organic compounds (mVOCs) on nitrification and denitrification activity in soil **(A,B)**. The mixing ratios of N_2O were either 0 or 10 ppm. To evaluate the effect of microbial volatiles (mVOCs) produced during nitrification on nitrification and denitrification activity, soils were exposed to the gas phase of soils during nitrification I, nitrification II, and nitrification III stages. Soil without exposure served as controls. After exposure, soils were incubated to determine nitrification **(C)** and denitrification **(D)** rates. The *x*-axis represents incubation period and the *y*-axis represents NO_3^{1-} concentration. Data points are arithmetic means and standard deviations of three replicates. Raman spectra of soil under the influence of nitrifying microbial volatiles **(E)**. Soils were exposed to nitrifying microbial volatiles (nitrification III) or not exposed (control). The *x*-axis represents wavenumber cm^{-1} and the *y*-axis represents Raman intensity. Data points are arithmetic means of three replicates.

TABLE 5 | Effect of microbial volatiles (mVOCs) produced during nitrification on the abundance of different microbial groups.

Nitrifying microbial volatiles	Nitrification	Eubacteria ($\times 10^6$ 16S rRNA gene copies g^{-1} soil)	Nitrifying bacteria ($\times 10^4$ bacterial amoA gene copies g^{-1} soil)	Nitrifying archaea ($\times 10^4$ archaeal amoA gene copies g^{-1} soil)
Un-exposed	Before nitrification	5.67 ± 0.57	4.33 ± 0.57	6.49 ± 0.65
	After nitrification	43.67 ± 4.50	103.33 ± 6.11	93.00 ± 8.54
Exposed to nitrifying volatiles (mVOCs)	Before nitrification	6.00 ± 1.00	4.67 ± 1.11	6.67 ± 1.15
	After nitrification	64.67 ± 3.51	195.67 ± 12.85	139.33 ± 16.16

Soils were exposed to the volatiles originating from nitrification over three successive ammonium additions. Soils without exposure served as an un-exposed control. After exposure to mVOCs, soils were amended with 10 mM NH_4–N and incubated. Microbial abundance was estimated before and after nitrification of this added ammonium. Values represent arithmetic means and standard deviations of three replicates.

It was observed that unlike other microbial activities, nitrification progressed steadily in spite of a constant increase in NO_3^{1-} concentration. Therefore, the formation of biogenic NO_3^{1-} may be a mechanism used by nitrifiers to block the feedback inhibition of NO_3^{1-} to nitrification. Production of CO_2 did not significantly vary with nitrification potential. However, N_2O production varied significantly among the treatments. Nitrous oxide was generally produced from nitrification, because active nitrification (continuous increase in the NO_3^{1-} concentration) was observed over the incubation period. N_2O production through denitrification cannot be ruled out, because some denitrification might have occurred in the soil microaggregates. However, NO_3^{1-} production from NH_4 was consistent and there was no decline in the NO_3^{1-} level. Therefore, the denitrification mediated N_2O production could be marginal. A follow-up experiment was carried out to determine the effect of N_2O on nitrification and denitrification. It was observed that there was no significant effect of N_2O on nitrification and denitrification.

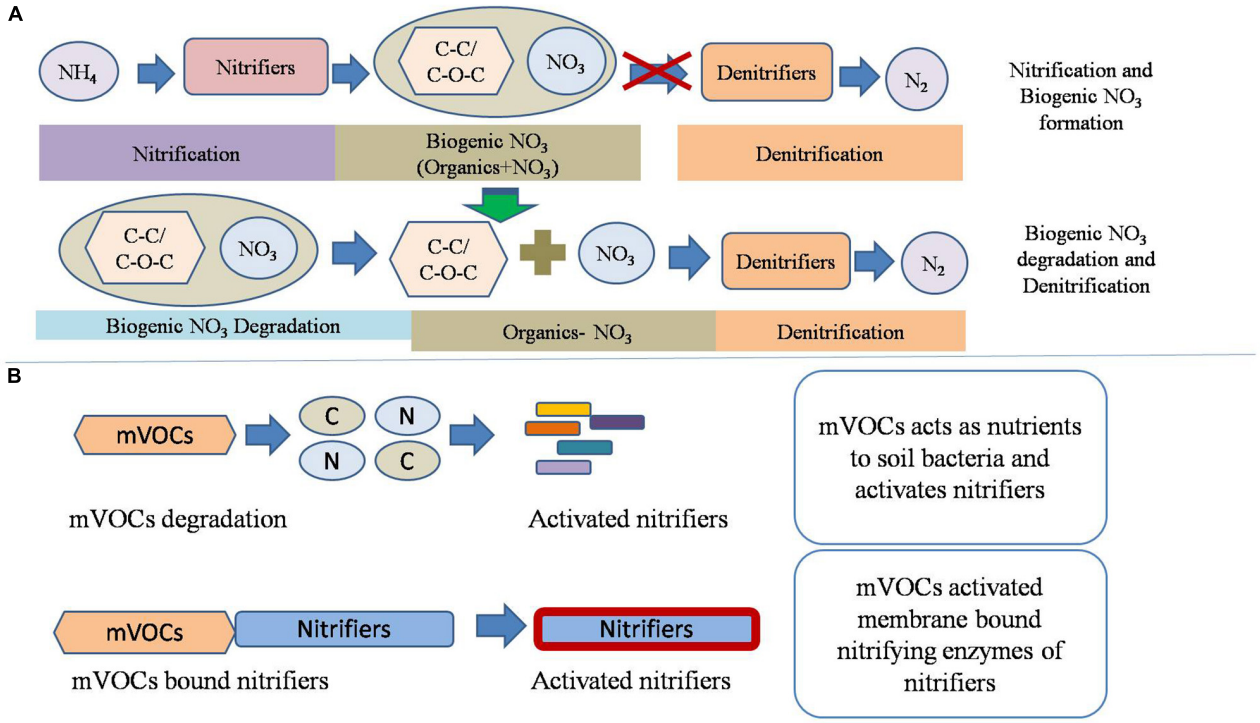

FIGURE 6 | Conceptual model of nitrification and its interaction with the redox metabolism. **(A)** The proposed mechanism of biogenic nitrate formation and of its interaction with denitrification. Biogenic nitrates are produced by the binding of NO_3^{1-} with extracellular organic compounds, possibly aliphatics. The biogenic nitrates are degraded before onset of redox metabolism under anaerobic conditions. **(B)** The proposed role of microbial volatile organic compounds (mVOCs) emitted by nitrifiers on soil nitrification. It is hypothesized that mVOCs acts as nutrients for proliferation of certain microbial groups and/or bind to cell membrane proteins to activate nitrifiers.

Apart from CO_2 and N_2O, other gaseous products emitted by soil microorganisms include mVOCs. Soil microbes produce VOCs including alkenes, alcohols, ketones, terpenes, benenoids, pyrazines, acids, and esters (Lemfack et al., 2013). Microbial volatiles act as signal molecules to other microorganisms, plants, and animals (Insam and Seewald, 2010). The composition of mVOCs originating from nitrification was beyond the scope of this research, which aims only to provide primary information about the influence of mVOCs on nitrification and denitrification. Based on the current study, conceptual models were developed depicting the potential interaction of mVOCs and nitrifiers (**Figure 6**). This experiment suggested that mVOCs stimulated nitrification, but no effect on denitrification. Probably, the mVOCs acted as signal molecules for the nitrifiers and stimulated their activity (nitrification). Exposure of soil to mVOCs did not increase the abundance of bacteria, nitrifying bacteria, and nitrifying archaea, suggesting that mVOCs stimulated the nitrifiers by increasing cell activity. Many bacteria decompose VOCs in soil (Tyc et al., 2016). The degraded products could have played important role in the activation of microbial population, resulting in high nitrification rates compared to the unexposed control. Soils after exposure to mVOCs were further tested by Raman spectrometer to evaluate if the volatiles altered chemical composition. However, mVOCs did not change the measured soil properties. We propose that the mVOCs stimulate nitrifiers by acting as signal molecules rather than altering the soil properties.

CONCLUSION

The current experiment addressed four key questions about nitrification. First, how does nitrification progress under repeated N amendment? Second, how does nitrification influence redox metabolism? Third, how does the nitrate produced from nitrification (biogenic nitrate) differ from inorganic nitrate? Fourth, do the nitrifiers communicate by means of VOCs? Nitrification activity was observed under three repeated N amendments. Nitrification increased steadily in respect to the NH_4–N amendment, due to increasing abundance of nitrifying bacteria and nitrifying archaea. After each nitrification stage, soils were incubated to undergo redox metabolism. An initial nitrification phase inhibited redox rates compared to the addition of an equivalent amount of inorganic NO_3^{1-} (KNO_3). Raman spectra of the nitrified soils revealed an increased concentration of aliphatics. Based on these observations, it was hypothesized that during nitrification, biogenic nitrates are produced by complex interaction (bonding) between NO_3^{1-} and the aliphatics, and that this biogenic nitrate is less reactive toward

denitrification than is inorganic nitrate. Nitrifiers emitted VOCs which stimulated nitrification. Nitrification was accelerated by both VOCs and biogenic nitrate. The current experiment mostly indicated the formation of biogenic nitrate and mVOCs by nitrifiers which regulate nitrification and redox metabolism. However, there is need of comprehensive studies on the biochemical characteristics of biogenic nitrate and mVOCs to better understand the nitrification. Further studies are also warranted with other soil types as well as under field conditions to verify complex interaction between biogenic nitrate, VOCs, and nitrification.

AUTHOR CONTRIBUTIONS

SRM conceptualized the experiments, and drafted the manuscript. MN executed experiments and performed most of the wet chemical analysis. RP assisted in setting up experiments. UA contributed in analyzing redox moieties of soil samples. AP facilitated experiments. GD performed qPCR reactions to quantify functional genes. BK analyzed data statistically and contributed in drafting and revising the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00772/full#supplementary-material

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REVIEW
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Controls and Adaptive Management of Nitrification in Agricultural Soils

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Agriculture is responsible for over half of the input of reactive nitrogen (N) to terrestrial systems; however improving N availability remains the primary management technique to increase crop yields in most regions. In the majority of agricultural soils, ammonium is rapidly converted to nitrate by nitrification, which increases the mobility of N through the soil matrix, strongly influencing N retention in the system. Decreasing nitrification through management is desirable to decrease N losses and increase N fertilizer use efficiency. We review the controlling factors on the rate and extent of nitrification in agricultural soils from temperate regions including substrate supply, environmental conditions, abundance and diversity of nitrifiers and plant and microbial interactions with nitrifiers. Approaches to the management of nitrification include those that control ammonium substrate availability and those that inhibit nitrifiers directly. Strategies for controlling ammonium substrate availability include timing of fertilization to coincide with rapid plant update, formulation of fertilizers for slow release or with inhibitors, keeping plant growing continuously to assimilate N, and intensify internal N cycling (immobilization). Another effective strategy is to inhibit nitrifiers directly with either synthetic or biological nitrification inhibitors. Commercial nitrification inhibitors are effective but their use is complicated by a changing climate and by organic management requirements. The interactions of the nitrifying organisms with plants or microbes producing biological nitrification inhibitors is a promising approach but just beginning to be critically examined. Climate smart agriculture will need to carefully consider optimized seasonal timing for these strategies to remain effective management tools.

Keywords: nitrification, global change, ammonia oxidizers, nitrite oxidizers, biological nitrification inhibition, agricultural management

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INTRODUCTION

Human activities have dramatically altered the global nitrogen (N) cycle by increasing the amount of reactive N in the biosphere (Kaiser, 2001; Fowler et al., 2013). The anthropogenic inputs of industrially produced N fertilizers and N fixation by crops now exceed the natural N inputs to terrestrial systems (Galloway and Cowling, 2002; Schlesinger, 2009; Fowler et al., 2013). Yet the N use efficiency (NUE) of our fertilizers in agricultural systems remains quite low, typically only about 50% or less of fertilizer N applied is taken up by the crop during the growing season (Raun and Schepers, 2008; Cavigelli et al., 2012). A better understanding of N cycling in agroecosystems is essential for intensifying sustainable food production while decreasing negative environmental impacts. Overall, improved management of nitrification may increase the NUE of fertilization while

reducing the transport of reactive N to rivers and groundwater and the emissions of greenhouse gases especially nitrous oxide (N_2O) (Smith et al., 2008; Robertson and Vitousek, 2009). These are important considerations for agricultural and environmental policy especially as global climate change intensifies (Schlesinger, 2009; Cavigelli et al., 2012; Robertson et al., 2014).

Agriculture is responsible for over half of the input of reactive N to terrestrial systems; however improving N availability through fertilization remains a primary management technique to increase crop yields in most regions. N fertility management is inherently complex because available N is temporally and spatially dynamic and subject to high rates of loss through diverse pathways. Mobility and availability of N from fertilizers and organic sources is the result of microbial enzymatic processes especially mineralization and nitrification operating within the physical and chemical constraints of the soil matrix (**Figure 1**). In many agricultural systems, large amounts of fertilizer N are lost from the root zone as nitrate through leaching and denitrification (Robertson et al., 2013). Avoiding the combination of high external inputs with low resource use efficiency remains a major concern for the sustainability of N in agroecosystems (Spiertz, 2010).

Nitrification is the biological oxidation of ammonia (NH_3) or ammonium (NH_4^+) to oxidized N in the form of nitrite (NO_2^-) and further to nitrate (NO_3^-). In the majority of agricultural soils, NH_4^+ is rapidly converted to NO_3^-, which may accumulate in the soil solution to high concentrations. Conversion of the cation, NH_4^+, to an anion (NO_2^- or NO_3^-) determines the movement of N through the generally negatively charged soil matrix and therefore strongly influences the fate of N in the soil. Nitrate is more likely than NH_4^+ to move rapidly via mass flow to plant roots, leach out of the root zone or be lost from the soil by denitrification. For these reasons, it is often desirable to manage agricultural soils to reduce nitrification, improve the match between available N supply and plant demand and increase N fertilizer use efficiency.

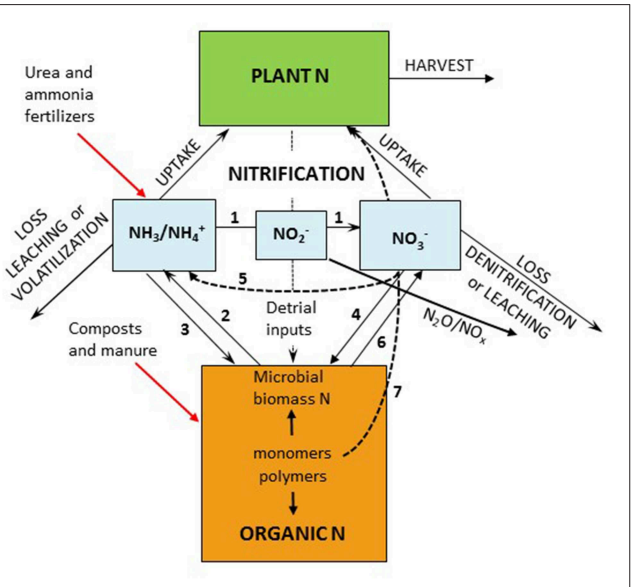

FIGURE 1 | The soil internal nitrogen cycle with (1) nitrification (including comammox), (2) mineralization (ammonification), (3) ammonium immobilization, (4) nitrate immobilization, (5) dissimilatory NO_3^- reduction to NH_3 (DNRA), (6) hetrotrophic nitrification, and (7) plant uptake of monomers.

In classical autotrophic nitrification, the conversion of N takes place in two steps: in the ammonia oxidizing bacteria (AOB) such as *Nitrosomonas* or *Nitrosospira,* NH_4^+ is converted to hydroxylamine and then to NO_2^- with a net outcome shown in Equation 1. Ammonia oxidizing archaea (AOA) such as *Nitrososphaera* have been shown to oxidize NH_4^+ to NO_2^- (Schleper and Nicol, 2010) although by a significantly distinct metabolism from the AOB (Kozlowski et al., 2016).

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+ \quad (1)$$

While the nitrite oxidizing bacteria (NOB) such as *Nitrobacter* or *Nitrospira* convert NO_2^- to NO_3^-

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad (2)$$

Recently certain *Nitrospira* bacteria have been found that mediate the entire reaction from NH_4^+ to NO_3^- within one organism in the Complete Ammonia **Ox**idation to nitrate known as Comammox (Daims et al., 2015; van Kessel et al., 2015).

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+ \quad (3)$$

Many AOB, AOA, NOB, and Comammox organisms gain energy from these reactions and then grow by the fixation of inorganic C into biomass and are therefore chemolithoautotrophs. While it is convenient to group and discuss organisms by the reactions that they mediate, our recent insights into the complexity and versatility of microbial metabolic modules catalyzing N transformations reminds us that this operational approach is not a static classification (Kuypers et al., 2018), the capability of organisms in their environment is the result of complex genetic potential shaped through their environmental and evolutionary past.

Chemolithotrophic bacteria and archaea that are energetically dependent on oxidizing NH_4^+/NH_3 and/or NO_2^- for their growth mediate the majority of nitrification in agricultural soils. For this reason the rate and extent of the nitrification process is closely linked to the abundance and functional ecotypes of these responsible organisms. Simulation modeling of nitrification processes may be improved by inclusion of microbial community or functional gene abundance data into predictive models (Bouskill et al., 2012; Graham et al., 2016; Le Roux et al., 2016; Breuillin-Sessoms et al., 2017). This review covers the main factors controlling nitrification rates in agricultural soils, agricultural practices that may reduce nitrification and associated fertilizer N loss and the potential interactions of nitrification rates and extent with climate change under agricultural management.

CONTROLS ON NITRIFICATION IN AGRICULTURAL SOILS

The main factors controlling the rates of nitrification in agricultural soils include the substrate supply, environmental conditions, organismal populations of nitrifiers and competitors, and the presence of nitrification inhibitors. These factors include those that act directly at the cell level and many that act indirectly affecting the soil habitat of the nitrifying organisms. The timescale for these factors spans from immediate change in rates at minutes to hours spanning to years and decades for changes in the soil organic matter pools and their turnover. There are significant interactions and feedbacks between controlling factors since the populations of active nitrifying microbes is determined by the substrates driving their metabolism and growth. Several mechanistic models simulate nitrification at various levels of complexity and these are compared for their treatment of some of these main controlling factors in **Table 1**.

Substrate Supply Effects on Nitrification

The substrate supply for energy yielding reactions (Equations 1–3) are important factors controlling nitrification in agricultural soils. The availability of ammonia/ammonium (NH_4^+/NH_3), NO_2^-, and O_2 often limits both the rate of nitrification and the size of the resultant nitrifier populations (Grant, 1994; Bouskill et al., 2012; Nowka et al., 2015; Venterea et al., 2015; Ouyang et al., 2018). Although O_2 is an important substrate for nitrification; its availability is closely linked to soil water status and thus O_2 availability will be discussed with environmental factors below. In agricultural soil environments, the substrate pool of NH_4^+/NH_3 is increased by (1) additions of urea and ammonical fertilizers, (2) deposition of animal wastes (urine and feces), (3) atmospheric deposition of NH_4^+, (4) biological N fixation, and (5) NH_4^+ production via mineralization. The competing consumptive processes including microbial assimilation (immobilization), plant assimilation, and ammonia volatilization decrease available NH_4^+/NH_3 (**Figure 1**).

Nitrification rates are often modeled as first-order with respect to NH_4^+/NH_3 pools (appropriate for lower concentrations) or using Michaelis-Menten equations (Norton and Stark, 2011; Bouskill et al., 2012; Inselsbacher et al., 2013; Breuillin-Sessoms et al., 2017). Often ammonia oxidation rates are assumed to limit the overall rate of nitrification and nitrite does not accumulate. Some important exceptions are described below. The

TABLE 1 | Simulation models including nitrification rate and their treatment of controlling factors.

	DayCent (Parton et al., 2001; Del Grosso et al., 2009, 2016; Abdalla et al., 2010)	**DNDC** (DeNitrification DeComposition) (Li et al., 1992, 2012; Giltrap et al., 2010; Gilhespy et al., 2014)	**Ecosys** (Grant, 1994, 2001, 2014; Grant and Pattey, 2003; Grant et al., 2006)	**MicroTrait–N** (Bouskill et al., 2012)
PARAMETER				
Nitrification rate	Nitrification rate is a function of NH_4^+, water content, temperature, pH, and texture	rate is a first order function of NH_4^+ concentration, nitrifier biomass, a temperature reduction factor, and a moisture reduction factor	substrate (NH_3) oxidation under non-limiting O_2 is calculated from active biomass and from NH_3 and CO_2 concentrations (same for NO_2^-)	Briggs Haldane kinetics for ammonia and oxygen for AO and for nitrite and oxygen for NO
Soil ammonia/ammonium	Model derived soil ammonium × maximum fraction nitrified	NH_4^+ concentration used in Michaelis-Menten kinetics	Solution NH_4^+/NH_3 drives rates	Dynamic solution NH_3 driven by pH and consumption
Mineralization	Net mineralization fraction (.20)	Submodel of decomposition	Submodel of decomposition	Inputs but not linked
Nitrite	Not modeled	Not modeled	Modeled explicitedly	Product of AO
Oxygen in soil	Limited at high WFPS, soil physical properties control gas diffusivity and 0_2 demand	DOC Anaerobic balloon concept	Consumption by microbial groups, O_2 uptake in competition with heterotrophs, roots; then diffusion to nitrifier	O_2 use by nitrification reactions
Temperature	Ts estimated based on heat flux and soil heat capacity, used as T factor	Ts estimated based on heat flux and heat flow used as a T factor compared to optimum	Uses modeled T_s applied through Arrhenius function	Different temperature optima across guilds Optimum set to 25°C
Water	Optimum WFPS about 55% if low scales down nitrification from moisture stress, high scaled down by DOC	Soil moisture content converted to WFPS, Moisture reduction factor, optimum at 90% WFPS	Water film thickness from modeled water potential	Assumed in water films
Nitrifier abundance	Not modeled	Nitrifier biomass, Nitrifier-bacterial growth and death rate are functions of DOC and a T factor.	(Active) Nitrifier biomass growth by double Monod functions of CO_2s and NH_3s AO and NO separately	Growth and death of biomass through C and N equations
Nitrifier denitrification (N gas from nitrification)	Fraction of N nitrified	Function of water-filled pore space and quantity of N nitrified	Process included when O_2 limits rate of NH_3 oxidation	Decomposition of hydroxylamine or detoxification of NO_2^- due to uncoupling

AO, ammonia oxidation; NO, nitrite oxidation; T, temperature; s, soil; WFPS, water filled pore space; DOC, dissolved oxygen concentration.

substrate for the crucial integral membrane protein ammonia monooxygenase (AMO) is generally accepted as solution NH_3 (Suzuki et al., 1974). All known substrates and competitive inhibitors of AMO are non-polar (Suzuki et al., 1974; Hooper et al., 1997; Arp et al., 2002) suggesting that the AMO active site is a non-polar environment. Rapid equilibration in aqueous environments means this solution NH_3 form is transient and seldom directly measured in soil environments. The determination of solution NH_4^+/NH_3 in soils is complicated by the microsite variability in pH and the sorption capacity of the soil (Venterea et al., 2015). These relationships are of particular importance after fertilization or urine deposition resulting in localized high concentrations of substrates. Many but not all AOB and AOA are capable of using urea and have genes encoding urease enzymes and urea transporters (De Boer and Laanbroek, 1989; Burton and Prosser, 2001; Koper et al., 2004; Tourna et al., 2011; Lu and Jia, 2013; Shen et al., 2013). Some comammox organisms and NOB also possess ureolytic activity (Koch et al., 2015, 2019; Palomo et al., 2018).

The injection of anhydrous ammonia and banding of urea fertilizers in soils results in temporarily extremely high NH_4^+/NH_3 concentrations and high pH as well. In these localized zones total NH_4^+/NH_3 may reach from several hundred up to 2,000 mg N/kg soil (Venterea et al., 2015). Under these episodic high concentrations, existing populations of ammonia oxidizers are operating at maximum capacity or even inhibited by high substrate (NH_3) or product (NO_2^-) concentrations.

In general, NO_2^- does not accumulate in soils except under transient conditions that have decreased the population or inhibited the activity of nitrite oxidizers. The intensive application of ammonical fertilizers (i.e., urea or anhydrous NH_3) may result in NO_2^- accumulation due to the inhibition of NO_2^- oxidation from the toxicity of high NH_3 levels in the application zone (Schmidt, 1982; Maharjan and Venterea, 2013; Giguere et al., 2017) or from subsequent localized lowering of pH and production of nitrous acid (Venterea and Rolston, 2000a). Any circumstance under which the rate of NH_3 oxidation exceeds that of NO_2^- oxidation will result in accumulation. This accumulation of NO_2^- is an important driver of N_2O/NO_x production by both biological and abiotic reactions (Venterea et al., 2015; Heil et al., 2016; Breuillin-Sessoms et al., 2017; Giguere et al., 2017). The interaction of soil pH, buffering capacity and ionization of NH_3 and NO_2^- may be useful predictors of NO_2^- accumulation and the associated increased production of N_2O/NO_x via nitrification and nitrifier-denitrification (Venterea and Rolston, 2000b).

The deposition or application of animal wastes due to grazing or amendments leads to local zones of high organic N, urea and NH_4^+/NH_3. Typically, over 70% of the N in ruminant urine is found as urea and localized deposition zones reach NH_4^+/NH_3 concentrations and elevated pH similar to those found in urea fertilizer bands. For intensively grazed pastures levels of deposition may reach up to 600–1,200 kg N ha^{-1} significantly exceeding uptake by pasture plants (Hamonts et al., 2013). Applications of manures and composts to agricultural lands adds urea, organic N and NH_4^+/NH_3 often stimulating nitrification rates in the receiving soils (Li et al., 2012).

Rates of NH_3 emissions are increasing with agricultural activities accounting for 80–90% of anthropogenic emissions. Increasing manure production and N fertilizer use drives NH_3 emissions and then subsequent deposition to land surfaces both globally and locally. Total N in wet and dry deposition approximately tripled during the last century (Simkin et al., 2016). Deposition typically occurs at a sustained elevated level in contrast to the large pulses of NH_4^+/NH_3 due to fertilization. These increased inputs can be expected to affect soil inorganic N pools for surface soils, most importantly in low fertility ecosystems.

Rates of NH_4^+/NH_3 production and consumption are important controls on the rate and extent of nitrification (Norton, 2008; Grant et al., 2016). Mineralization is the general term for the conversion of organic N to inorganic N as either NH_4^+ or further to NO_2^-/NO_3^-, ammonification is the conversion of organic N to the NH_4^+ form while immobilization is the assimilation of inorganic N to organic N generally mediated by microorganisms. Mineralization-immobilization turnover (MIT) refers to the combined transformations between organic and inorganic N that accompanies the growth and death of the soil biota. The supply of NH_4^+ for nitrification depends upon the balance of mineralization to immobilization and the quality and quantity of substrate for decomposition. Soil organic C and N pool size are effective predictors of soil mineralization rates when considered over continental scales (Booth et al., 2005). In tightly coupled N cycles the pool size of NH_4^+ does not reflect the supply of this substrate. Plant uptake may compete directly for NH_4^+. Assessment of the true inorganic N supplying capacity of the soil, i.e., gross ammonification, may better represent the absolute flux of inorganic N produced by soil N mineralization (Van Groenigen et al., 2015). The fraction of the mineralized N that is nitrified or the ratio of gross nitrification to mineralization (GNR/GMR) (**Table 2**) is considered an index of the nitrifying capacity of soils (Booth et al., 2005; Habteselassie et al., 2006). Nitrification potentials that measure short-term nitrite/nitrate production in shaken soil slurries with non-limiting substrate supply, are useful indicators of the enzymatic potential for nitrification but are not necessarily predictive of *in-situ* rates (Hart et al., 1994; Norton and Stark, 2011). Soils that have received repeated applications of composts and manures typically show increases in the ratio of gross nitrification rate to nitrification potential (GNR/NP) because high rates of mineralization continuously supply substrate NH_4^+ (**Table 2**) (Habteselassie et al., 2006; Ouyang et al., 2016). Relationships of mineralization to nitrification rates are best assessed through the determination of gross rates using isotope pool dilution and modeling approaches. These comparisons of gross and net nitrification rates are evidence that net nitrification measurements are poor predictors of gross nitrification rates for many soils (Stark and Hart, 1997; Burger and Jackson, 2003, 2004; Habteselassie et al., 2006; Norton and Stark, 2011; Han et al., 2012).

Environmental Conditions–Temperature, Soil Moisture, Aeration, and pH

Temperature

The response of nitrification to temperature has been evaluated in a diverse range of soils, and the optimum temperature for

TABLE 2 | Ratios of gross and net N transformation rates for an agricultural soil under silage corn that received ammonium sulfate (AS), dairy waste compost (DC), and dairy liquid waste (LW) at 100 and 200 kg available N ha^{-1} for 6 years.

Treatment	NNR[a]/GNR	GNR/GMR	GNR/NP
AS100	0.38[ba]	0.59[b]	0.10[b]
AS200	0.36[ba]	0.66[b]	0.09[b]
DC100	0.16[b]	1.36[ab]	0.58[a]
DC200	0.15[b]	1.88[a]	0.64[a]
LW100	0.46[ab]	0.78[ba]	0.18[b]
LW200	0.69[a]	0.66[b]	0.22b[a]

GNR/GMR, and GNR/NP values are means for year 1999 to 2002 (Habteselassie et al., 2006). Numbers followed by same letter within a column are not significantly different at $P < 0.05$.

[a]From laboratory incubation measurements.

NNR, net nitrification rate; GNR, gross nitrification rate; GMR, gross mineralization rate; NP, nitrification potential.

nitrification has been found to be environment specific (Stark, 1996; Parton et al., 2001; Lu et al., 2018). Across a range of North American ecosystems, the community composition of AOB was correlated with temperature as indicated by mean annual temperature (Fierer et al., 2009). The temperature optimum for nitrification in an AOA dominated soil has also been found to be increased under selective warming pressure and to have selected for temperature optima related to the environment (Daebeler et al., 2017). Overall soil microbial communities tend to be temperature generalists since they are adapted to wide swings of temperature in surface soil habitats (Wallenstein and Hall, 2012). Generally, the optimum temperature for maximum short-term nitrification rates (i.e., V_{max}) may exceed the temperatures normally experienced at the site under consideration and may exceed the temperature optimum for growth of nitrifier biomass (Stark and Firestone, 1996; Taylor et al., 2017). Cultured AOB from soils generally have temperature optimum between 25 and 30°C (Jiang and Bakken, 1999), but *N. cryotolerans* from the Arctic Ocean has a temperature optimum for growth of 22°C and can grow at 0°C (Koops et al., 1991). There is evidence for soil nitrifier activity under similarly cold temperatures typical of winter season soils (2–10°C) (Cookson et al., 2002) and for nitrification in AOA dominated Artic soils (Alves et al., 2013). Recent evidence suggests that certain groups of acid tolerant AOA may also be adapted to lower temperatures regimes (Gubry-Rangin et al., 2017). The temperature response of nitrification has been modeled using the Arrhenius equation (Grant, 1994), a Poisson density function (Stark, 1996; Ouyang et al., 2017), square root (SQRT) function or using macromolecular rate theory (MMRT) (Taylor et al., 2017). Studies performed with pure cultures and with mixed environmental consortia from temperate agricultural soils consistently indicate that AOA activity has a higher temperature optima and higher temperature minimum than AOB activity (**Figure 2**) (Ouyang et al., 2017; Taylor et al., 2017; Lu et al., 2018). Modeled temperature response parameters may be useful for trait based modeling linking microbial populations to nitrification rates (Bouskill et al., 2012; Breuillin-Sessoms et al., 2017).

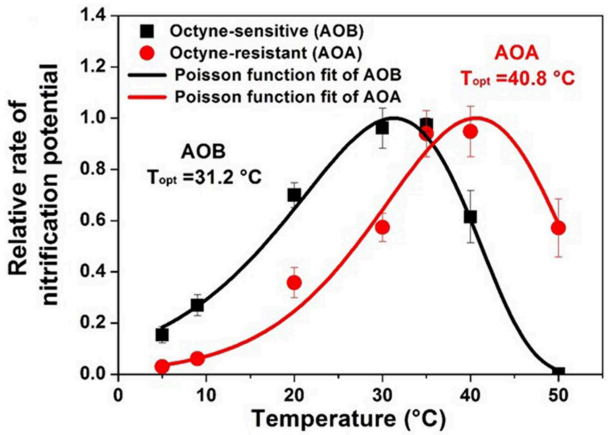

FIGURE 2 | Temperature response of the relative potential nitrification rates for AOB (octyne-sensitive) and AOA (octyne-resistant) from a calcareous agricultural soil in Utah, USA. Rates are normalized to the fraction of maximum nitrification potentials at optimum temperature. Lines predicted by generalized Poisson density equation (Adapted from Ouyang et al., 2017).

Moisture/Aeration

Soil moisture affects nitrification rates through several confounding influences of substrate availability of both ammonium and oxygen by diffusion and direct effects of dehydration at very low water potentials. These interdependent factors often confound experiments to determine the role of soil drying and wetting on nitrification rates under field conditions (Stark and Firestone, 1995; Placella and Firestone, 2013). Optimum water filled pore space (WFPS) for nitrification is around 55% for fine textured soils and around 40% WFPS for coarse textured soils (Parton et al., 2001) see **Supplemental Figure 1**). Nitrification in soils saturated with water (i.e., water potential approaching 0 kPa) is inhibited due to lack of available oxygen. Nitrification nearly halts in very dry soils (<-3.0 MPa), such as found under seasonal dry xeric or aridic soil climates. In general, the diffusion of substrates limits nitrification activity most near optimum water potentials, whereas the adverse physiologic effects associated with cell dehydration will be the most limiting factor at very low water potentials (Stark and Firestone, 1995). In the nitrification submodel of DayCent (Del Grosso et al., 2012) nitrification is limited by moisture stress when soil water-filled pore space (WFPS) is too low and by O_2 availability when WFPS is high based on soil textural class (**Supplemental Figure 1**). In the highly detailed model Ecosys, O_2 availability is based on water film thickness and the wide range of competing microbial processes consuming O_2 (Grant and Pattey, 2003).

Soil pH

The soil pH is one of the most important factors controlling rates and product accumulation from nitrification see **Supplemental Figure 1** (Parton et al., 2001; Kyveryga et al., 2004). Rates of both ammonia and nitrite oxidation are generally favored by neutral to slightly alkaline soils and it is in these soils that the largest losses or accumulations of NO_3^- generally

occur. Management of agricultural soil pH by liming is common practice in vast areas of crop production and is often necessary to offset acidification due to fertilizers. Currently ∼40% of the world's arable soils are acidic and this area has recently been increasing (Kunhikrishnan et al., 2016). High rates of nitrification and leaching of NO_3^- further acidify agricultural soils (Schroder et al., 2011). During the Twentieth century, observations that nitrification was occurring in acids soils from both natural and agricultural ecosystems continued to accumulate with observations from tea plantations, heath soils and coniferous forests (De Boer and Kowalchuk, 2001). During this same time frame the available isolates of AOB were fairly intolerant of acidity and their nitrification rates decreased dramatically as pH decreased (De Boer and Kowalchuk, 2001). These observations were partially explained by the known low concentrations of NH_3 (NH_4^+/NH_3 couple has a $pK_a = 9.25$) and the contention that NH_3 is the actual substrate for ammonia oxidizers (Suzuki et al., 1974). The use of urea as a substrate, microsite variability of soil pH and heterotrophic nitrification were able to explain some portion of nitrification observed in acid soils (Burton and Prosser, 2001). Since the role of AOA in ammonia oxidation in the soil environment was revealed (Treusch et al., 2005; Leininger et al., 2006; Nicol et al., 2008) the importance of AOA in the ammonia oxidation of acid soils has gained increasing support (Nicol et al., 2008; Gubry-Rangin et al., 2010; Yao et al., 2011, 2013; Prosser and Nicol, 2012; Li et al., 2018). Members of the AOA *Nitrosotalea* lineage are abundant and widely distributed in acidic soils globally (Gubry-Rangin et al., 2011). An obligate acidophilic isolate, Ca. *Nitrosotalea devanaterra*, is unable to grow at neutral pH (Lehtovirta-Morley et al., 2011) and exhibits specialized genomic inventory for functioning under acid conditions (Lehtovirta-Morley et al., 2016b). Soil pH has also been observed to affect the nitrite oxidizer community (Han et al., 2017).

Effects of Abundance and Community Structure of Nitrifiers on Rates

There is a complex interaction between the soil environment, plant community and management (especially fertilization) that determines the community structure of nitrifiers in agricultural soils (Bertagnolli et al., 2016; Han et al., 2018). The abundance and ecotypes of the ammonia and nitrite oxidizers present in the soil may control the immediate rate of nitrification especially when substrate is in excess. Generally, when fertilizers are applied the existing populations respond relatively quickly to the transient increased substrate availability dependent upon favorable environmental conditions. Comparisons between the responses of AOB and AOA to fertilizers suggest that the kinetics of their responses to substrate are distinct (Prosser and Nicol, 2012). An example from Utah agricultural soil comparing the AOB and AOA response shows that AOA reached a lower Vmax at a much lower substrate availability (**Figure 3**) (Ouyang et al., 2017). Nitrification driven by AOA was also found to be saturated at relatively low NH_4^+ in a range of Oregon soils (Giguere et al., 2015). These observations explain why some studies have observed a positive correlations between the abundance of AOB and nitrification potential rates performed at relatively high NH_4^+ (1 mM) but little or no correlation with potential rates and AOA abundances (Jia and Conrad, 2009; Taylor et al., 2012; Ouyang et al., 2016). In contrast to these

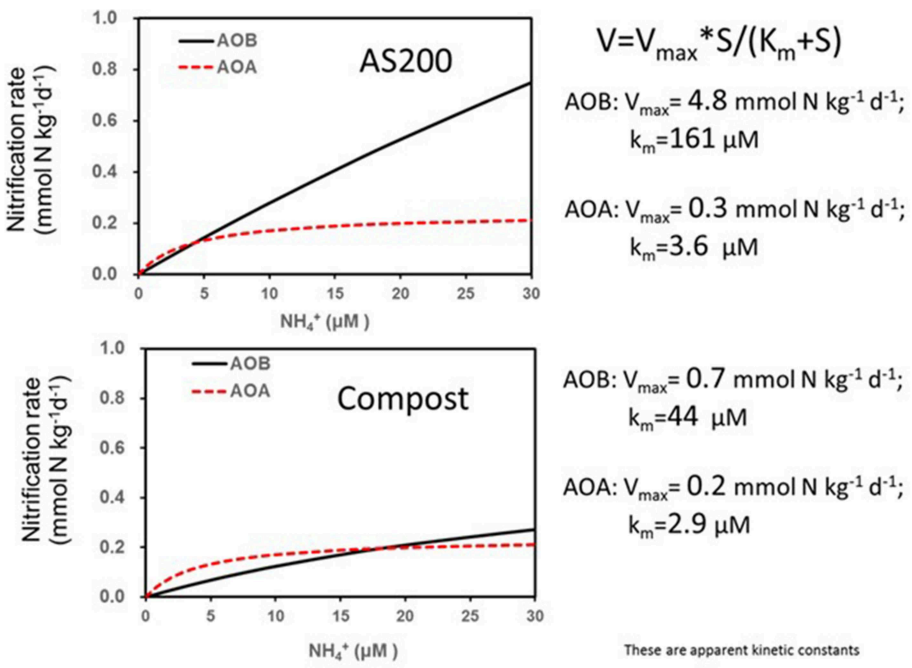

FIGURE 3 | Nitrification rate kinetic models based on substrate concentrations for a calcareous agricultural soil from Utah treated for 3 years with either ammonium sulfate or steer waste compost at 200 kg N/ha. Soils were sampled 28 days after fertilization (adapted from Ouyang et al., 2017).

observations, under conditions such as acid soils that favor AOA, nitrification rate is often proportional to AOA gene abundance (Yao et al., 2011). In Scotland, a survey that included nitrification potentials, AOB and AOA abundances and their communities found that specific phylotypes of AOA and AOB were linked to soil niches described by combinations of soil pH and fertilization (Yao et al., 2013). In general, acidic soils from non-fertilized systems exhibited lower rates and were dominated by distinctive AOA phylotypes. In calcareous agricultural soils from Utah, nitrification potentials (at 1 mM NH_4^+) were dominated by activity of AOB related to *Nitrosospira* even though the AOA were more abundant. However, in these same soils, net and gross nitrification rates were mediated by AOB in the first weeks following fertilization then, after ammonium was depleted, the activity was dominated by AOA **Figure 4** (Ouyang et al., 2017). Candidatus *Nitrososcosmicus franklandus* is an AOA strain (*Archaea*, *Thaumarchaeota, Nitrososphaerales*) isolated from circum-neutral pH, fertilized soil in Scotland (Lehtovirta-Morley et al., 2016a) has an overlapping ammonia tolerance to known AOB soil isolates. In the acidic red soils of China after 16 years of contrasting fertilizer treatments (He et al., 2007), the AOA remained dominant but both AOA and AOB abundances were increased by organic and inorganic fertilizers, both AOA and AOB played a role in nitrification activity. The abundance of AOA and AOB has been suggested as a bioindicator for soil monitoring based on their differential responses to soil management and relative ease of quantification by real-time PCR (Wessen et al., 2010; Wessén and Hallin, 2011).

Enrichment and pure culture studies of the AOB suggest that substrate kinetics and growth response of AOB differ even within a genus (Jiang and Bakken, 1999; Koops and Pommerening-Roser, 2001; Bollmann et al., 2002; Webster et al., 2005; Bouskill et al., 2012; Sedlacek et al., 2019). Different ecophysiology within the AOA is also indicated by pure culture work (Gubry-Rangin et al., 2011; Martens-Habbena and Stahl, 2011; Hatzenpichler, 2012; Kits et al., 2017).

Fewer assessments have been made of the NOB communities of agricultural soils. Several studies suggest nitrite oxidation in agricultural soils is primarily catalyzed by NOB communities with members from *Nitrospira* and *Nitrobacter* (Freitag et al., 2005; Xia et al., 2011; Pester et al., 2014). Higher potential rates of nitrite oxidation have been found to be associated with *Nitrobacter* vs. *Nitrospira* dominated communities and shifts toward *Nitrobacter* types are often associated with changes in management such as nitrogen fertilization and tillage (Attard et al., 2010; Le Roux et al., 2016; Han et al., 2018). The growth of *Nitrobacter* populations as indicated by *nxrA* gene copies has been associated with rapid nitrite use and lowered N_2O emissions (Venterea et al., 2015). *Nitrobacter vulgaris* was also found to quickly decrease accumulated nitrite and prevent N_2O emissions in several Oregon soils (Giguere et al., 2017). Pure cultures of *Nitrospira* spp. generally exhibit higher affinities and lower Vmax than *Nitrobacter spp.* (Nowka et al., 2015). Kinetics of the comammox bacterium, *Nitrospira inopinata,* suggest an oligotrophic lifestyle as well (Kits et al., 2017).

Feedback between fertilizer application and abundance occurs because both the activity and the abundance of nitrifying organisms increase following fertilization with ammonical N fertilizers (He et al., 2007; Ouyang et al., 2016, 2018; Xiang et al., 2017; Orellana et al., 2018). A recent meta-analysis examined the impact of N fertilization on the abundance of N cycling genes in agricultural soils showed that the positive effect size was significant for both the AOA and AOB from a survey of ∼100 samples each (Ouyang et al., 2018). In an earlier meta-analysis (Carey et al., 2016) the AOB abundance was found to be more responsive to N fertilization than that of AOA for the majority of observations. AOB abundance was associated with increased nitrification potentials in fertilized soils. In Utah soils, both the abundance and the community of ammonia AOB

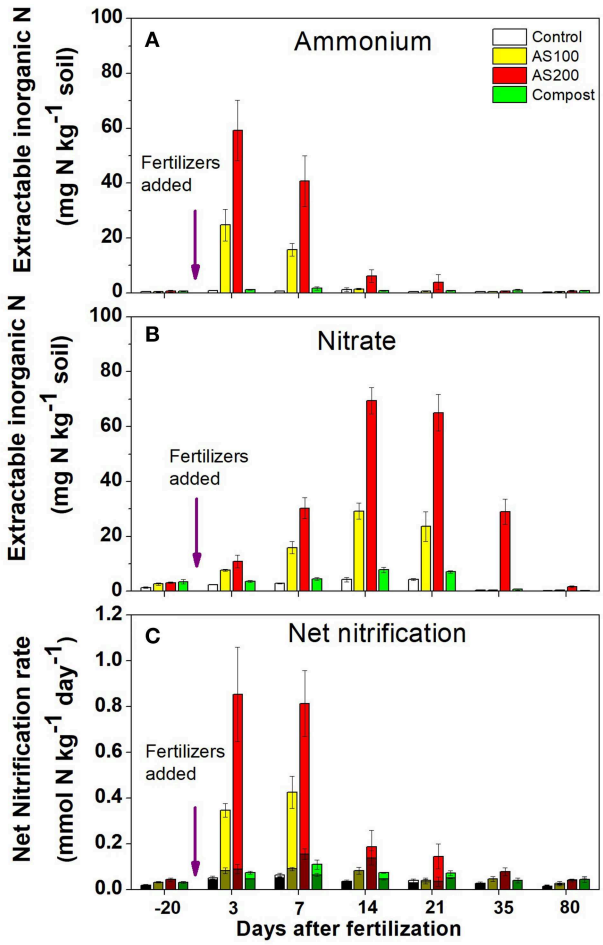

FIGURE 4 | Inorganic N pools and net nitrification rates for a calcareous agricultural soil from Utah. Field plots had been treated for four previous years with either no N fertilizer (control), ammonium sulfate 100 kgN/ha (AS 100), ammonium sulfate 200 kgN/ha (AS 200) or steer waste compost at 200 kg N/ha (Compost) under silage corn production. Observations for 2015 growing season for: **(A)** ammonium N pool size, **(B)** nitrite +nitrate N pool size, **(C)** net nitrification rate for octyne-sensitive (AOB) net nitrification, octyne-resistant (AOA) net nitrification, and total net nitrification. Octyne-resistant nitrification rates (AOA) are the shaded bottom portion of each bar, octyne-sensitive nitrification rates (AOB) are the lighter top portion of each bar (Adapted from Ouyang et al., 2017).

were more responsive than those of AOA to repeated annual applications of ammonium sulfate fertilizer (Ouyang et al., 2016). Generally, nitrification potential activities were more sensitive to agricultural management practices and environment disturbance than to the abundance and diversity of nitrifiers. For example, in our Utah soil, nitrification potentials were significantly increased by N fertilizers after the first fertilization, while *amoA* gene abundance and diversity showed no significant difference among treatments (Ouyang et al., 2016). Similarly, we found that the nitrite oxidation potentials were significantly stimulated by fertilizers while *nxrB* abundances were not affected (Ouyang, 2016). These *amoA* and *nxrB* gene measurement were based on soil DNA, while the rate of ammonia oxidation may be more related to the relationship among transcription, translation, and enzyme function (Nicol et al., 2008; Myrold et al., 2014; Rocca et al., 2015).

Regulation of transcription of nitrification related genes has been examined both in pure cultures (Sayavedra-Soto et al., 1998, 2015; Bollmann et al., 2005; Hawkins et al., 2007; Starkenburg et al., 2008; Park and Ely, 2009; Radniecki and Lauchnor, 2011), and in soil or sediment environments (Tourna et al., 2008; Di et al., 2010; Gubry-Rangin et al., 2010; Abell et al., 2011; Herrmann et al., 2012; Placella and Firestone, 2013) primarily targeting *amoA* transcription. In a meta-analysis of functional genes and transcript abundance and their relationship to process rates (Rocca et al., 2015) there was less correlation between transcript level (mRNA) and process rates than with gene abundance and process rate. This lack of relationship between transcription and process rate is not surprising considering differences in transcript stability (turnover), transient and episodic rates of transcription and subsequent translation and difficulties with methods for determining transcript abundance in environmental matrices.

A proteomic approach might be appropriate for explaining short-term changes in nitrification activity. The ideal method is to extract and purify key enzymes such as AMO and NXR directly for assays in soils, but the membrane-bound feature of these enzymes makes this strategy difficult (Arp et al., 2002; Kerou et al., 2016). However, the recent study on activity-based protein profiling of AMO in *Nitrosomonas europaea* may pave a way to indirectly quantifying active AMO fluorescently in soils (Bennett et al., 2016). Nitrification is likely the soil N cycle process for which we are approaching a level of understanding when we may include some nitrifier community characteristics into process models using trait-based modeling approaches (Bouskill et al., 2012; Le Roux et al., 2016).

Plant and Microbial Interaction With Nitrifiers

Plants take up and assimilate both NH_4^+ and NO_3^-, but often shows substantial differences in preference for one inorganic N form (Marschner, 2011). This NH_4^+ or NO_3^- preference of plant species could exert differential effects on nitrifiers (Patra et al., 2006; Skiba et al., 2011; Thion et al., 2016). Plant often stimulates soil N transformation processes by releasing C into the rhizosphere either as root exudates or as direct transfers to mycorrhizal fungi (Phillips et al., 2011; Shi et al., 2016; Meier et al., 2017). A meta-analysis summarized that N transformation processes were significant higher in rhizosphere than bulk soil, including net and gross N mineralization and net nitrification (Finzi et al., 2015). Rhizosphere interactions have been observed to decrease nitrification (net and gross rates) by favoring plant and microbial assimilation of NH_4^+ (Hawkes et al., 2007). Some plants are able to produce nitrification inhibitors in their root exudates, and therefore suppress nitrifier activities (Subbarao et al., 2013, 2015; Coskun et al., 2017). While competition for N between plants and microbes is very strong in the rhizosphere, it is not clear if nitrifiers will outcompete heterotrophic microbes in the rhizosphere (Kuzyakov and Xu, 2013).

Evidence from pure cultures indicate that *Nitrosomonas* spp. are weak competitors for NH_4^+, compared to heterotrophic bacteria (Verhagen and Laanbroek, 1991; Verhagen et al., 1994; Bollmann et al., 2002). In many agricultural soils, gross nitrification rates are often 1–75 fold higher than rates of microbial NH_4^+ assimilation indicating that soil nitrifiers are strong competitors for NH_4^+ (Burger and Jackson, 2003; Booth et al., 2005; Inselsbacher et al., 2010). Heterotrophic microbes may assimilate nitrate as well especially under high organic matter and high C availability. The balance between organic C and NH_4^+ availability will likely determine the fate of NH_4^+ during competition in agricultural soils.

Arbuscular mycorrhizal fungi (AMF) may play an important role in mediating availability of NH_4^+ to nitrifiers. AMF could directly compete for NH_4^+ (Veresoglou et al., 2011, 2012; Chen et al., 2013; Storer et al., 2018), but also likely exert indirect influences on nitrifiers via the plant (Chen et al., 2013; Veresoglou et al., 2018). AOA community composition was altered more than the AOB community by AMF (Chen et al., 2013). Ectomycorrhizal fungi produce many extracellular enzymes for N mineralization and may increase the availability of soil N (Courty et al., 2010). Interestingly, ectomycorrhizal fungi inoculation changed AOA, but not AOB communities in an acid soil (Li et al., 2019).

The biological interaction between soil microfauna and microorganisms in the soil food web also mediates soil N cycling (Xiao et al., 2010; Jiang et al., 2014; Trap et al., 2016; Zhu et al., 2018). For example, Xiao et al. (2010) found the presence of bacterivorous nematodes significantly stimulated nitrification activity and changed the community composition of AOB. Interestingly, Zhu et al. (2018) showed bacterivorous nematodes significantly reduced the abundance of AOB, but increased AOA, irrespective of the nematode species in the soil. There may also be a role for bacterial predators such as *Micavibrio* that have been observed in wastewater systems to prey upon *Nitrospira* (Dolinšek et al., 2013). The knowledge of potential environmental interactions between viruses and nitrifiers is limited although genomes of AOB have shown evidence of prophage (Chain et al., 2003; Stein et al., 2007; Norton et al., 2008). More recently prophage induction by stress followed by lysis was demonstrated in *Nitrosospira multiformis* (Choi et al., 2010). The outcome of these complex interactions in agricultural soils is driven by the timing and intensity of organic C and available N and their distribution by mass flow and diffusion through the soil fabric.

MANAGING NITRIFICATION IN AGRICULTURAL SOILS

Meeting world food demand while reducing surplus N lost to the environment will require substantial increases in the NUE of agricultural systems (Zhang et al., 2015). Management strategies are needed that minimize the risk of N loss even in high productivity systems that necessarily require high N inputs. As the demand for food production increases globally, the production and use of N fertilizer will likely continue to increase from ∼110 Tg N in 2013 up to 120 Tg by 2018 (FAO, 2015). The vast majority of N fertilizers applied to soils are in the ammonical forms including urea (57% in 2013 and increasing) and are therefore subject to nitrification after application. In the United States, ∼50% of this N fertilizer is used on maize (corn) crops (USDA, ERS, 2018). As agriculture intensifies, there will be higher levels of N applied to reach the yield potential of the most productive varieties if current conventional management continues. Common principles for N management include the "4Rs" approach of applying the right source, at the right rate, at the right time in the right place (Clarke and Beegle, 2014). Many appropriate technologies are currently available to reduce nitrification, greenhouse gas (GHG) emissions and N losses but these may require appropriate incentives for farmers to adopt (Robertson et al., 2013). Complex models such as DayCent (Del Grosso et al., 2012) that are used for the estimation of the flux of N_2O from agricultural soils include nitrification submodels. The outcomes of management activities may be simulated and assessed with these tools.

China has some of the most intensive use of N fertilizers and associated high levels of N loss. In a meta-analysis of Chinese agriculture, management practices designed to minimize N loss were assessed including: the application of controlled-release N fertilizers, nitrification inhibitors (NI) and urease inhibitors (UI), higher splitting frequency of fertilizer N application, lower basal N fertilizer (BF) proportion, deep placement of N fertilizer, and optimizing N rate based on soil N test (Xia et al., 2017). These knowledge-based N fertilization practices were generally effective at reducing N loss by leaching, runoff and GHG emission while showing some increases in economic return. Split applications of N and the use of enhanced efficiency fertilizers including those with polymer coatings and urease and nitrification inhibitors will make increased economic sense if they are used selectively under those environmental conditions where the potential N loss is high (Motavalli et al., 2012).

Management practices that improve or maintain soil health such as disturbing the soil less (reduced tillage), growing greater diversity of crops (in rotation and as diverse mixtures of cover crops), maintaining living roots in the soil as much as possible (with crops and cover crops), and keeping the soil covered with residue at all times will increase the resiliency of agroecosystems and decrease N losses (Zhang et al., 2015). These practices will likely result in decreased net nitrification while maintaining yields. The implementation of this knowledge to build more resiliency into our agricultural systems will need support from socioeconomic policy research.

Management to Control Ammonium Substrate Availability

The goal of N fertilizer rate recommendations is to estimate the gap between the N supplied by the soil and the N required for the crop to reach an optimum yield. In the United States and Europe, regional yield response curves and the fertilizer-crop price ratio are often used to provide recommendations to farmers on economically optimal N application rates (Sawyer et al., 2006; Morris et al., 2018). Decreasing this basal N fertilizer rate will logically decrease N availability to nitrification but risks reducing yields enough to be an economic disadvantage and even increase overall environmental impacts. Therefore, rather than decrease overall N rate, approaches designed to improve NUE while maintaining yields may mitigate the risks associated with nitrification. Strategies for controlling NH_4^+ substrate availability include timing of fertilization to coincide with rapid plant uptake, formulation of fertilizers as slow release forms and/or with inhibitors (e.g., urease inhibitors), keeping plants growing continuously to assimilate N, and increasing microbial N immobilization (**Figure 5**).

Timing of Fertilization to Coincide With Rapid Plant Uptake

Generally, N fertilizers can be applied either before planting, as a sidedress, or as a split preplant-sidedress fertilizer treatment. Nitrogen is used more efficiently if applied during the growing season prior to the time of maximum plant uptake rate, as compared to application before the crop is planted (Sawyer et al., 2006). The timing of this split application may be based on crop stage or other plant or soil testing indicators such as the presidedress nitrate test (PSNT). Sidedress fertilization has been observed to reduce yield scaled N_2O by 60% vs. fall fertilization (Abalos et al., 2016) and often results in improvements in NUE (Ma et al., 2010). Unfortunately, there remain large areas in the US Midwest and Canada where convenience favors anhydrous ammonia application to drier soils during the fall preceding spring planting. This approach is based on the principle that cold soil temperatures will slow nitrification sufficiently to retain fertilizer in the soil. Fall applications typically reduce NUE and must be timed carefully to wait until soil temperatures decrease enough to postpone nitrification activity until spring. Nitrification inhibitors are often combined with fall applications to delay nitrification but these may not remain effective through to the following spring.

Keep Plants Growing Continuously to Assimilate N

Competition with plants for available N can decrease nitrification and decrease nitrate accumulation. In many non-agricultural systems, plant N uptake occurs across seasons and N is retained in organic forms and in plant roots. Additionally, even when nitrification is occurring, there may be little net nitrification measurable because of nitrate use by plants and heterotrophic microbes (Stark and Hart, 1997; Norton and Stark, 2011). A range of N conserving mechanisms have evolved in natural

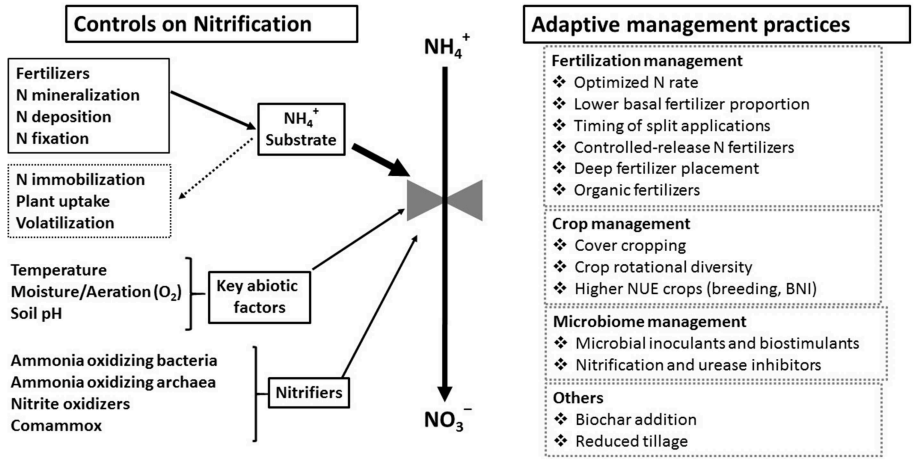

FIGURE 5 | Relationship of controlling factors for nitrification to adaptive management practices promoting systems with higher nutrient use efficiency.

ecosystems including direct uptake of organic N by plants (by short-circuiting mineralization) and suppression of nitrification. These mechanisms essentially close the N cycle and facilitate soil organic N accumulation. The use of cover crops, living mulches and catch crops keeps living plant roots in the soil, adds organic matter to the system, and decreases nitrate accumulation and potential leaching (Abdalla et al., 2019). Cover crops must be managed carefully especially in drier climates to avoid decreases in the productivity of the primary crop due to water or nutrient uptake while promoting soil nitrate recycling.

Controlled-Slow Release Fertilizers

Slow/controlled release fertilizers are designed to better match the timing of nutrient release to the plant demand. Because of cost factors, their use in agricultural settings is limited although they are widely used in horticultural applications. Urea is one of the most widely used fertilizers in agriculture and is extremely soluble. Slow release coatings may be applied to limit solubility and delay urea hydrolysis and subsequent nitrification. Urea coatings include organic polymer coatings and inorganic coatings such as sulfur, their characteristics and merits of these materials have been reviewed recently (Naz and Sulaiman, 2016).

Intensify Soil Internal N Cycling

The use of inorganic fertilizers simplifies the soil internal N cycling process, leading to a high-nitrifying agricultural system (**Figure 6A**). Nitrate is often a dominant N pool, especially shortly after fertilization, in these agricultural soils. When the proportion of N supply to the plant by N fixation and N mineralization is increased relative to fertilizers sources then a low-nitrifying agricultural system is favored that reduces N loss and improves NUE (**Figure 6B**). Increased diversity of N cycling functional groups may also help retain N in soil. Intensified internal N cycling may be accomplished by the addition of high C organic amendments such as compost, manure, and biochar (Paustian et al., 2016); and by direct inoculation of N-fixation and mineralization promoting bacteria and AMF (Hu and He, 2018).

Inhibit Nitrifiers Directly

Nitrification Inhibitors

Nitrification inhibitors (NIs) slow the microbial conversion of ammonium-N to nitrate-N (nitrification), reducing the risk of loss through leaching or denitrification and thereby increasing the NUE of fertilizers. Many synthetic NIs act on the ammonia monooxygenase enzyme often as competitive suicide subtrates (for example acetylene) (McCarty, 1999). Several nitrification inhibitors that are widely used in agriculture include: (1) 2-chloro-6-(trichloromethyl) pyridine (nitrapyrin), (2) dicyandiamide (DCD), and (3) 3,4-dimethylepyrazole phosphate (DMPP). Urease inhibitors, such as N-(n-butyl) thiophosphoric triamide (NBPT), are used to decrease urea hydrolysis and volatilization. Meta-analyses report that the application of urease and nitrification inhibitors significantly reduced inorganic N leaching (−48%), N_2O emission (−44%), and NO emission (−24%) (Burzaco et al., 2014; Qiao et al., 2015; Thapa et al., 2016) while increasing crop yield (7.5%) and NUE (12.9%) (Abalos et al., 2014). The beneficial effect of nitrification inhibitors may depend on the environment (e.g., soil pH and texture) and other management factors (e.g., irrigation and N fertilizer rate) (Abalos et al., 2014). The longevity of the inhibitors under soil conditions as affected by temperature is of key importance for their effectiveness (Menéndez et al., 2012; Guardia et al., 2018). Reaching the optimum balance between N oxides and greenhouse gas losses, N efficiency and crop yields often indicates the use of nitrification inhibitors with liquid organic sources such as manure slurries (Guardia et al., 2017). However, the use of nitrification inhibitors also increases cost, potential for NH_3 emission and the risk of environmental contamination (Kim et al., 2012; Qiao et al., 2015). Recently, nitrapyrin has been detected in streams, suggesting off-site transport of this N stabilizing compound (Woodward et al., 2016) and DCD residues were detected in milk in New Zealand resulting in the suspension of DCD use in pastures (Thapa et al., 2016). Chemical nitrification inhibitors are not permitted in certified organic management systems, so organic alternatives are

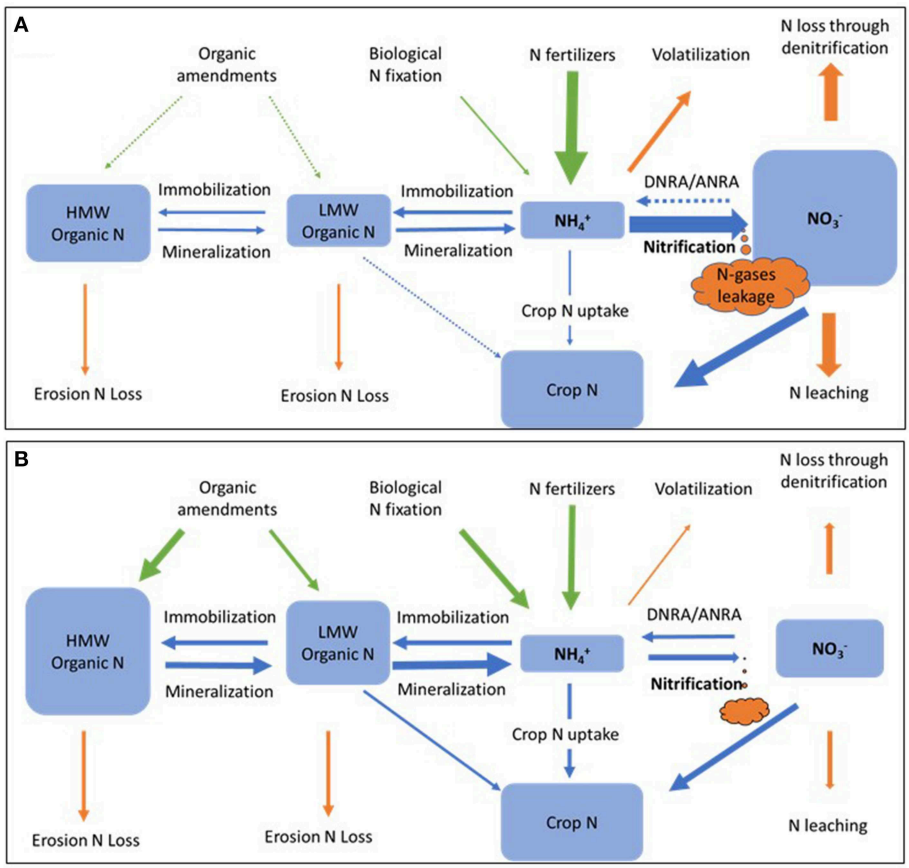

FIGURE 6 | Hypothetic nitrogen pools and flows of high-nitrifying **(A)** and low-nitrifying **(B)** agricultural systems. Arrows represent nitrogen inputs (green), losses (orange), and transformations (blue). HMW, high molecular weight; LMW, low molecular weight; DNRA, dissimilatory nitrate reduction to ammonium; ANRA, assimilatory nitrate reduction to ammonium.

needed for management of nitrification and the use of neem seed oil (Opoku et al., 2014) has been suggested for this purpose.

Biological Nitrification Inhibition

Biological nitrification inhibition (BNI) is the ability of certain plant roots to impede soil nitrification through the production of biological inhibitors (Subbarao et al., 2013, 2017; Byrnes et al., 2017; Coskun et al., 2017). If BNI may be exploited to reduce nitrification in high nitrifying, low NUE systems then fertilizer use and loss may be decreased with associated decreases in GHG production. Some BNI's have been isolated from tropical pasture grasses that are adapted to low-N environments, in particular *Brachiaria* spp. have high BNI-activity in root systems and among field crops, sorghum (*Sorghum bicolor*) has been observed to produce biological nitrification inhibitors (Subbarao et al., 2015). Incorporation of these crops into rotations or pasture systems may help to retain N in these soils systems and increase soil N pools. If BNI traits from these plants could be transferred to grain crops, there may be potential benefits to NUE but unknown but likely tradeoffs to productivity. The search continues for biological nitrification inhibitors for the major grain crops especially maize.

Both plants and microbes may produce chemical compounds to inhibit nitrification to compete for ammonium in the rhizosphere. While most studies on BNI focus on plant root exudates; microbes could also produce compounds that inhibit nitrification. Soil microbes produce a wide array of signaling molecules and hydrocarbons including ethylene (Ladygina et al., 2006) that might be exploited for their inhibitory effects.

MANAGING NITRIFICATION UNDER A CHANGING CLIMATE

The goal of reducing N losses from agricultural systems under changing climatic conditions is inherently complex spanning from technical through socio-economic approaches. Management that promotes shifting toward low nitrifying agricultural systems is part of a potential solution. Reducing the residence time and amount of inorganic N pools in agricultural soils while maintaining sufficient N fertility will require system based management. Reductions in the seasonal use of bare fallow, use of cover crops, increases in crop rotational diversity and

perennial crops may increase the capacity for N retention in agricultural soils (**Figures 5**, **6**). Unfortunately, projected impacts of changing climate may make our current mitigation efforts less effective (Le Roux et al., 2016; Bowles et al., 2018). Climate change affects nitrification in agriculture primarily through raising temperatures and the amount and intensity of rainfall (Robertson et al., 2013; Bowles et al., 2018). This combination of factors will increase the propensity for nitrification and subsequent N loss through leaching and denitrification. The controlling factors for nitrification described above have been used as drivers for the rate of nitrification in the process based models DayCent (Del Grosso et al., 2012) and DNDC (Li, 2007) (**Table 1**). In both of these systems nitrification is a function of NH_4^+ availability, water content, temperature, pH, and texture (Grant et al., 2016) although DNDC more explicitly drives microbial reactions by consideration of the redox balance in the soil and the volumetric fraction of the soil that is anaerobic (Li, 2007). Trait-Based models of nitrification predict that there may be changes in ammonia and nitrite oxidizer communities driven by global change contributing to feedback effects (Bouskill et al., 2012; Le Roux et al., 2016). Some factors that are under the control of land managers include: amount, form and application timing of N fertilizers, the use of nitrification inhibitors, and the amount and timing of water application in irrigated systems (**Figure 5**). These factors may be used to parameterize farm-scale (Del Grosso et al., 2016; Paustian et al., 2018) or trait-based models to advise management. However, factors such as the timing and intensity of rainfall, extreme drought events, and the timing of mineralization remain challenging management targets. In the future under a changing climate, elevated temperature and more variable precipitation will likely increase N mineralization and nitrification leading to even more urgent need to manage nitrification and prevent N losses from agriculture (Bowles et al., 2018).

SUMMARY AND FUTURE DIRECTIONS

We review the controlling factors on the rate and extent of nitrification common in agricultural soils from temperate regions including substrate supply, environmental conditions, abundance and diversity of nitrifiers, and plant and microbial interaction with nitrifiers. Two main strategies for managing nitrification are to control ammonium substrate availability or inhibit nitrifiers directly. We propose four key future directions: (1) focus on enzymes involved in nitrification using proteomics—direct extraction of enzymes or fluorescently labeling key enzymes, (2) link ecophysiology in soil to sequence variants for trait-based modeling, (3) discover novel nitrification inhibitors, survey rootzone microbes and cultivars of major crop plants for inhibitory compounds, and (4) improve nitrification management modeling, especially for changing climate scenarios.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

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Cropping With Slag to Address Soil, Environment, and Food Security

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The effective utilization of slag fertilizer in agriculture to neutralize soil acidity, improve crop productivity, mitigate greenhouse gas emissions, and stabilize heavy metals in contaminated soils turns it into a high value added product in sustainable agriculture. These effects could be due to the shift in microbial metabolism and/or modification of microbial habitats. At the system level, soil microorganisms play an integral role in virtually all ecosystem processes. There is a growing interest to reveal the underlying mechanisms of slag-microbe interactions and the contribution of soil biota to ecosystem functioning. In this perspective, we discuss the possible driving mechanisms of slag-microbe interactions in soil and how these slag-microbe interactions can affect crop yield, greenhouse gas emissions, soil carbon sequestration, and heavy metal stabilization in contaminated soils. In addition, we discuss the problems and environmental concerns in using slag in agriculture. Emphasis has been given for further research to validate the proposed mechanisms associated with slag-microbe interactions for increasing soil quality, crop productivity, and mitigating environmental consequences. While evaluating the slag amendment, effects on agriculture and environment, the potential risks, socio-economics, techno-economics, and ethics should be assessed.

Keywords: microbial dynamics, silicate fertilization, slag, greenhouse gas emissions, carbon sequestration

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INTRODUCTION

Over the past decades, with the rapid growth of industrialization, the higher volume of byproducts (slag) generated from iron/steel production draw attention to the need for its recycling in an increasingly efficient way. With the increase in population, the available land to dispose of large amounts of slag in landfill sites is reduced and the disposal cost is becoming increasingly higher. Moreover, the land filled with disposed slag has become an important source of pollution of air, water, and soil, which further adversely affects vegetation and human health (Branca and Colla, 2012). The entry of heavy metals/metalloids into the food chain is a critical issue of current public health (Chand et al., 2015). From the perspective of natural resource conservation, environmental protection, and human health safety measures, recycling

of slag has drawn the attention of scientists, environmentalists, and policymakers in recent years. The increase of slag recovery and use in different fields of application, such as agriculture, is an imperative way for sustainable development (Ito, 2015).

Slag consists mostly of mixed oxides of elements such as silicon, sulfur, phosphorus, and aluminum, and products formed in their reactions with furnace linings and fluxing substances such as limestone (Yildirim and Prezzi, 2011; Piatak et al., 2015). Since slag is rich in lime (CaO), silicic acid (SiO_2), phosphoric acid (P_2O_5), magnesia (MgO), Mn, and Fe, these properties of the slag can be exploited to make use of fertilizer (Ito, 2015). Notably, steel-making slag and blast furnace slag have been extensively utilized as raw materials for fertilizer production, mostly in Japan, Korea, and China. Fertilizers made of slag are categorized as slag silicate fertilizer, lime fertilizer, slag phosphate fertilizer, and iron matter of special fertilizer (Ito, 2015). In recent years, several studies have revealed that the slag-based fertilizer amendment in agriculture has great promise to improve crop productivity (White et al., 2017; Gwon et al., 2018), alleviate soil acidification (Ning et al., 2016), mitigate greenhouse gas (GHG) emissions (Wang et al., 2015; Gwon et al., 2018), and stabilize heavy metals in contaminated soils (Ning et al., 2016), which turns it into a high value added product for sustainable agriculture. These beneficial effects of slag fertilization largely rely on the changes in soil microbial habitats and microbial activities. In fact, at the system level, soil microorganisms play a vital role in virtually all ecosystem processes and provide ecosystem services crucial for the maintenance of soil quality and productivity (Das et al., 2017). In this perspective, we discuss the driving mechanisms of slag-microbe interactions in soil, and slag-microbe interaction effects on crop yield, greenhouse gas reduction, soil carbon storage, and heavy metal stabilization in contaminated soils. Lastly, we discuss environmental concerns about the use of slag in agriculture and the future perspectives.

DRIVING MECHANISMS OF SLAG-MICROBE INTERACTIONS IN SOIL

The shift in soil microbial community and activities in response to slag fertilizer amendment may depend on the type of slag fertilizer (e.g., silicate fertilizer, lime fertilizer, slag phosphate fertilizer, and iron matter of special fertilizer), which modify soil properties and soil microbial habitats. With advances in omic techniques, soil microbial communities and community-level molecular characteristics have been exploited as early indicators of ecosystem processes for sustainable soil management and agricultural productivity (Shokralla et al., 2012). In recent years, extensive research has been conducted to obtain a mechanistic understanding of the contribution of microbial communities to ecosystem functioning under various agronomic management practices. Unfortunately, few studies have focused specifically on understanding the changes in soil microbial community and function under slag fertilizer amendment in cropping systems. Since the mechanisms of slag-microbe interactions in soil are still not clear, this perspective focuses on the synthesis of several possible mechanisms based on published research. The influence of slag fertilizer on the soil microbiome are diverse and the possible mechanisms of slag-microbes interactions can be as follows: (1) slag fertilizer supplies nutrients not only to the plant but also to soil microorganisms; (2) slag fertilizer modifies soil microbial habitats by improving soil properties (e.g., increasing soil pH) (Gwon et al., 2018), which is essential for nutrient mobilization and microbial growth; (3) silicate fertilizer increases plant photosynthesis (Detmann et al., 2012) and likely increases belowground carbon allocation through root exudates, which eventually triggers soil microbial proliferation and activities; and (4) steel slag enhances heavy metal immobilization in soil (Ning et al., 2016) and thus reduces their bioavailability and toxicity to microbes. Besides, the slag fertilizer amendment may induce changes in soil enzyme activities that affect soil nutrient mobilization and microbial dynamics. In **Figure 1**, we show the proposed mechanism of slag-microbe interactions in soil. The proposed mechanisms of slag-microbe interactions need to be experimentally verified and intensive research needs to be conducted to explore the microbial role in soil processes and agricultural productivity. The potential effects of slag on crop plants have been described in the separate subheading and also shown in **Figure 2**.

SLAG-MICROBE INTERACTION EFFECTS ON CROP YIELD

Among fertilizers made from slag, the use of silicate fertilizer, particularly in rice cropping systems has been gaining awareness and demand (Meharg and Meharg, 2015). Rice is a high Si accumulating plant. Intensive rice cultivation to meet the growing food demand chronically depletes Si from soil, thus degrades soil quality and decreases the crop yield (Branca and Colla, 2012). This necessitates silicate fertilizer amendment in rice cropping systems for sustainable rice cultivation. Studies have indicated an increase (0.16–47.2%) in rice grain yield in lowland rice fields following the addition of slag-based silica fertilizer (**Supplementary Table S1**). The increase in grain yield, however, mostly depends on slag type, application rate, soil type, and agronomic management. Ali et al. (2009a) reported that the silicate fertilizer amendment in no-tillage submerged paddies could improve the crop yield up to 47.2%. Wang et al. (2015) suggested that the silicate fertilization is not significantly effective in improving the rice yield. Higher grain yields in response to silicate fertilization could be attributed to the fact that Si preferentially deposits in the epidermal cell wall and increases physical strength of leaves and leaf-sheaths and help plants to sustain yield by counteracting various biotic and abiotic stresses and increasing plant biomass (Luyckx et al., 2017). Besides silicate fertilizer, lime fertilizer has been widely used in acidic soil to neutralize the soil, which helps plants to protect themselves against soil pathogens. In addition, Ca content in slag fertilizer makes the roots strong and promotes the absorption of K, which is important for plant growth. Slag phosphate fertilizer has been used to provide adequate

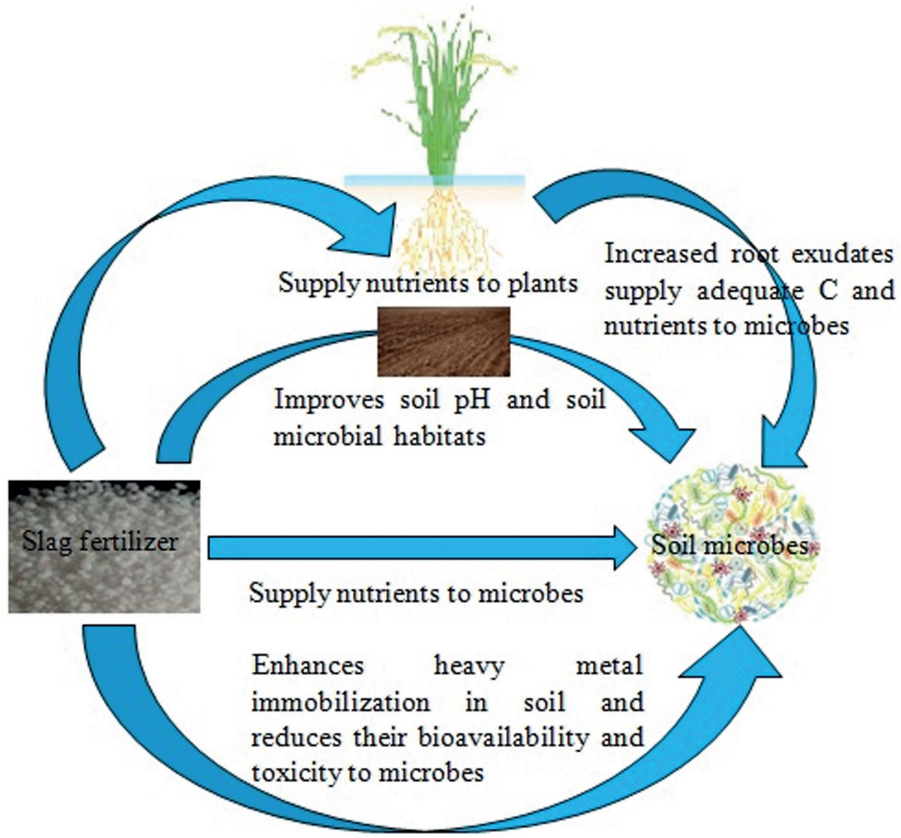

FIGURE 1 | The proposed mechanism of slag-microbe interactions in soil.

phosphorus to the plant, which improves plant growth and fruiting. Likewise, the iron matter of special fertilizer has been used to mitigate the toxicity of heavy metals in soil as well as in the plant. Noteworthy, the increased yield under slag fertilization is largely regulated by microbial decomposition of organic matter and nutrient mobilization. It can be postulated that slag fertilizer amendment not only increases soil nutrients *per se,* but also enriches soil microorganisms that have a beneficial role in nutrient mobilization (e.g., carbon and nitrogen mineralization, phosphorus solubilization, nitrogen fixation, etc.). Identification and elucidation of functional roles of keystone soil microbes that sustain plant health and productivity under slag fertilization could provide a technological breakthrough for a sustainable use of slag in agricultural productivity.

SLAG-MICROBE INTERACTION EFFECTS ON GREENHOUSE GAS EMISSIONS

Agriculture significantly contributes to the emission of methane (CH_4) and nitrous oxide (N_2O), which are two of the most important greenhouse gases responsible for global warming (Das and Adhya, 2014). Methane emission from soils is regulated by CH_4-producing archaea, i.e., methanogens, and CH_4-consuming bacteria, i.e., methanotrophs, while N_2O emission is mostly regulated by nitrifying and denitrifying bacteria (Singh et al., 2010). Soil amendment that reduces methanogen abundance and activity, and/or increases methanotroph abundance and activity could be effective to mitigate CH_4 emissions from the soil. Slag fertilizers, in particular, iron/steel slag fertilizers are rich in iron. Iron acts as an alternative electron acceptor in anoxic soil and its application decreases CH_4 emissions by stimulating iron-reducing bacteria at the expense of methanogens (Gwon et al., 2018). Ali et al. (2009b) showed that 4 mol of Fe^{3+} prevent the generation of 1 mol of CH_4. Moreover, silicate fertilizer amendment can increase root biomass and O_2 transport from the plant to root by enlarging arenchyma gas channels (Liang et al., 2007), which in turn suppresses CH_4 production and stimulates CH_4 oxidation. Studies conducted in Korea, Japan, China, Indonesia, and Bangladesh indicated the potential of slag fertilizer amendment to decrease CH_4 emissions by 0.6–56.0% from lowland rice paddies (**Supplementary Table S1**). The extent of CH_4 emissions reduction depended on the slag fertilizer type, rate of application, soil type, and agronomic practices (**Supplementary Table S1**). Wang et al. (2018a) showed that the application of slag fertilizer (8 Mg ha^{-1}) with biochar (8 Mg ha^{-1}) reduced CH_4 emission up to 38.6% in early rice in China; however, Lee et al. (2012) reported that the silicate fertilization is not effective in reducing CH_4 emissions in green

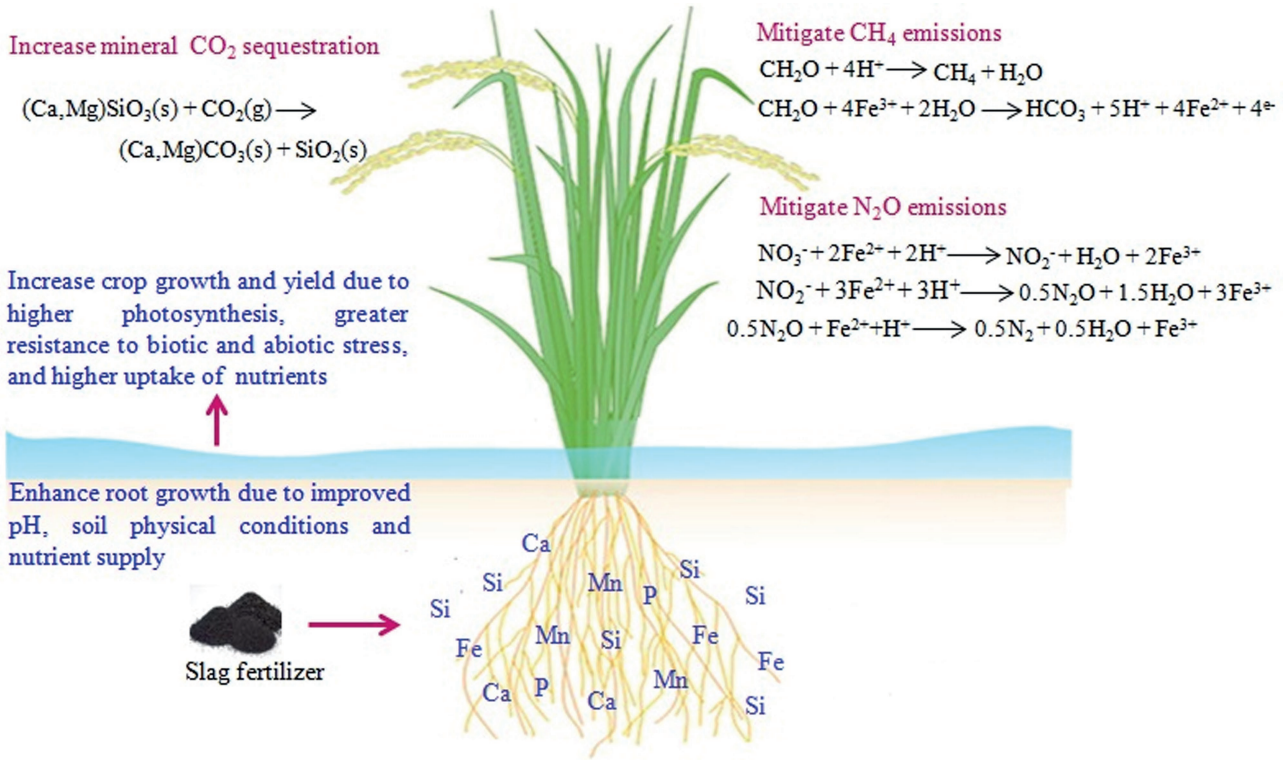

FIGURE 2 | Summary of potential effects of slag on crop plants.

manure amended paddy soils probably due to the enhanced decomposition of added organic matter by the silicate liming effect. Elucidation of methanogen and methanotroph diversity and their functional changes in response to slag fertilizer amendment will improve our mechanistic understanding of CH_4 dynamics in relation to slag fertilization.

Unlike CH_4 emissions, the slag fertilizer effects on N_2O emissions from rice cropping systems are contradictory. Some studies suggest slag fertilizer decreases N_2O emissions (Susilawati et al., 2015; Wang et al., 2015), while other studies suggest slag fertilizer increases N_2O emissions (Huang et al., 2009; Liu et al., 2012). Wang et al. (2015) indicated that a 99% reduction in N_2O emissions could be achieved in an intermittent irrigated rice paddy using silicate fertilizer at the rate of 8 Mg ha^{-1}. The decrease in N_2O emissions have been attributed to lower N availability and higher Fe availability with Si fertilization. Iron oxidation coupled to denitrification can occur in anoxic soils, which can lead to N_2O production (Melton et al., 2014); however, under conditions where Fe is highly available, the electrons donated by Fe(II) exceed the electron demand for N_2O production, which leads to complete denitrification to N_2 and thus a suppression of N_2O emissions (Wang et al., 2016). Increases in N_2O emissions with Si fertilization have been attributed to: (1) Si fertilization acting to lower soil C decomposition, which would alleviate immobilization of fertilizer N thereby making more mineral N available to nitrification and denitrification; and (2) Si fertilization improving soil pH and Eh, which are two factors important to N_2O emissions (Huang et al., 2009; Liu et al., 2012). In a recent study, Song et al. (2017) reported that the silicate fertilizer amendment significantly decreased denitrification potential and *nirS* and *nirK* gene abundance in paddy soils. Owing to the contradictory results, the mechanism underlying N_2O emissions and changes in the genetic potential of nitrifying and denitrifying bacteria under slag fertilization needs further investigation.

SLAG-MICROBE INTERACTION EFFECTS ON SOIL CARBON STORAGE

Carbon dioxide sequestration in soils is well recognized as an avenue to mitigate climate change. Mineral carbonation of CO_2 (mineral CO_2 sequestration) occurs spontaneously on geological time scales and has a high potential for CO_2 sequestration (Oelkers et al., 2008). It typically involves the dissolution of silicate minerals and subsequent precipitation of stable carbonate minerals (e.g., $CaCO_3$, $MgCO_3$, and $FeCO_3$). Mineral carbonation reactions require combining CO_2 with metals to form stable carbonate minerals. With few exceptions, the required metals are divalent cations, including Ca^{2+}, Mg^{2+}, and Fe^{2+}, and the most abundant cation source are silicate minerals. Although mineral carbonation is thermodynamically favorable, it proceeds very slowly (Oelkers et al., 2008). Research is going on worldwide to enhance mineral weathering processes and to accelerate mineral carbonation reactions (Salek et al., 2013). There are

only few reports concerning the effects of the slag fertilizer amendment on carbon sequestration in cropping systems. Wang et al. (2018) reported that the addition of steel slag and biochar in subtropical paddy fields could decrease active SOC pools and enhance soil C sequestration only in the early crop, but not the late crop. Since slag fertilizers are a rich source of silicon minerals and alkaline in nature, their application in agricultural soil may potentially increase soil carbon sequestration. The use of slag fertilizer instead of agricultural lime (limestone) to increase soil pH would eliminate the dissolution of lime as an important source of agricultural CO_2 emissions. It is well recognized that the enzyme carbonic anhydrase (CA) participates in silicate weathering and carbonate formation and thus plays an important role in the biomemetic CO_2 sequestration (Bose and Satyanarayana, 2017). Bio-inoculation of bacteria possessing CA activity in slag fertilized agricultural systems could accelerate silicate weathering and enhance CO_2 sequestration. Likewise, the introduction of plant growth promoting bacteria possessing CA activity in agriculture could have the dual benefit of increased crop yield and CO_2 sequestration. In a recent review it is postulated that farming with rock could have a great promise in sequestering carbon in soils (Beerling et al., 2018). There is an urgent need to evaluate the fate of soil carbon and carbon sequestration potential of slag fertilizer in field conditions.

SLAG-MICROBE INTERACTION EFFECTS ON HEAVY METAL STABILIZATION IN CONTAMINATED SOILS

The stabilization technique aims at reducing heavy metal and metalloid (e.g., As, Cr, Cu, Pb, Cd, and Zn) bioavailability in contaminated soil. The technique is based on amendments to change the soil physicochemical properties through adsorption, precipitation, ion-exchange techniques, redox potential technology, and pH control technology that change the existing forms and speciation of heavy metals/metalloids and thus, reduce their toxicity (Mosa et al., 2016). There are several examples, as follows: as can be stabilized by sorption on Fe oxyhydroxide and also by the formation of amorphous Fe(III) arsenates; Cr can be stabilized by the reduction from more mobile and toxic Cr(VI) to less toxic and stable Cr (III); Cu can be stabilized by precipitation of Cu carbonates and oxyhydroxides, iron exchange and formation of ternary cation-anion complexes on the surface of Fe and Al oxyhydroxides; and Pb and Zn can be immobilized by phosphorus amendments (Branca and Colla, 2012). The slag fertilizer amendment markedly affects the soil solution composition through acid–base, precipitation, and sorption reactions. Owing to its suitable chemical and mineralogical properties, slag fertilizer has been used as a stabilizing agent to minimize metal and metalloid contamination in soil (Ning et al., 2016). Moreover, the adequate Si supply through slag silicate fertilizer amendment causes competitive inhibition of As(III) uptake by crop plants (Meharg and Meharg, 2015). The effects of slag fertilizer amendment on the biogeochemical cycling of soil elements that are regulated by soil microbes need to be investigated. A combination of slag fertilizer and microbial remediation strategies could be proposed for effective remediation of soil contaminants.

ENVIRONMENTAL CONCERNS ABOUT THE USE OF SLAG IN AGRICULTURE

The main concerns regarding the use of slag in agriculture are the potential for heavy metal accumulation in soil and the risks related to liming of soil (Chand et al., 2015). Slags contain traces of heavy metals, but the concentrations of heavy metals might not be enough to pose environmental risks (Gwon et al., 2018); however, it is believed that the long-term application of slag fertilizer in agriculture may accumulate heavy metals/metalloids in soil and may cause health risks. Several studies reveal that metal contamination in soil and metal uptake by plants are not adversely affected by short-term slag fertilizer amendment in cropping systems (Ali et al., 2008, Gwon et al., 2018). In addition, long-term experiments in Germany showed that steel slag fertilizer amendment did not increase bio-available Cr content in soil and Cr uptake by plants (Hiltunen and Hiltunen, 2004). Kuhn et al. (2006), however, revealed that the long-term application of converter slag significantly increased Cr and V contents in the cultivated layer of soil. For a better understanding of the long-term effects of the slag fertilizer amendment in agriculture, further research under diverse soil types and agronomic management practices need to be carried out. Due to the high reactivity of CaO and MgO and high pH (i.e., 12.5) of $Ca(OH)_2$, repeated application of slag may make the soil excessively alkaline, which may decrease the bioavailability and uptake of macronutrients such as P and micronutrients such as Fe, Cu, and Zn by the plant and likely hinder plant growth and productivity (Chand et al., 2015). Another demerit of slag fertilizer is that it contains small proportions of N and K, and P (in some slag fertilizer), which are essential nutrients for plant growth. Therefore, slag fertilizer should be applied together with a chemical fertilizer that contains adequate amounts of N, P, and K.

CONCLUSIONS AND FUTURE DIRECTIONS

With the rapid increase in steel production, steel industries are under pressure for effective and eco-friendly recycling of slag. While in the past, steel-making processes were exclusively designed for the production of specific quality and quantities of iron and steel, one of today's goals for steel making is to design and develop technologies to produce high-quality slag according to the market requirements. Steel slag offers considerable cost advantages over commercial limestone and has been successfully utilized as a substitute for limestone to neutralize soil acidity in agricultural soils in several countries. Owing to its high Si content, the use of slag as silicate fertilizer is gaining demand. The term "slag" is used in the specifications of slag

silicate fertilizer and slag phosphate fertilizer in the Fertilizer Control Law. The slag can be mixed with livestock wastes to make compost, so that both slag and livestock waste can be effectively utilized in agriculture. However, to secure the reliability of the slag as fertilizer, it is quite necessary to conform to the regulations on hazardous heavy metals provided by the Fertilizer Control Law and the soil environmental standards provided by the Basic Law for Environmental Pollution Control.

Understanding the effects of slag fertilizer on soil microbial communities and functions is essential to address some critical agro-environmental issues, such as whether the slag fertilizer amendment would be useful to increase crop productivity, reduce GHG emissions, increase soil carbon sequestration, and stabilize heavy metals in contaminated soils. The recent advances in omic techniques, e.g., high-throughput sequencing, metatranscriptomic analysis, and DNA/RNA-based stable isotope probing (SIP) will no doubt be imperative to uncover the hidden dimensions of slag-microbe interactions in ecosystem functioning.

AUTHOR CONTRIBUTIONS

SD wrote the manuscript. All authors contributed to the intellectual input and provided assistance to the manuscript preparation.

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Reduction in Methane Emissions From Acidified Dairy Slurry Is Related to Inhibition of *Methanosarcina* Species

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Liquid dairy manure treated with sulfuric acid was stored in duplicate pilot-scale storage tanks for 120 days with continuous monitoring of CH_4 emissions and concurrent examination of changes in the structure of bacterial and methanogenic communities. Methane emissions were monitored at the site using laser-based Trace Gas Analyzer whereas quantitative real-time polymerase chain reaction and massively parallel sequencing were employed to study bacterial and methanogenic communities using 16S rRNA and methyl-coenzyme M Reductase A (*mcrA*) genes/transcripts, respectively. When compared with untreated slurries, acidification resulted in 69–84% reductions of cumulative CH_4 emissions. The abundance, activity, and proportion of bacterial communities did not vary with manure acidification. However, the abundance and activity of methanogens (as estimated from *mcrA* gene and transcript copies, respectively) in acidified slurries were reduced by 6 and 20%, respectively. Up to 21% reduction in *mcrA* transcript/gene ratios were also detected in acidified slurries. Regardless of treatment, *Methanocorpusculum* predominated archaeal 16S rRNA and *mcrA* gene and transcript libraries. The proportion of *Methanosarcina*, which is the most metabolically-diverse methanogen, was the significant discriminant feature between acidified and untreated slurries. In acidified slurries, the relative proportions of *Methanosarcina* were $\leq$ 10%, whereas in untreated slurries, it represented up to 24 and 53% of the *mcrA* gene and transcript libraries, respectively. The low proportions of *Methanosarcina* in acidified slurries coincided with the reductions in CH_4 emissions. The results suggest that reduction of CH_4 missions achieved by acidification was due to an inhibition of the growth and activity of *Methanosarcina* species.

Keywords: dairy manure, greenhouse gas, manure acidification, methane, methanogens

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INTRODUCTION

Livestock production is a significant source of methane (CH_4) emissions (e.g., 119.1 $\pm$ 18.2 Tg in 2011) (Wolf et al., 2017), mainly from enteric fermentation and manure management of dairy farming operations (Laubach et al., 2015; Jayasundara et al., 2016; Wolf et al., 2017). The large volumes of manure produced annually from intensive dairy farming operations are

usually stored in slurry form (VanderZaag et al., 2013), which create environments conducive to CH_4 production (Grant et al., 2015; Petersen, 2018). To reduce CH_4 emissions from such storage systems, strategies such as reduction of aged manure (inoculants), crust development for potential aerobic CH_4 oxidation, and manure acidification using sulfuric acid (H_2O_4) have been reported (Petersen et al., 2012; Sommer et al., 2017; Habtewold et al., 2018). Sulfuric acid-based acidification of liquid dairy manure has primarily been used to abate ammonia (NH_3) emissions, but can also reduce CH_4 emissions (Ottosen et al., 2009; Petersen et al., 2012; Fangueiro et al., 2015; Sommer et al., 2017). For instance, CH_4 emissions from cattle slurry were reduced by 68% by acidification to pH 5.5 with H_2SO_4 (Sommer et al., 2017). More than 90% reduction of CH_4 emissions from acidified pig slurry were also reported by Petersen et al. (2014). In fact, acidification of stored liquid dairy manure has already been implemented at farm-scale in some countries such as Denmark. In slurries, H_2SO_4 itself is expected to be converted to plant-available sulfate sulfur (Eriksen et al., 2008), and H_2SO_4 would not be found in the slurry after acidification has already occurred. However, there are no data available about the effects of manure acidification on the activities of microbial communities in stored liquid dairy manure.

In stored liquid dairy manure, complete degradation of complex organic matter involves different groups of microbial communities (hydrolytic, acidogenic, acetogenic, and methanogenic). The pH range can impact the growth and activity of these microbial groups differently, i.e., hydrolytic and acidogenic bacteria generally grow best at a pH of around 6 whereas most methanogens and acetogens have pH optima of around 7 (Lay et al., 1997; Angelidaki et al., 2003, 2011; Pind et al., 2003). Thus, slurry acidification may result in upsetting the anaerobic biodegradation processes and reduce methanogenic activity. In this study we investigated structure and activity responses of bacterial and methanogenic communities to the addition of H_2SO_4 to stored liquid dairy manure.

In various manure related environments, culture independent investigations of bacterial and methanogenic communities often involve using phylogenetic and/or functional gene markers (e.g., 16S rRNA and *mcrA* genes) (Petersen et al., 2014; Pandey et al., 2018). However, our previous study indicated that *mcrA* transcripts were more relevant to methane CH_4 emissions than *mcrA* genes (Habtewold et al., 2018). Particularly with slurry acidification, where significant number of bacterial and methanogenic communities could be dormant or dead, DNA-based studies of these microbes may not reflect activities. Unlike DNA-based studies, changes in the transcriptional levels of phylogenetic and functional marker genes and transcript/functional gene ratios are strong indicators of growth and activity of microbial communities (Freitag and Prosser, 2009; Ma et al., 2012; Blagodatskaya and Kuzyakov, 2013; Wilkins et al., 2015). Hence, in the current study, we aimed to investigate abundance, activity, and diversity responses of bacterial and methanogenic communities in acidified liquid dairy manure by targeting 16S rRNA and *mcrA* genes and transcripts.

MATERIALS AND METHODS

Methane Measurements and Manure Sampling

The study was conducted during the summer season (25 June through 23 October 2017) at the Dalhousie University's Bio-Environmental Engineering Center (BEEC) in Truro, NS, Canada (45°45′ N, 62°50′ W). Six pilot-scale rectangular outdoor manure storage tanks covered with flow-through steady-state chambers were used. This site has been previously described by Wood et al. (2012). Fresh dairy slurry obtained from a commercial farm was loaded (10.5 m^3) to each tank. Using duplicate tanks per treatment, 70% H_2SO_4 (1.4 L or 2.4 L L^{-1} slurry) or water (2.4 L L^{-1} slurry) were injected (with simultaneous mixing) across the depth of slurries. During storage, gas samples were drawn continuously from the inlet (ambient air) and outlet of each tank using polyethylene tubing, and CH_4 concentrations were determined at the site using TGA 100A tunable diode laser trace gas analyzer (Campbell Scientific Inc., Logan, UT, United States). Methane flux (g m^{-2} s^{-1}) was calculated as described by Wood et al. (2012), and emissions were then converted into daily averages.

For the microbial study, slurry samples were collected before (fresh manure) and after acidification. After acidification, manure samples were collected bi-weekly from the top (10 cm from the surface) and bottom (20 cm from floor) sections of each tank (1.8 cm). From each sampling location, nine slurry samples (on coordinates of a grid) were collected from across the surface and pooled in a clean bucket. Then, two grams subsamples (in duplicate) were collected from each pool in 15 mL Falcon tubes containing 5 mL LifeGuard™ Soil Preservation Solution (MoBio Laboratories Inc., Carlsbad, CA, United States). Samples were then transported to the lab cold and stored in a −20°C freezer until nucleic acid extractions. Based on daily CH_4 fluxes, manure samples were selected after 20, 50, and 100 days of storage to assess changes in the structure of microbial communities before, during and after peak CH_4 fluxes, respectively. Sub-samples of appropriate volume were also collected to analyze pH, dry matter (DM), and volatile solid (VS) contents, which were analyzed at the Nova Scotia Department of Agriculture's Laboratory Services (Harlow Institute, Bible Hill, NS, United States) using standard methods.

Nucleic Acid Extractions and Quantitative Real-Time PCR

Slurry samples stored with LifeGuard™ Soil Preservation Solution were thawed and centrifuged (4000 × g for 10 min). Pellets were then used to co-extract total RNA and DNA using RNA PowerSoil Total RNA Isolation with DNA Elution Accessory Kits (MoBio Laboratories, Inc., Carlsbad, CA, United States) following the manufacturer's protocol. Based on information from the manufacturer and our experience, this RNA isolation kit can be used to efficiently isolate RNA and DNA from manure samples as it does for different soil types. As there was little difference in the abundances of bacteria and methanogens between the top and bottom sections of slurries,

DNA or RNA samples from these locations were pooled to have one representative sample per tank. RNA samples were reverse transcribed into complementary DNA (cDNA) using Maxima^{TM} H Minus First Strand cDNA Synthesis Kit (Thermo Scientific^{TM}) following the manufacturer's protocol with few modifications. Briefly, 1 μl each of 10× dsDNase Buffer and dsDNase were added to 2 μl (0.3-1 μg) RNA, gently mixed and spun, and incubated at 37°C for 5 min in a preheated thermocycler with lid temperature adjusted to 37°C. After chilled on ice and briefly centrifuged, 4 μl Maxima cDNA H Minus Master Mix (5×) and 6 μl nuclease-free water were added, and gently mixed and centrifuged. For cDNA synthesis reactions, which were performed in a thermocycler with lid temperature adjusted to 50°C, thermal conditions were: 25°C for 10 min, 50°C for 15 min, and 85°C for 5 min. Prior to further downstream analyses, both cDNA and DNA samples were diluted and assessed for potential inhibitory effects as described previously (Habtewold et al., 2017). Diluted DNA (50×) and cDNA (100×) were then used as templates for quantitative real-time polymerase chain reaction (qPCR). Reaction ingredients, conditions, and thermal cycling of qPCR were as described by Habtewold et al. (2017). Known copies of plasmid standard curves for *mcrA* (10e7 to 10e1) and bacterial 16S rRNA (10e9 to 10e1 copies) genes and transcripts were prepared from *Methanosarcina mazei* (ATCC 43340) and a pure culture of *Clostridium thermocellum*, respectively. Efficiency, r^2, and slope of plasmid standard curve for *mcrA* gene were 98.5 ± 2.8%, 0.99, and −3.34 ± 0.04, whereas for 16S rRNA gene, these values were 98.5 ± 2.7%, 0.99, and −3.36 ± 0.07, respectively. CFX Manager software version 3.1 (Bio-Rad Laboratories, Inc., Hercules, CA, United States) and GraphPad prism v.7 (GraphPad Software, Inc) were used to analyze the qPCR data.

Amplicon Library Preparation and Sequencing

Methane fluxes from all acidified slurries were very low, thus slurries treated with 2.4 L 70% H_2SO_4 m^{-3} slurry (acidified slurries) and untreated slurries were selected to study the effects of acidification on community structure of bacteria and methanogens. Polymerase chain reaction (PCR) primers (515FB-806RB) that target the V4 region of bacterial and archaeal 16S rRNA genes were used to prepare 16S rRNA gene and transcript libraries (Walters et al., 2016). To study methanogens, the gene encoding the alpha subunit of methyl coenzyme M reductase (*mcrA*) which is a key enzyme in methanogenesis was targeted using mlas-mod and mcrA-rev primers (Angel et al., 2011). On both 16S rRNA and *mcrA* gene primers, Illumina adapter sequences A and B (**Supplementary Table S1**) were added to the 5′-ends of the forward and reverse primers, respectively. For both genes, amplicons were prepared in two PCR steps with a total of 35 cycles. First, duplicate 25 μL PCR reactions per sample were prepared by adding 5 μL of 5X Phusion HF buffer, 0.25 μL of Thermo Scientific^{TM} Phusion^{TM} Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific), 0.5 μL of 10 mM dNTPs (Thermo Scientific), 0.5 μL of each of the forward and reverse primers (10 μM), 2 μL of diluted DNA (10–50 ng/μL) or cDNA, and 16.25 μL nuclease free water. Thermal cycling for both genes were as follows: initial denaturation at 98°C for 3 min, followed by 25 cycles of dissociation at 98°C for 10 s, primer annealing (50°C and 55°C for 30 s, for 16S rRNA and mcrA gene/transcript, respectively), extension at 72°C for 30 s, and a final extension for 10 min. Duplicate PCR reactions were pooled and products were cleaned using silica spin columns (Wizard® SV Gel and PCR Clean-Up System; Promega) following the recommended protocol. The second step PCR was performed for 10 cycles to attach Illumina index tags to the ends of the amplicons that were obtained from the first-step PCR. For each sample, a different combination of the Index primers 1 (N7xx) and Index primers 2 (S5xx) of Illumina's Nextera® XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, United States) were used to perform PCR. This was performed in a single 50 μL reaction mix per sample, and same proportion of reagents and thermal cycling conditions were used as the first-step PCR except the 4 μL purified amplicons template DNA. PCR products were then purified by magnetic beads (Agencourt AmPure XP; Beckman Coulter, Brea, CA, United States) and re-suspended in 25 μL. Purified PCR products were tested for correct amplicon length using gel electrophoresis and submitted to the University of Guelph Advanced Analysis Centre, Genomic Facility (Guelph, ON, Canada) for sequencing. Prior to sequencing, libraries were normalized by Sequalprep (Thermo Fisher Scientific, Hampton, NH, United States) and library quality was assessed from 6 randomly selected samples using Bioanalyzer DNA1000 chip (Agilent, Santa Clara, CA, United States). Multiplexed sample sequencing was conducted using MiSeq v3 600 cycle reagent kit (Illumina Inc., San Diego, CA, United States) producing 2 × 300 bp. Unprocessed FASTQ files were received for subsequent analysis.

Sequence Data Analysis

Raw sequence data of 16S rRNA genes and transcripts were processed and analyzed in Mothur v.1.39.5 (Schloss et al., 2009) following the recommended pipeline (Kozich et al., 2013). Briefly, forward and reverse reads of each sample were merged, target-specific primer sequences removed, and sequences were screened for ambiguity and length. Then, sequences were aligned against the Silva reference sequence (release 132), further screened for length and homopolymer, overhangs and common gaps filtered, and pre-clustered to further denoise sequencing errors. After removal of potential chimeric sequences, Mothur-formatted version of the RDP's 16S rRNA reference (version 16) was used to classify sequences into phylotypes at 80% cut-off in which undesirable targets that might have been picked by primers were filtered. Finally, purified sequences were clustered into operational taxonomic units (OTUs) at 0.03 cut-off (97% similarity), and phylotypes of OTUs identified using the RDP's 16S rRNA reference database. The *mcrA* gene and transcript sequences were processed similarly except that non-target reads and potential frameshift errors were removed or corrected using the FrameBot function of the RDP's Functional Gene and Repository Pipeline tool (Fish et al., 2013; Wang et al., 2013). OTU-based alpha diversity (e.g., rarefaction, coverage, Chao1, and Inverse Simpson diversity

estimate) and beta diversity (e.g., non-metric multidimensional scaling) analyses were performed in Mothur. Significance of differences in diversity, richness, and composition microbial community between treatments were tested in Mothur, STAMP (statistical analysis of taxonomic and functional profiles), and GraphPad prism v.7 GraphPad Software, Inc.) (Schloss et al., 2009; Parks et al., 2014).

Sequence Accessions

Raw reads of 16S rRNA and *mcrA* genes and transcripts have been deposited in NCBI's short read archives as FASTQ files under the accession number SRP119447.

RESULTS

Manure Characteristics, CH_4 Flux and Microbial Abundance

Initially, the pH of fresh dairy manure used in the current study was 7.5. Twenty days after addition (and mixing) of 1.4 L or 2.4 L 70% H_2SO_4 per cubic meter of dairy slurry, mean pHs of slurries were 6.5 ± 0.1 and 5.9 ± 0.01, respectively (**Figure 1A**), while the pH of untreated slurries was 6.8 ± 0.07. After 50 days of storage, slurry pH gradually increased in all tanks by 0.35 ± 0.2. Nevertheless, pH increases in acidified slurries were small when compared with untreated control. Regardless of treatments, VS contents of slurries declined during storage (**Figure 1B**). Total and ammoniacal nitrogen contents (in %) of the fresh manure were 0.46 ± 0.01 and 0.18 ± 0.01, respectively. At the end of the storage period, these values were reduced to 0.37 ± 0.07 and 0.16 ± 0.02, and 0.42 ± 0.06 and 0.16 ± 0.03, in the untreated and acidified slurries, respectively. Unlike the untreated slurries, where peak CH_4 fluxes (76–52 g m^{-2} d^{-1}) were detected between 50 and 60 days of storage (**Figure 1C**), fluxes from the acidified slurries were consistently low (<10 g m^{-2} d^{-1}) throughout the storage period. Addition of 1.4 L and 2.4 L 70% H_2SO_4 m^{-3} slurry resulted in 69–84% reduction of cumulative CH_4 emissions when compared with untreated slurries (**Figure 1D**).

Fresh manure (<1-day old) had a large number of bacteria, where copy numbers (Log10) of 16S rRNA genes and transcripts were 10.3 and 13.3 g^{-1} dry manure, respectively (**Figure 1E**). After 20 days of storage, the abundance of bacteria decreased in both acidified and untreated slurries. However, there were no significant differences (Kruskal–Wallis followed by Dunn's test) in the abundance of bacteria between acidified and untreated

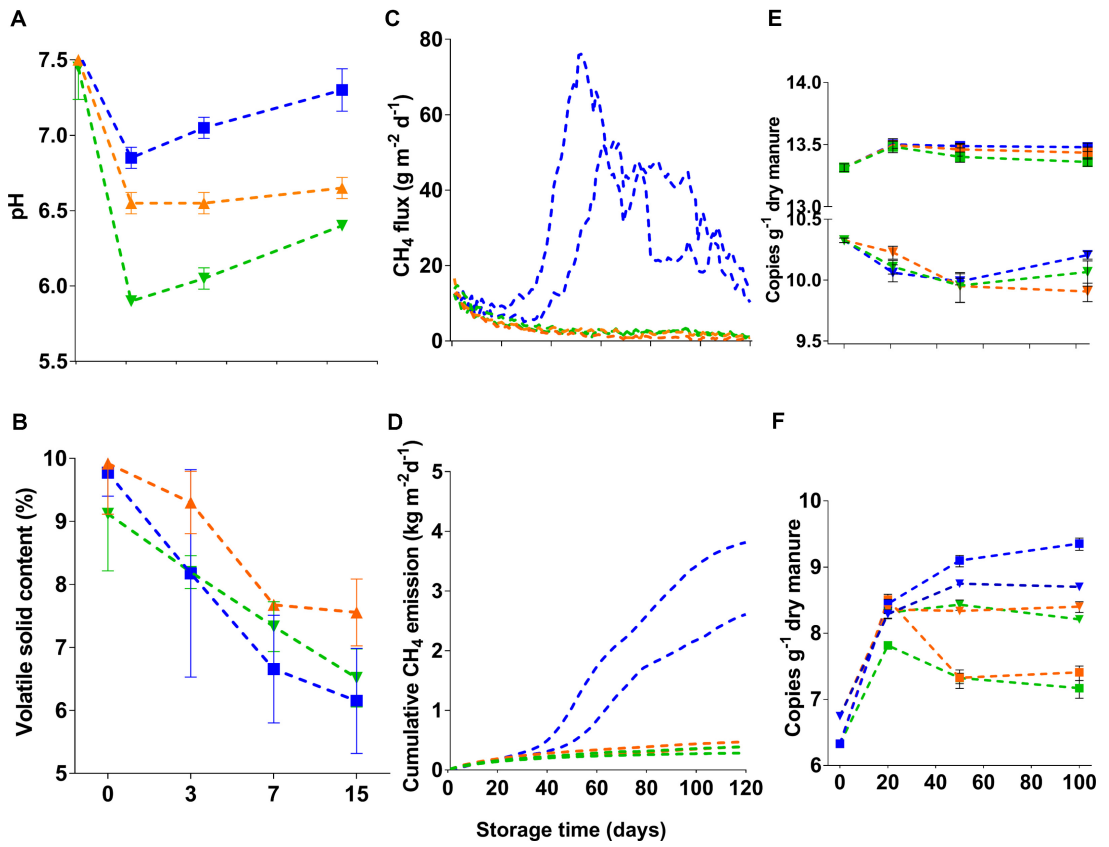

FIGURE 1 | Manure characteristics (**A**: pH; **B**: volatile solid content), CH_4 emissions (**C**: daily flux; **D**: cumulative emissions), and abundance of microbial communities (**E**: bacteria: **F**: methanogens). In **(E,F)**, filled down triangle and square symbols indicate gene and transcript copies, respectively. The blue color in all figures indicates untreated manure whereas the yellow-brown and green colors indicate manures that received 1.4 L and 2.4 L 70% H_2SO_4, respectively.

slurries (**Figure 1E**). For instance, when CH_4 flux peaked in untreated slurries (after 50 days of storage), differences in the copy numbers of 16S rRNA genes in acidified and untreated slurries were only about 0.4%. These small differences slightly increased (up to ∼3%) after 100 days of storage, but were not statistically significant. Thus, storage time had a greater impact on bacterial abundance than slurry acidification. Similarly, the activity of bacteria (as estimated from 16S rRNA transcript copies g^{-1} dry manure) showed little variation (up to 1%) with slurry acidification (**Figure 1E**). These results indicated that neither abundance nor activity were altered with manure acidification (pH up to 5.9).

Unlike bacteria, slurry acidification negatively affected the abundance and activity methanogenic populations. Fresh manure had 6.74 ± 0.05 copies (Log10) of *mcrA* genes g^{-1} dry manure (**Figure 1F**). After 20 days of storage, the *mcrA* gene copies showed significant increases (∼30% and ∼25% in untreated and acidified slurries, respectively; Dunn's test, $p < 0.0001$). The effect of slurry acidification however was not noticeable after 20 days of storage (**Figure 1F**). After 50 days of storage, there were significantly lower numbers (4–4.7%; Dunn's test, $p = 0.047$) of *mcrA* gene copies (Log10 transformed) in acidified slurries. These differences slightly increased (5–6%; Dunn's test, $p = 0.006$) after 100 days of storage. The effect of slurry acidification on the activity of methanogens (estimated from *mcrA* transcript copies g^{-1} dry manure) was more significant. After 20 days of storage, slurries that received ∼2.4 L 70% H_2SO_4 m^{-3} slurry showed ∼6.5% lower copies of *mcrA* transcripts, while *mcrA* transcript/gene ratios were reduced by ∼1.7% (**Supplementary Figure S2**). Significant reductions (up to 32%; Dunn's test, $p < 0.0001$) of *mcrA* transcript copies (Log10) were detected after 50 and 100 days of storage. During these time periods, *mcrA* transcript/gene ratios in acidified slurries were also reduced by ∼21 and 25%, respectively (**Supplementary Figure S2**).

Effects of Manure Acidification on the Diversity of Bacteria and Methanogens

After quality inspections of raw MiSeq sequencing data, 1863088 quality reads of 16S rRNA gene and transcript (an average of 71657 per sample) were obtained. Similarly, 524245 quality reads of *mcrA* gene and transcript (an average of 20163 per sample) were obtained. Diversity and community composition of bacteria and methanogens were analyzed after singletons were removed from 16S rRNA and *mcrA* genes and transcripts reads (Auer et al., 2017). Rarefaction plots for both gene and transcript indicated sufficient sampling efforts that might have covered most bacterial and methanogenic communities in the manure (**Supplementary Figures S1a,b**).

Shifts in the diversity of bacteria and methanogens due to acidification and/or storage time were shown using Inverse Simpson diversity index. Bacterial and methanogen diversity in 16S rRNA and *mcrA* gene libraries were higher at the beginning of storage (**Table 1**). However, diversities in the corresponding transcript libraries were reduced by half. These differences were consistent throughout the storage period, which might indicate the inability of several bacterial taxa from fresh manure to adapt to storage conditions. Unlike bacteria, there were significant differences (*t*-test, $p < 0.05$) in the diversity of methanogens between acidified and untreated slurries (**Table 1**). Diversity in *mcrA* gene and transcript libraries of untreated slurries also increased with storage period. These results indicated that

TABLE 1 | Richness and diversity analysis of bacteria and methanogens from dairy manure.

Days	Treatment	S_{obs}	S_{chao}	Invsimpson	S_{obs}	S_{chao}	Invsimpson
		16S rRNA gene			***mcrA* gene**		
0		**2951**	**3653**	**50**	**49**	**49**	**2.93**
20	Control	3052 ± 192	4383 ± 99	40 ± 3	41 ± 3	54 ± 10	1.41 ± 0.09
50		2955 ± 83	4571 ± 23	45 ± 1	47 ± 5	57 ± 11	1.8 ± 0.15
100		2910 ± 13	3997 ± 216	42 ± 7	43 ± 3	48 ± 4	2.38 ± 0.3
20	Acidified	3088 ± 111	4651 ± 42	38 ± 1	45 ± 3	65 ± 11	1.64 ± 0.03
50		3205 ± 150	4670 ± 204	41 ± 6	39 ± 1	73 ± 48	1.39 ± 0.08
100		2721 ± 510	3861 ± 536	39 ± 26	42 ± 1	51 ± 6	1.33 ± 0.03
		16S rRNA transcript			***mcrA* transcript**		
0		**1985**	**3044**	**27**	**16**	**23**	**1.49**
20	Control	3030 ± 223	4506 ± 157	26 ± 5	19 ± 1	20 ± 2	1.39 ± 0.4
50		2890 ± 695	4385 ± 723	22 ± 3	17 ± 1	17 ± 1	2.06 ± 0.03
100		2938 ± 397	4250 ± 542	22 ± 4	25 ± 2	36 ± 1	2.51 ± 0.1
20	Acidified	3113 ± 424	4596 ± 321	19 ± 1	15 ± 2	17 ± 3	1.38 ± 0.24
50		2739 ± 806	4407 ± 836	19 ± 2	10 ± 1	16 ± 6	1.37 ± 0.28
100		3058 ± 113	4474 ± 357	22 ± 9	8 ± 2	11 ± 7	1.45 ± 0.08

Mean and standard deviation of biological replicates (n = 2) are shown in the table. S_{obs}, S_{chao}, and Invsimpson, indicate Species observed, Species estimated, and Inverse Simpson index, respectively.

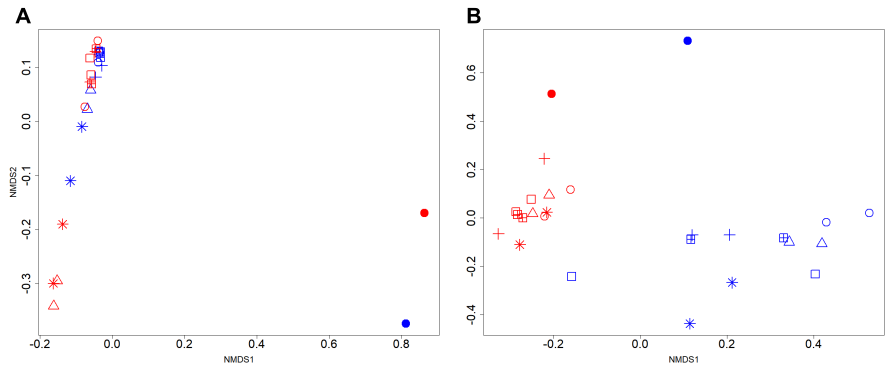

FIGURE 2 | Two-dimensional non-metric multidimensional scaling (NMDS) of **(A)** bacterial and **(B)** methanogenic communities in stored liquid dairy manure. Samples from day 0, 20, 50, and 100 of untreated slurries were indicated by the filled circle, open circle, open triangle, and asterisk, respectively. Acidified slurries from day 20, 50, and 100 were indicated by the plus, square plus, and open square, respectively. Blue and red colors indicated gene and transcripts libraries, respectively.

manure acidification results in stronger impacts on methanogens when compared with bacteria.

The effects of manure acidification on the community structure of bacteria and methanogens were indicated by NMDS plots (**Figures 2A,B**). With a good fit of ordination (2D Stress = 0.09; $r^2 = 0.97$), the NMDS plot did not show distinct clustering of bacterial communities from acidified and untreated slurries (**Figure 2A**). In line with the Inverse Simpson diversity estimates, bacterial communities in 16S rRNA gene and transcript libraries showed significant separation (AMOVA, $p < 0.001$) regardless of treatments. Methanogens from acidified and untreated slurries particularly after 50 days of storage showed distinct NMDS clustering patterns (2D Stress = 0.07; $r^2 = 0.99$) which supported the diversity estimates. While the methanogens in *mcrA* transcript libraries of untreated slurries clustered separately from those in *mcrA* gene libraries (AMOVA, $p < 0.05$), no significant separation was observed for the acidified slurries (**Figure 2B**).

Effects of Acidification on Relative Proportions of Bacterial and Methanogenic Phylotypes

In this study, 95.5% of the 16S rRNA gene and transcript reads were related to bacteria (**Supplementary Figure S3a**). In fresh manure, phylum Firmicutes and Bacteroidetes predominated (55–57% and 32–24%, respectively) 16S rRNA gene and transcript libraries (**Supplementary Figure S3b**). Relative proportions of the predominant bacterial phyla did not vary with slurry acidification. Regardless of treatments, 16S rRNA gene libraries from stored slurries were dominated by Firmicutes (35 ± 8%), Bacteroidetes (25 ± 4%), and Spirochaetes (15 ± 4%) whereas Firmicutes (59 ± 5%) and Bacteroidetes (21 ± 3%) predominated the 16S rRNA transcript libraries (**Supplementary Figure S3b**). At the genus level, *Sphaerochaeta* was the most abundant (15 ± 4%) bacteria in 16S rRNA gene libraries (**Figure 3A**). Uncultured members of Bacteroidetes (8 ± 2%), *Turicibacter* (6 ± 4%), and *Romboutsia* (6 ± 3%) were also predominant in both acidified and untreated slurries. In 16S rRNA transcript libraries of both acidified and untreated slurries, many members of Firmicutes (e.g., *Romboutsia*, *Turicibacter*, uncultured Clostridiales, *Clostridium*_XI, uncultured *Ruminococcaceae*) and uncultured Bacteroidetes accounted for 46 ± 4% and 13 ± 2%, respectively (**Figure 3A**).

Analysis of differences in mean proportions of 16S rRNA gene and transcript reads between acidified and untreated slurries (White's non-parametric *t*-test with Bonferroni correction, CI = 95%, $\alpha = 0.05$) indicated that manure acidification with H_2SO_4 did not alter the composition of bacterial communities. Regardless of treatments, 16S rRNA gene and transcript communities were significantly different (White's non-parametric *t*-test, $p = 0.015$; **Figure 3B**), which was in line with the NMDS analysis. Genera from different bacterial phyla (e.g., *Spirochaeta*, *Petrimonas*, and *Sedimentibacter*) and members of the Firmicutes (e.g., *Romboutsia*, *Clostridium*_XI, and uncultured members *Ruminococcaceae*) were represented differently in the 16S rRNA gene and transcript communities.

Archaea accounted for 4.5% of 16S rRNA gene and transcript reads (**Supplementary Figure S3a**), and all were methanogens. The most abundant genus in fresh manure was *Methanobrevibacter* (76%), but *Methanocorpusculum* was predominant (92 ± 1% and 86 ± 2% in archaeal 16S rRNA gene and transcript libraries, respectively) in stored slurries of all treatments (**Figure 4**). While the proportion of *Methanosarcina* in 16S rRNA gene and transcript libraries of untreated slurries gradually increased (up to 5 and 18%, respectively), its proportions in acidified slurries was consistently below 1%. Thus, *Methanosarcina* seemed strongly inhibited by manure acidification.

Like in archaeal 16S rRNA gene libraries, *Methanobrevibacter* predominated the *mcrA* gene library from fresh manure (**Figure 5A**). Although *Methanocorpusculum* represented < 1% of the methanogens in fresh manure, it represented 83 ± 3% of the *mcrA* gene and transcript reads from acidified slurries. *Methanocorpusculum* was also dominant in untreated slurries but gradually declined (84 to 59% and 84 to 46% in the *mcrA* gene and transcript libraries, respectively) with storage time. In

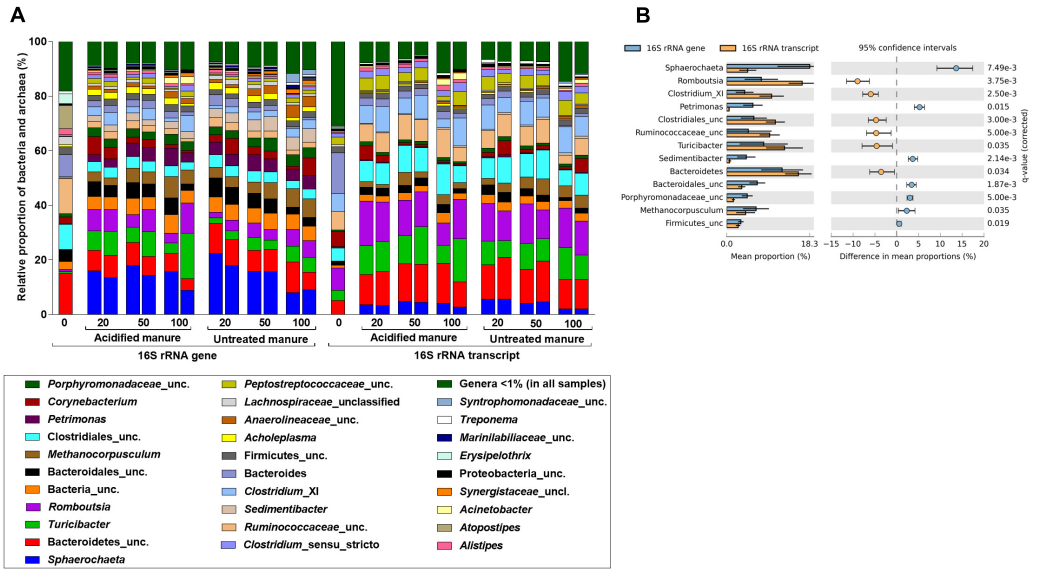

FIGURE 3 | Effects of manure acidification on bacterial and archaeal phylotypes as indicated by **(A)** relative proportions of abundant genera in fresh, acidified and untreated slurries **(B)** extended error bar plots illustrating significantly abundant bacteria (Effect size = 2, White's non-parametric *t*-test with Benjamini–Hochberg multiple test correction, *q*-values < 0.05) in acidified and untreated slurries. Numbers on the X axis indicate storage time in days. Except fresh manure, all have biological replicates (n = 2).

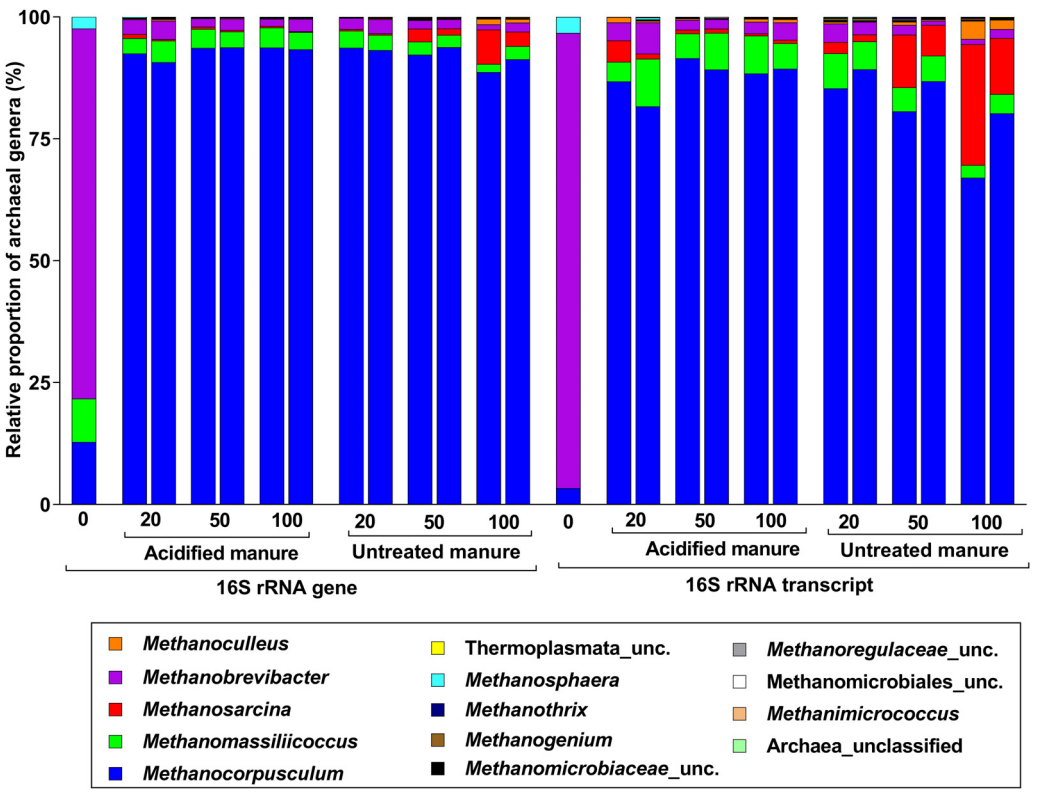

FIGURE 4 | Taxonomic distribution of archaeal phylotypes identified from 16S rRNA gene and transcript reads. Numbers on the X-axis show storage time in days. Except fresh manure, all have biological replicates (n = 2).

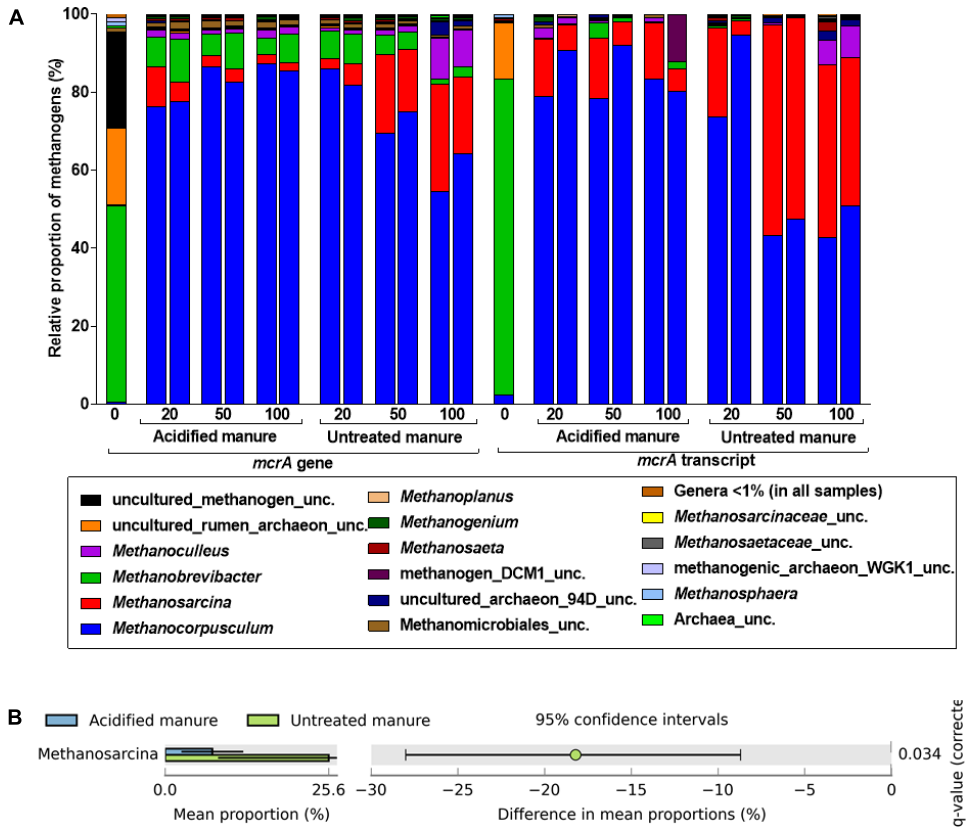

FIGURE 5 | Effects of manure acidification on methanogens as indicated by **(A)** a stacked bar showing relative proportions of methanogenic genera in fresh, acidified and untreated slurries **(B)** extended error bar plots illustrating significantly abundant methanogens (Effect size = 2, White's non-parametric *t*-test with Benjamini–Hochberg multiple test correction, *q*-values < 0.05) in acidified and untreated slurries. Numbers on the *X* axis indicate storage time in days. Except fresh manure, all have biological replicates ($n = 2$).

contrast, the relative proportion of *Methanosarcina*, increased (4 to 25% and 13 to 41% in the *mcrA* gene and transcript libraries, respectively) in untreated slurries, but in acidified slurries the population remained stable. Increased amounts of CH_4 emitted from untreated slurries coincided with the increased abundance of *Methanosarcina*.

Analysis of differences in mean proportions of *mcrA* gene reads between acidified and untreated slurries indicated that *Methanosarcina* ($p = 0.05$) was differentially enriched in untreated slurries (**Figure 5B**), indicating the negative impacts of manure acidification on methanogens related to the genus *Methanosarcina*.

DISCUSSION

Stored liquid dairy manure is a point source of CH_4. Studies have demonstrated that H_2SO_4-based acidification of liquid dairy manure (reducing pH to ∼5.5) can reduce CH_4 emissions from 67 to 90% (Petersen et al., 2012, 2014; Misselbrook et al., 2016; Sommer et al., 2017). In line with these studies, 69–84% reductions of cumulative CH_4 emissions were detected in this study after acidification of dairy manure to pH 6.5 and 5.9. Thus, small changes in slurry pH were able to disrupt methanogenesis that has pH optima around 7 (Lay et al., 1997; Liu et al., 2008; Weiland, 2010; Mao et al., 2017); however, reduction in CH_4 emissions could also be related to the toxicity of hydrogen sulfide that could be accumulated as a result of potential sulfate reduction (Petersen et al., 2012). Throughout the storage period, CH_4 fluxes from acidified slurries remained low, consistent with levels observed during the lag phase (the first 20 days of storage, **Figure 1C**). The lag phase observed in the current study was also observed in our previous studies that did not involve acidification (Habtewold et al., 2018). A previous study from our lab has linked shifts in methanogens to methane emissions (Habtewold et al., 2018), therefore we predicted that the communities would shift after acidification. Using qPCR-based quantification and deep sequencing (Illumina MiSeq) of phylogenetic and functional marker genes and transcripts, we demonstrated that slurry acidification (to pH 5.9) did not affect the community structure of most anaerobically-degrading microorganisms except methanogens closely related to the genus *Methanosarcina*. As major players in CH_4 production (Conrad, 1999), impacts on methanogens related to *Methanosarcina* can have a drastic effect on methanogenesis and CH_4 emissions.

With slurry acidification (mean pHs 6.5 and 5.9), shifts in the abundance and activities of bacterial communities, as estimated from 16S rRNA gene and transcript copies, were not significant. Thus, acidification might have little effect on growth and activities of bacterial communities involved in the anaerobic degradation of organic matter (e.g., hydrolytic, acidogenic, and acetogenic bacteria) in manure (Lin et al., 2013; Kuruti et al., 2017). As acidification may reduce aggregation of slurry particles (Fangueiro et al., 2015; Gomez-Munoz et al., 2016; Regueiro et al., 2016), substrate availability for hydrolytic and acidogenic bacteria in slurries may increase. Gradual reductions in the volatile solids contents of both acidified and untreated slurries indicated that these communities were active. Microbial consumption of volatile solids in manure typically increases the amount of organic acids (e.g., acetic, propionic, and butyric acids) and methanogenic substrates (e.g., CO_2, H_2, acetate, formate, and alcohol). Thus, pH reductions observed during the first 20 days of storage (regardless of treatment) might be related to accumulation of organic acids. Although pH reductions due to organic acid accumulation might be obscured in acidified slurries, reductions in total solids contents in these slurries might indicate microbial activities. Regardless of treatments, slurry pH gradually increased after 50 days of storage which was in line with other studies (Patni and Jui, 1985; Sommer et al., 2017). However, pH increases (often due to consumption of organic acids by acetogens and methanogens) in acidified slurries were lower when compared with untreated slurries. These small changes in pH coincided with low CH_4 flux from acidified slurries, indicating negative impacts of acidification with H_2SO_4 on methanogens.

Acidification had little effect on the abundance of methanogenic populations. This was in line with a study by Petersen et al. (2014) where the abundance of methanogens in pig slurry did not shift with slurry acidification (pH down to 5.5). However, the authors detected more than 90% reductions in CH_4 emission which indicate the negative impacts of slurry acidifications with H_2SO_4 on methanogenic processes. Ottosen et al. (2009) also detected significant reductions (>98%) in microbial processes (oxygen consumption rate, methanogenesis and sulfate reduction) in acidified pig slurry. As DNA-based studies of *mcrA* genes provide information about all methanogens (active, dormant, and dead), in this study we used instead mRNA of the *mcrA* genes (*mcrA* transcript) to specifically study changes in physiological status of methanogens and methanogenic processes. Unlike population abundance, the reduced copy numbers of *mcrA* transcripts in acidified slurries with negligible CH_4 emissions might reflect the negative effect of manure acidification on the activities of methanogens. However, some or most methanogens might still grow and function in acidified slurries as the abundance and activities of methanogens in it were higher when compared with fresh manure. This would account for the residual methane emission observed.

With little impacts of acidification on the abundance and activity of bacteria, accumulated intermediary compounds including propionate, butyrate, and valerate could be converted into acetate by the acetogens (Demirel and Scherer, 2008), making stored liquid dairy manure rich in acetate (Barret et al., 2013; Habtewold et al., 2018). Although acetoclastic methanogenesis (using acetate as substrate) is the major contributor of CH_4 produced in many environments (Conrad, 1999), CH_4 production in environments with high concentration of acetate has been found to drastically reduce as pH decline (Van Kessel and Russell, 1996), although the exact mechanism is not yet clear.

Consistent with the qPCR data, the diversity and relative proportions of bacterial communities were not altered with slurry acidification (to pH 5.9). Regardless of treatments, *Sphaerochaeta* was predominant in the 16S rRNA gene libraries. These bacteria are enriched with fermentation and carbohydrate metabolism genes (Caro-Quintero et al., 2012), but it is unclear why they represented lower proportions in the 16S rRNA transcript libraries where several fermentative bacteria (e.g., *Turicibacter*, *Bacteriodetes*, and *Romboutsia*) (Bosshard et al., 2002; Thomas et al., 2011; Gerritsen et al., 2017) were predominant. The abundance of these bacteria, particularly in the 16S rRNA transcript libraries of acidified slurries, might indicate availability of methanogenic substrates. Regardless of treatments, methanogens closely related to the genus *Methanocorpusculum* that are known to perform hydrogenotrophic methanogenesis (reducing CO_2 to CH_4 using hydrogen) predominated the archaeal 16S rRNA and *mcrA* gene and transcript libraries. This was in line with our previous pilot-scale studies conducted using manure imported from the same commercial farm (Habtewold et al., 2017, 2018). However, CH_4 emissions were significant only in untreated slurries where the proportion of methanogens closely related to the genus *Methanosarcina* had significantly increased. In contrast to many other methanogens, *Methanosarcina* has been reported to grow under high concentrations of ammonia and VFA (Demirel and Scherer, 2008). However, the current study indicated that these methanogens were apparently impacted by the acidification with H_2SO_4 and perhaps by products of sulfate reduction (e.g., H_2S). Compared to acidified slurries, the predominance of *Methanosarcina* was high in untreated slurries which coincided with increased CH_4 emissions. *Methanosarcina* species are metabolically the most diverse and have higher efficiency in CH_4 production (e.g., 3× when glucose is used as substrate) when compared with *Methanocorpusculum* (Conrad, 1999; Kotsyurbenko et al., 2004), thus any effect on these methanogens might result in significant reduction of CH_4 production.

In stored liquid manure, reductions in CH_4 emissions might also be related to potential methanotrophy, which is presumed to occur in the surface crusts of slurries where oxygen is freely available for methanotrophs (Petersen and Ambus, 2006). In the current study, no crust was formed, and no known methanotroph was detected in both the 16S rRNA gene and transcript libraries of all treatments. Thus, the contribution of methanotrophy to the reduction of CH_4 emissions detected in the current study were less likely.

With the use of H_2SO_4 for manure acidification, slurries can be enriched with sulfate which is an important substrate for sulfate-reducing bacteria that have high affinity to available hydrogen (Kristjansson and Schönheit, 1983). Although the relative proportions of sulfate-reducers detected in the current study (e.g., *Desulfatibacillum*, *Desulforhopalus*, *Desulfuromonas*, and *Desulfobulbus*) were low, together with potential homoacetogenic bacteria (e.g., *Acetobacterium* and *Blautia*), they might still compete hydrogenotrophic methanogens for available substrates (Weijma et al., 2002). Methanogens related to the genus *Methanosarcina* can perform all three pathways of methanogenesis (hydrogenotrophic, acetoclastic, and methylotrophic), thus may compete favorably by changing substrates. Although sulfate-reducing bacteria can also compete for acetate, this substrate is highly abundant in stored liquid dairy manure (Barret et al., 2013; Habtewold et al., 2018), and acetate consumption rates in methanogens are relatively higher when compared to sulfate reducers (Bhattacharya et al., 1996). Hydrogen sulfide, which could be accumulated in slurries as a result potential sulfate reduction, might also suppress the activities of methanogens except *Methanosarcina* (Demirel and Scherer, 2008). Thus, differential enrichment of *Methanosarcina* in untreated slurries indicated manure acidification with H_2SO_4 had more impact on these methanogens when compared to *Methanocorpusculum*.

CONCLUSION

H_2SO_4-based acidification of stored liquid dairy manure (mean pH 6.5 and 5.9) could reduce cumulative CH_4 emissions by $76 \pm 7\%$ and $78 \pm 6\%$, respectively. Slurry acidification (pH down to 5.9) with H_2SO_4 coincided with significant reduction of VS contents of slurries in all treatments, but did not significantly impact the abundance, activity or community structure of bacteria. Regardless of treatments, *Methanocorpusculum* was the predominant methanogenic genus. *Methanosarcina*, while representing a minor proportion of the methanogens in this dairy slurry, was relatively lower in acidified slurries, and this coincided with significant reductions in CH_4 emissions. Thus, we propose that manure acidification with H_2SO_4 reduced CH_4 emissions by inhibiting growth and activities of *Methanosarcina*, the most metabolically diverse methanogen.

AUTHOR CONTRIBUTIONS

All the authors were involved in the planning of the work and revision of the manuscript. JH conducted the molecular work, data analysis, interpretations, and prepared the manuscript with the guidance of KD and RG.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02806/full#supplementary-material

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REVIEW
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Subsoil Arbuscular Mycorrhizal Fungi for Sustainability and Climate-Smart Agriculture: A Solution Right Under Our Feet?

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With growing populations and climate change, assuring food and nutrition security is an increasingly challenging task. Climate-smart and sustainable agriculture, that is, conceiving agriculture to be resistant and resilient to a changing climate while keeping it viable in the long term, is probably the best solution. The role of soil biota and particularly arbuscular mycorrhizal (AM) fungi in this new agriculture is believed to be of paramount importance. However, the large nutrient pools and the microbiota of subsoils are rarely considered in the equation. Here we explore the potential contributions of subsoil AM fungi to a reduced and more efficient fertilization, carbon sequestration, and reduction of greenhouse gas emissions in agriculture. We discuss the use of crop rotations and cover cropping with deep rooting mycorrhizal plants, and low-disturbance management, as means of fostering subsoil AM communities. Finally, we suggest future research goals that would allow us to maximize these benefits.

Keywords: arbuscular mycorrhiza, subsoil, soil depth, agriculture, sustainability, climate-smart

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INTRODUCTION

Assuring food and nutrition security has long been one of the greatest challenges for humanity and given current population growth and climate change scenarios, this is an increasingly challenging task. Some of the latest estimates predict the need to increase agricultural productivity by at least 70% by 2050, and the focus shifts increasingly to the role of soil biodiversity in general (Bender et al., 2016) and particularly arbuscular mycorrhizal (AM) fungi (Thirkell et al., 2017), in achieving this in a sustainable way. Moreover, agricultural productivity needs to become more resistant and resilient to the increasingly common and severe extreme climate events, that is, agriculture needs to get climate-smart (Lipper et al., 2014).

Arbuscular mycorrhizal fungi are a monophyletic, widespread group of fungi that form a mutualistic relationship with most land plants, including many agricultural crops (Smith and Read, 2008; Brundrett and Tedersoo, 2018). While predominantly known for their ability to increase plant nutrient uptake and productivity (Smith and Smith, 2011), they influence a wide range of ecosystem processes (Rillig, 2004; Powell and Rillig, 2018). AM fungal biomass abundance (Higo et al., 2013), spore numbers (Jakobsen and Nielsen, 1983; Oehl et al., 2005; Muleta et al., 2008; Säle et al., 2015), and root colonization levels (Sutton, 1973; Jakobsen and Nielsen, 1983) typically decline with increasing soil depth,

but over 50% of AM fungal total biomass can be found below 30 cm (Higo et al., 2013), and outside of agriculture, AM roots have been reported as deep as 8 m (de Araujo Pereira et al., 2018). AM fungal communities below 30 cm have also been shown to differ from those in topsoil both in spore morphology-based studies (e.g., Oehl et al., 2005; Muleta et al., 2008; Säle et al., 2015) and sequencing studies, with some phylotypes being exclusively detected in subsoil (Moll et al., 2016; Sosa-Hernández et al., 2018a). There is also growing evidence for subsoil ecological specialization in some AM fungal taxa (Sosa-Hernández et al., 2018b). Moreover, in an elevated CO_2 experiment by Rillig and Field (2003), AM root colonization increased in subsoil (here 15–45 cm) but not in topsoil, suggesting that topsoil and deeper soil AM communities might respond differently to environmental changes. Altogether, AM fungal communities below the plow layer are often overlooked but probably highly relevant components of agroecosystems that hold opportunities for management. In this paper, we review the different potential benefits of subsoil AM for agriculture, summarize the knowledge about them, and provide suggestions for future research on this topic.

SUBSOIL AND CLIMATE-SMART AGRICULTURE

In agriculture, the term subsoil refers to the soil beneath the Ap horizon, i.e., beneath the tilled or formerly tilled horizon. Considering that tillage depth is usually 20–30 cm, the vast majority of the volume of agricultural soil can be defined as subsoil, which makes even more remarkable the comparatively scarce knowledge we have and attention we pay to it as compared to topsoil. Subsoil contributions to plant nutrition range between 10 and 80%, and are expected to increase when topsoil is dry or nutrient depleted (Kautz et al., 2013). Unsurprisingly, several studies have shown no yield increase after fertilization even in nutrient-poor soils, as nutrient availability is typically characterized in topsoil and potential nutrient delivery from subsoil was not considered (Kautz et al., 2013). Guaranteeing plant access to the subsoil nutrient and water reservoir greatly increases the resistance of the system, making a greater pool of resources available and allowing the plant to avoid detrimental conditions in the topsoil, e.g., during a drought event.

Biodiversity is assumed to stabilize ecosystem functioning under fluctuating environmental conditions, known as the insurance hypothesis (Yachi and Loreau, 1999), and Isbell et al. (2015) showed that biodiversity adds to the resistance of ecosystem productivity under climate extremes. We now also start realizing the potential impacts of soil biodiversity loss or alteration on human health (Wall et al., 2015) and food properties and quality (Rillig et al., 2018). While microbial abundances commonly decrease with increasing soil depth, subsoils can also be a microbial biodiversity reservoir and harbor unique taxa (Fierer et al., 2003), and subsoil communities have been hypothesized to contribute to the recolonization of topsoil after perturbation (An et al., 1990; Verbruggen et al., 2012), adding resilience to the system.

SUBSOIL ARBUSCULAR MYCORRHIZAL FUNGI FOR SUSTAINABLE AGRICULTURE

General Aspects

Subsoil AM fungi communities can be abundant (Wortmann et al., 2008; Higo et al., 2013) and unique (Moll et al., 2016; Sosa-Hernández et al., 2018a) and they likely contribute to plant performance and ecosystem functioning in an underappreciated manner. In contrast with topsoil, subsoils are typically characterized by higher bulk densities and compaction, reduced pore spaces, and lower oxygen concentrations (Lynch and Wojciechowski, 2015; Weil and Brady, 2016), altogether representing a suboptimal environment for roots. Although we still lack empirical evidence of subsoil AM fungal-specific traits, it is a fair assumption that they are adapted to these environmental conditions. Among the hypothesized traits of these subsoil-specialized AM fungi would be an increased ability to colonize even the smallest soil pores, enhanced tolerance to anaerobic conditions, and, due to the general scarcity and uneven distribution of roots, greater persistence in time in the form of resting structures or long-lived mycelium. All these traits could be well-matched to the intrinsic problems a plant faces in subsoil, and could become particularly important under certain circumstances, such as present in clay soils, soils with high compaction, or soils with aeration problems. Moreover, applying a competitor-stress tolerator-ruderal (CSR) framework to AM fungi (Chagnon et al., 2015), subsoil AM fungi are expected to follow a stress tolerator life strategy. As such, deeper soil AM fungal phylotypes are expected to exhibit greater resource use efficiency and production of long-lived biomass, representing an advantageous carbon cost/benefit investment for the plant. These slow-growing communities would initially represent a carbon sink for the plant with little immediate benefits, but once the fungal network has been established, a long-lasting mycelium would provide its services to the plant at perhaps relatively little additional cost. Following the same rationale, the observed decrease in AM fungal spores with depth (e.g., Oehl et al., 2005; Muleta et al., 2008; Säle et al., 2015) might be less related to a decrease in abundance than to a change in both environment and life history strategy. AM fungal spores can be dispersed by wind (Egan et al., 2014), small mammals (Janos et al., 1995), earthworms (Reddell and Spain, 1991), or arthropods (McIlveen and Cole Jr., 1976), but all these vectors seem unlikely to be relevant in subsoils, with perhaps the exception of earthworms. With less disturbance and decreased microbial activity, probably a long-lived mycelium is in itself the best option for dispersal in time, and at larger time scales, also in space. This again represents a potential advantage for the plant symbiont, since AM fungal spores are particularly large and filled with lipids and carbohydrates

with a high metabolic cost (Giovannetti, 2000), and ultimately it is the plant that provides this carbon and energy. While the same holds true for the production of mycelium, plants obtain a direct profit from this carbon investment, because it is the mycelium that explores the soil and captures and transports nutrients to the plant. Summing up, plants may receive greater returns for every unit of carbon they provide to AM fungi in subsoil, as compared to in topsoil.

Last but not least, subsoil arbuscular mycorrhizae may have a significant role in the very formation of soil. The importance of the biological component in pedogenesis has long been identified (Jenny, 1994) and while bacteria tend to have greater geochemical capabilities, fungi can weather rocks too, especially mycorrhizal fungi (Hoffland et al., 2004). In fact, it is difficult to understand pedogenesis throughout earth's history without considering the coevolution of plant roots and mycorrhizal fungi (Leake and Read, 2017). The ability of ectomycorrhizal (EM) fungi to release low-molecular weight organic chelators in soil, which enhances mineral weathering, remains to be shown in AM fungi. However, AM fungi affect mineral weathering through various indirect pathways, including increased respiration, soil stabilization, enhanced evapotranspiration and exudation (Taylor et al., 2009), and differences in the mineral weathering abilities of AM and EM roots might be less pronounced than previously assumed (Koele et al., 2014). When it comes to deeper soil layers, biological activity is generally lower and despite potential accumulation of clay minerals from upper horizons, usually, it comprises larger amounts of primary minerals, posing great potential for mineral weathering and nutrient release. AM fungi greatly expand the volume of soil under the influence of the symbiosis, often referred to as the mycorrhizosphere (Linderman, 1988), and in subsoil, this likely means fostering microbial activity in a greater volume of soil. This combined action of roots, AM fungi, and the associated microbial community has the potential to favor soil development, and in shallow soils where the parent material or the bedrock is close to the surface, this process could increase soil formation and deepening (**Figure 1A**).

Efficient Fertilization

Probably the most widely appreciated contribution of AM fungi to plant performance is their ability to increase plant nutrient uptake, particularly of P (Smith and Smith, 2011). Harnessing the nutrient supply by AM fungi, the amount of applied fertilizer and the energy linked to its production can be reduced. A major issue in optimizing efficient fertilization is reducing the amount of nutrients lost to the system *via* leaching. AM fungi decrease nutrient leaching not only expanding the nutrient

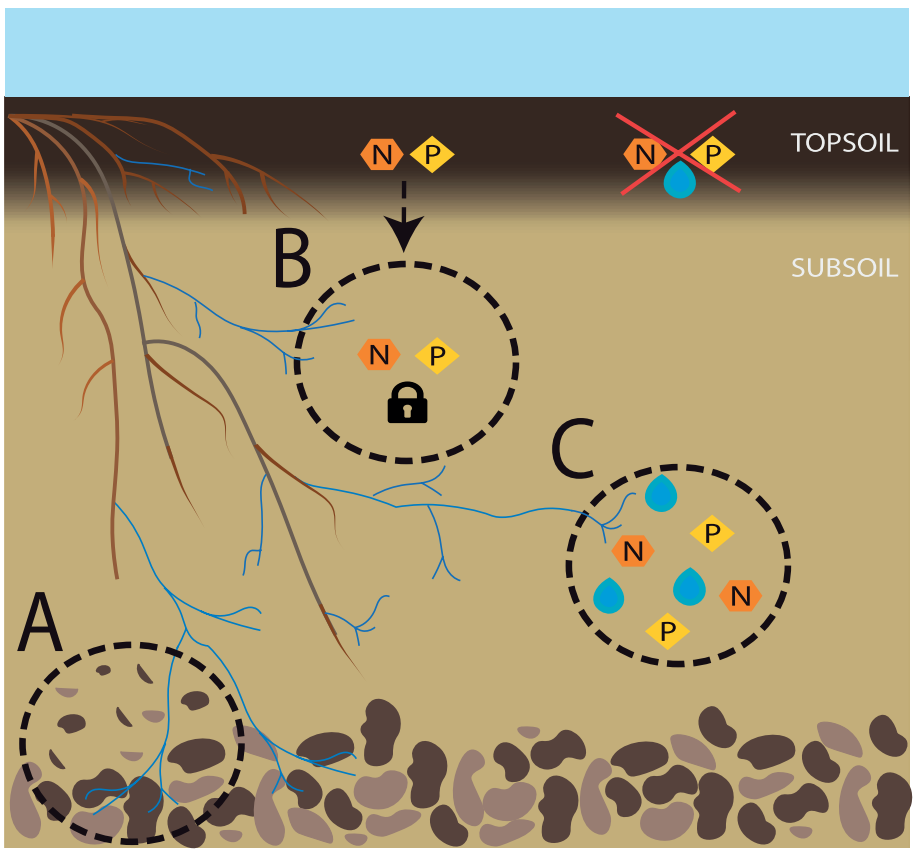

FIGURE 1 | Subsoil AM fungi for sustainable agriculture. Overview of the contributions of subsoil AM fungi to a sustainable agriculture. **(A)** Enhanced soil formation; **(B)** reduction of nutrient leaching; **(C)** access to deep nutrient and water pools, particularly when suboptimal conditions prevail in the topsoil.

interception zone due to the development of a mycorrhizosphere, but also thanks to increased nutrient uptake, enhanced soil structure and fostering of the microbial community with associated nutrient immobilization (Cavagnaro et al., 2015, **Figure 1B**). Köhl and van der Heijden (2016) demonstrated that different AM fungal species differ in their ability to decrease nutrient leaching, highlighting the potential importance of AM fungal diversity. In fact, the observed increase in nutrient leaching in highly fertilized agroecosystems may be explained not only due to greater soil nutrient content, but also due to a typically reduced abundance and diversity of AM fungi (van der Heijden, 2010).

AM fungi have been shown to stabilize community productivity across gradients of nutrient availability, and to reduce plant tissue nutrient content variability along such gradients in a grassland (Yang et al., 2016). If transferable to agricultural systems, these effects would be crucial in achieving food and nutrition security particularly in regions where access to fertilizers might be limited or irregular. Moreover, expanding the available soil nutrient pool to deep soil further increases resistance, allowing for instance the maintenance of plant growth under drought conditions, where nutrients in topsoil might be present but not accessible (positionally unavailable) for the roots (**Figure 1C**). Altogether, with the continuously increasing prices of fertilizers and their predicted scarcity in a near future, making the most out of these resources is the only way forward and subsoil and subsoil AM fungi may prove important in this task.

Nitrogen

Nitrogen (N) applied in agricultural fields can be lost *via* leaching or in form of gaseous emissions. The influence of AM fungi on gaseous loss of N will be discussed later in this article, in the context of greenhouse gas emissions. As for leachate N, it occurs mostly in form of dissolved nitrate (NO_3^-), a particularly mobile form of N in soil. AM fungi promote soil aggregation (Leifheit et al., 2014) by improving soil structure and therefore increasing soil water-holding capacity. Additionally, AM fungi take up N preferentially in the form of ammonium (NH_4^+), reducing the pool of N available for nitrification and consequently reducing the mobility of N. In subsoil, AM fungi could intercept N that migrated down the profile and immobilize it or deliver it to the plant, thus avoiding N losses (**Figure 1B**). Moreover, the proportion of NH_4^+ to other N sources increases in subsoil (Kautz et al., 2013), increasing the potential role of subsoil AM fungi in mobilizing and delivering this N to the plant, assuring access to a previously unavailable pool and reducing the need for N fertilization (**Figure 1C**).

A particularly relevant role of subsoil AM fungi might be the capture and delivery to the plant of N weathered from rocks. Recently, Houlton et al. (2018) demonstrated that bedrock weathering might be a significant source of active N in various terrestrial environments. When this weathering occurs in deep soil layers, a big proportion of this N may be released to groundwater and ultimately to the sea (Houlton et al., 2018). In such scenarios, the presence of an active microbial community, together with deep soil root proliferation, is crucial to capture this N before it is lost from the system. Due to their unique ability to capture and transport nutrients from the soil directly to plant roots, including N (Smith and Smith, 2011), AM fungi are promising candidates for maximizing the benefits obtained from this previously ignored resource, both reducing the need of N input and avoiding the contamination of groundwater.

Phosphorus

When it comes to P, it is generally assumed that due to its low mobility in soils, leaching is of no importance and most effort has been spent on avoiding P loss and P-mediated eutrophication *via* topsoil erosion. However, we now know that excessive manuring, the existence of preferential pathways, or a sandy soil texture can lead to significant P leaching (Djodjic et al., 2004; Schoumans, 2015), with its associated economic and environmental consequences. The role of AM fungi in P uptake has been extensively researched (Smith and Smith, 2011), and they can reduce the need of heavy manuring due to increased and efficient P uptake. As for subsoils, here AM fungi can again increase water-holding capacity, reducing the risk of leaching; but these fungi can also intercept P that has migrated down the profile and deliver it to the plant (**Figure 1B**). Inputs of organic P in subsoil, mostly *via* roots but also with direct injection of organic matter, can remain inaccessible to the plant due to decreased decomposition and mineralization rates. The role of subsoil AM fungi may be particularly important in acquiring this otherwise unavailable P (**Figure 1C**). Moreover, Wang et al. (2017) found some evidence that AM fungi in subsoil might contribute more to plant P nutrition than topsoil AM fungi, under heavy P fertilization. Consequently, subsoil AM fungi have potential to be of great relevance in the avoidance of P loss, particularly in sandy soils or when the topsoil is P saturated.

Re-allocation of Nutrients

More generally, fostering the proliferation of roots and AM fungi in deeper soil layers expands the volume of biologically active soil, increasing nutrient mineralization and immobilization rates. Thanks to their unparalleled ability to penetrate even the smallest soil pores such as in high-density environments like subsoil, these fungi reach nutrients beyond the rhizosphere and transport them to the plant and topsoil again. This notwithstanding, no microorganism can increase the net content of nutrients in soil, with the exception of N-fixing bacteria. Therefore, even the most sustainable and efficient agricultural practices will eventually need to resupply nutrients to the soil. The same applies to subsoils: gaining access to this nutrient pool does not exempt farmers from the need to eventually replenish it. Natural migration of nutrients from topsoil to subsoil typically occurs *via* root exudates, dead roots, the action of anecic earthworms, and the deposition of nutrients dissolved in water that reach subsoil through preferential flow pathways (Kautz et al., 2013). Therefore, enhancing the formation and maintenance of biopores is crucial for a proper replenishment of the subsoil. Additionally, the presence of an extensive mycorrhizosphere with its associated exudates can foster the return of some

nutrients to the subsoil. Apart from these natural processes, direct inclusion of nutrients in deeper layers, such as injection of organic matter into subsoil, should be considered. Recent studies have shown positive effects of the admixing of organic matter in subsoil on the performance of barley (Jakobs et al., 2017), but understanding the long-term effects of these on subsoil diversity and sustainability requires further research.

GREENHOUSE GAS EMISSION IN AGRICULTURE

Modern agriculture is responsible for an estimated 12% of the global anthropogenic greenhouse gas emissions (Linquist et al., 2012). Some of these emissions are associated with fertilizer production and the use of heavy machinery, but most of them occur in the form of direct emissions from the field. The potential benefits of subsoil AM fungi in alleviating emissions related to fertilizer application were discussed in the previous section. Next, we will address the role of subsoil AM fungi in reducing the release of two important greenhouse gasses associated with agriculture: carbon dioxide (CO_2) and nitrous oxide (N_2O).

Carbon Dioxide: Subsoil Arbuscular Mycorrhizal Fungi and Carbon Farming

The traditional view of very stable carbon in subsoil is questioned in recent findings. Stable subsoil carbon may be readily decomposed when fresh carbon is added. We propose that AM fungi have the potential to counteract this phenomenon due to their function in soil structure and in the capture of nutrients.

The Traditional View of Carbon in Subsoil

In depths of up to 3 m, the pedosphere stores more carbon (C) than the biosphere and the atmosphere combined (Jobbágy and Jackson, 2000). With increasing depth throughout the soil profile, the mean residence time of C increases, reaching up to 10,000 years (refs. 2–4 in Fontaine et al., 2007). In the past, it was generally assumed that the age of C is connected to its stability, i.e., older C is also more stable.

Indeed, we do find very stable compounds in the subsoil that have much slower turnover times than compounds in the topsoil [Spielvogel et al., 2008; Rumpel and Kögel-Knabner, 2011; Balesdent et al., 2018 (and refs. 16–19 therein)]. This could be attributed to several reasons:

(1) Subsoils usually have reduced amounts of energy sources and nutrients, especially N and P, which limit microbial activity and thus the turnover of OM. (2) Subsoils have a higher soil density with smaller pore volumes that decrease overall habitat space for soil organisms, thus reducing their abundance. (3) Subsoils often show a change in texture, i.e., increased amounts of clay that can bind organic matter (OM) in organo-mineral complexes with stable bonds resulting from, e.g., ligand exchange or polyvalent cation bridges. As environmental conditions such as temperature and moisture are usually more stable in subsoil (Weil and Brady, 2016), the importance of soil mineral chemistry for OM stabilization becomes more pronounced. (4) In subsoil, a greater proportion of OM is located in microaggregates as compared to topsoil, allowing for slower turnover times (Torres-Sallan et al., 2017).

Recent Findings Question the Stability of C in Subsoil

However, in more recent studies, the stability of old C in the subsoil has been questioned and a number of studies have shown that subsoil C is susceptible to decomposition when fresh C is added to the soil (e.g., Fontaine et al., 2007; Hobley et al., 2017). The majority of these studies extracted the soil for use in pot studies, where single and sometimes easily degradable substances were added to the soil. The soil extraction represents a massive disturbance, changes temperature, soil density, and moisture conditions, which strongly boost microbial activity and thus degradation of OM (Rumpel and Kögel-Knabner, 2011). Therefore, the instability of subsoil OM might have been overestimated due to methodological flaws and could be much less in the field under realistic conditions.

The Role of Arbuscular Mycorrhizal Fungi in Subsoil Carbon Cycling–Soil Structure

One factor usually not included in previous experiments considering subsoil C cycling is AM fungi. In numerous studies, they have been shown to improve soil aggregate stability through hyphal enmeshment of soil aggregates and the production of extracellular polymers (Rillig and Mummey, 2006). Compared to topsoil, subsoil is subject to less disturbance that can disrupt hyphal networks, leading to a longer residence time of aggregate-protected OM (Lehmann et al., 2017). Therefore, stabilization of soil aggregates by mycorrhizal hyphae in the subsoil can contribute substantially to the protection and thus sequestration of soil organic matter (SOC) (**Figure 2A**). A better soil structure also improves soil pore connectivity, leading to increased interactions between soil microbes, and, consequently, likely increased competition for nutrients. If AM fungi could outcompete decomposers for nutrients, they would be able to indirectly reduce decomposition activity and thus potential loss of added or stabilized carbon (**Figure 2B**).

The Role of Arbuscular Mycorrhizal Fungi in Subsoil Carbon Cycling–Nutrient Additions

More specifically, competition for nutrients can also be induced directly by AM fungi, as they acquire nutrients and water for themselves, thereby reducing the nutrient and water availability for other microbes, which could reduce the activity of decomposers due to nutrient or water deficiency (Verbruggen et al., 2013; Jansa and Treseder, 2017, **Figure 2B**). However, nutrient additions, as single or combined additions of N and P increase SOC decomposition, an effect called priming (Kuzyakov, 2010). Meyer et al. concluded that both the current soil nutrient conditions and microbial nutrient demand must be considered when predicting the effect of N addition on SOC turnover. According to the

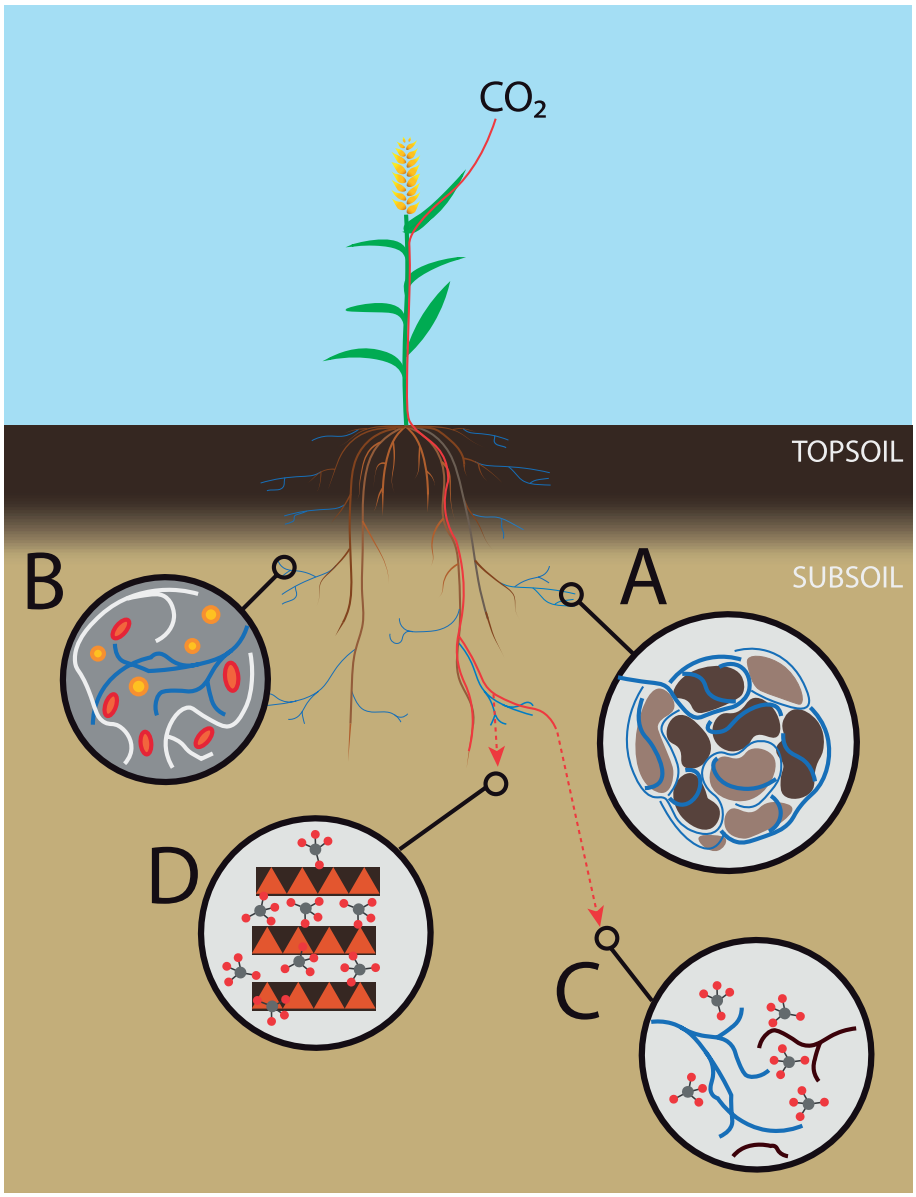

FIGURE 2 | Subsoil AM fungi and carbon farming. Overview of different benefits of subsoil AM fungi on carbon sequestration. **(A)** Improvement of the soil structure, leading to aggregate-protected organic matter. **(B)** Competition with saprotrophic bacteria and fungi, thus reducing decomposition rates. **(C)** Increased carbon input in subsoil *via* mycelial exudates and turnover. **(D)** Formation of highly stable mineral-associated organic matter fractions.

authors, the importance of the subsoil as a long-term C sink is unclear when there is also increased input of additional N and P. The nutrient capture by AM fungi could be important for minimizing the stimulating effect that additional nutrients have on decomposition of SOM, particularly after the admixing of organic matter in subsoil, as performed by Jakobs et al. (2017).

In addition to capturing nutrients, AM fungi can reduce the availability of carbon compounds in the rhizosphere, because plants provide carbon to AM fungi in exchange for nutrients delivered (Jones et al., 2004). In the absence of AM, higher rhizodeposition would stimulate microorganisms in the rhizosphere, and thereby possibly stimulate SOM decomposition as microorganisms mine for nutrients in stabilized SOM. AM fungi receive up to 20% of a plants' assimilates (Bago et al., 2000), which they first use for their own metabolism, before mycelial exudates are released. In this way, the mycorrhizal extraradical mycelium can be an important pathway of C to the SOM pool, when they exude mycelial organic compounds to soil parts more distant from the root system, but also *via* mycelium turnover (**Figure 2C**). In topsoil, the C input by mycorrhiza can sometimes exceed the input of leaf litter and fine root turnover. In a boreal forest, Clemmensen et al. (2013) found that in subsoil, up to 70% of soil C can be root-derived, especially when root densities were high in deep horizons.

In this study, and several others, mycorrhizal and other endophytic fungi dominated the subsoil, but decomposer fungi were only abundant in upper soil horizons. This suggests that decomposition processes controlled by microbial community composition *in situ* might be dominant in topsoil but subordinate in subsoil.

The Role of Arbuscular Mycorrhizal Fungi in Subsoil Carbon Cycling–Litter Decomposition

Although AM fungi may increase litter decomposition in short-term laboratory experiments, they probably have positive long-term effects on soil C. In the short term, AM fungi are able to enhance OM degradation through the stimulation of decomposers, but we do not know whether this stimulation is permanent. Moreover, microbial metabolites are not necessarily lost, they can be integrated into very stable compounds such as mineral-associated SOM fractions, which have the longest mean residence times in soil (**Figure 2D**). Indeed, subsoil OM contains more microbial-derived compounds compared to topsoil and microbially processed sugars seem to better associate with the mineral phase than plant-derived OM (Rumpel and Kögel-Knabner, 2011). This stabilization mechanism could be especially important in subsoil, because here, the amount of clay minerals and sesquioxides increases, representing a great potential for long-term stabilization of (fresh) C.

The Potential Contribution of Arbuscular Mycorrhizal Fungi in Subsoil Carbon Storage

Some efforts are made to find ways to increase SOC storage, e.g., in subsoil by increasing the presence of plants throughout the year with catch crops, by the use of undersown crops or deep rooting plants (Kell, 2011; Jakobs et al., 2017). Without further management, however, this could stimulate soil microbial activity and thus also decomposition of freshly added OM as well as stabilized OM (Kong, 2018). To counteract this effect, AM fungi could be fostered in order to reduce rhizodeposits, by including mycorrhizal crops together with a low management intensity (e.g., no tillage) and adapting a low fertilization level, as mycorrhizal fungi are more abundant in no-tillage systems and their effects are more pronounced in nutrient-limited systems (Jansa et al., 2002, 2006). However, the interaction of plants, AM fungi, and other microbes in relation to SOC storage in soil particles or microbial biomass is still not very well understood. For instance, although AM fungi have been observed to induce smaller priming effects on SOM than roots, they might still promote soil respiration and thus increase SOC losses. Therefore, future research should adopt a comprehensive approach for studying plant—fungal-mediated processes in C cycling, considering the influxes (e.g., photosynthetic assimilation, root exudation, mycelial exudation, litter fall, soil organism detritus and fecal residues), effluxes (e.g., all parts of soil respiration, decomposition, leaching), as well as immobilization and storage of C in SOM and microbial biomass. These processes are especially interesting to study with respect to long-term C gains, e.g., through plant growth promotion effects, soil aggregation, or the production of microbial products.

Data on the sensitivity of stored deep C are limited; we need further on-site research (with a low level of disturbance and alteration of environmental conditions) to evaluate the impact and importance of management strategies such as deep rooting plants, and effects of microbial community properties.

Nitrous Oxide Emissions

Agriculture is a major source of anthropogenic N_2O emissions (Linquist et al., 2012), a potent greenhouse gas with tremendous global warming potential 280–310 higher than CO_2 and a lifetime in the atmosphere that ranges from 118 to 131 years (IPCC, 2001; Fleming et al., 2011). Multiple pathways of N_2O production co-occur in soil and their relative contribution to its emission is poorly understood. Ammonia oxidation, dissimilatory nitrate reduction to ammonium (DNRA), and various denitrification pathways have been identified as microbially mediated processes with significant contributions to N_2O emission in agricultural soils (Baggs, 2011; Zhu et al., 2013, **Figure 3**). Under low oxygen concentrations, such as those expected in subsoil, typically anaerobic processes, such as denitrification or DNRA (**Figures 3A,B**), are expected to prevail (Baggs, 2011), with significant denitrification rates having been reported in subsoil (Cleemput, 1998; Clough et al., 2005). Since NO_3^- is the primary substrate for both processes, we can expect that the reduction in NO_3^- leachate arriving at the subsoil due to the effect of AM fungi would also have a negative impact on DNRA and denitrification rates in subsoil. Furthermore, in grassland subsoil, the addition of easily available C increased N_2O production, suggesting again that the reduced secretion of simple carbohydrate exudates in an AM root would further

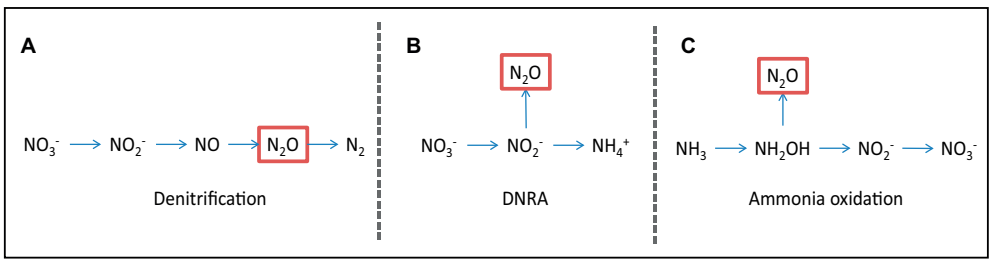

FIGURE 3 | Nitrous oxide emissions. Simplified overview of N_2O (nitrous oxide)-producing processes that can be influenced by AM fungi. **(A)** Denitrification, **(B)** dissimilatory nitrate reduction to ammonium (DNRA), **(C)** ammonia oxidation.

reduce this process. Ammonia oxidation is an aerobic process mediated by autotrophic organisms, in which the concentrations of oxygen and the substrate ammonia (NH_3) influence process rates (**Figure 3C**). AM fungi were shown to have a direct negative effect on N_2O emission following N fertilization in a pot trial using agricultural soil, and the competition with nitrifiers for NH_4^- was identified as the main driver (Storer et al., 2018). While the presence of high NH_4^- concentrations in subsoil is unlikely due to its limited mobility, this might not be the case following the mineralization of admixed organic matter in subsoil. Under such scenarios, where additionally considerably less anaerobic conditions prevail due to the deep tillage, the presence of subsoil AM fungi to readily take NH_4^- up and outcompete nitrifiers would be potentially important.

POTENTIALS AND LIMITATIONS TO PROMOTE BENEFICIAL EFFECTS OF ARBUSCULAR MYCORRHIZAL FUNGI IN THE SUBSOIL BY AGRICULTURAL MANAGEMENT

Achieving food security at a global scale is a complex task requiring multiple approaches. As for increasing and securing agricultural productivity, climate-smart agriculture offers the best perspectives for success (Lipper et al., 2014). Much more research is needed to fully understand the role of subsoil and subsoil AM fungi in plant performance and to what extent we can manage them for sustainable intensification. This notwithstanding, evidence begins to accumulate pointing at particular agricultural practices that may help make our yields more sustainable and climate-smart (**Table 1**). First and foremost, we need to approach these challenges in a well-informed and integrated way, as optimizing only some aspects of productivity while ignoring others will certainly be counterproductive (Rillig et al., 2016). In fact, there is no one-size-fits-all solution and required management components are highly context dependent. This is why sustainable intensification has been defined as an increase of knowledge per hectare (Buckwell et al., 2014), stressing the importance of fine-tuned information.

TABLE 1 | Suggested management approaches to foster subsoil AM fungi.

Management	Aim
Crop rotation	Including deep rooting and mycorrhizal plants in crop rotations to increase deep soil root proliferation and AM abundance
Catch crops and cover crops	Catch crops and cover crops can increase AM abundance through the profile, increasing AM colonization for the next crop
Crop breeding and selection	Plant breeding and selection of crops with a focus on mycorrhizal responsiveness and deep rooting traits
Reduced/no till	Reduced and no-till systems typically increase AM abundance
Deep plowing	In the presence of a plow pan that restricts root growth into subsoil, deep plowing can allow for subsoil root and AM fungal proliferation

Plant Breeding and Choice

Clearly, a fundamental prerequisite for the exploitation of subsoil is the presence of deep roots. Thus, crop rotation or catch cropping with deep rooting plants is essential to access deep soil resources and to create biopores that subsequent crops can use to grow into subsoil (Kautz et al., 2013). For instance, deep rooting and mycorrhizal plants, such as wheat, have been shown to increase AM fungal abundance through the soil profile (Higo et al., 2013). The use of cover crops has also been identified as a means of increasing AM fungal inoculum in soil (e.g., Galvez et al., 1995; Boswell et al., 1998; White and Weil, 2010; Lehman et al., 2012). Additionally, crop breeding and crop selection can be done considering a set of traits that favor the plant's abilities to access subsoil, as reviewed by Bishopp and Lynch (2015) and Lynch and Wojciechowski (2015). However, it is very unlikely that any one given cultivar will possess all the traits required to fully optimize the use of subsoil. Therefore, while developing crop rotations or intercropping systems, it is desirable to look closely at the roots and select for a varied and balanced set of traits that better suits our goals (Rillig et al., 2015), aiming not only for a diversity of aboveground characteristics but a diversity of root architectures and abilities that can sustain the desired ecosystem services (Bardgett et al., 2014; Bardgett and van der Putten, 2014). Plant domestication has produced high-yielding and resistant phenotypes that perform better than their wild relatives in the context of high-input agriculture. This selective breeding has often come at a cost of neglected impacts on the soil microbiome (Pérez-Jaramillo et al., 2016). In the particular case of AM fungi, an extensive analysis comparing domesticated plants with their wild relatives found that under limited P availability, both phenotypes profit from AM colonization, but under high P fertilization regimes (such as in conventional agriculture), the symbiosis was less efficient in domesticated plants (Martín-Robles et al., 2018). In addition to deep rooting traits, we recommend accounting for mycorrhizal responsiveness in future plant breeding efforts to assure that crops can benefit the most from the local AM fungal communities (Rillig et al., 2016).

Subsoil Management

Access to subsoil can be limited by physical properties, such as the existence of a hard plow pan that prevents root growth. The benefits of deep tillage and other subsoil tillage management options can be controversial and highly context dependent; but on average, given the existence of a plow pan, yields can be substantially increased after deep plowing (Schneider et al., 2017). The existence of subsoil-specific AM fungal phylotypes and their inability to survive soil mixing events, however, calls for precaution and the general avoidance of any method that inverts the soil profile (Sosa-Hernández et al., 2018b). Intensive tillage has been identified as a major factor reducing AM fungal abundance and diversity in agriculture (Kabir, 2005). Recently, Säle et al. (2015) compared the effects of reduced and conventional tillage, down to 40 cm in the soil profile using spore-based community analysis. Their results confirm the expected shifts in spore abundance and diversity in topsoil but those effects

were not significant in deeper layers, despite a shift in community composition. The absence of spore abundance shifts does not necessarily imply a lack of effect on hyphal abundance or colonization rates, but changes in subsoil community composition highlight that tillage can affect AM fungi in deeper layers, with unknown consequences for their functionality. No-till or reduced till systems however typically face another set of problems that may include increases in bulk soil density, limited nutrient mobility through the profile, or the use of agrochemicals for weed control, plus a set of economic and technical constraints that are more pronounced on small farms (Giller et al., 2015).

Arbuscular Mycorrhizal Fungal Inoculum

Assembling the right consortia of plant phenotype and rhizosphere microbiome has also been postulated as one of the means for a new underground revolution that aims at an ecological intensification in agriculture (Bender et al., 2016). This approach is very promising but holds intrinsic associated risks (Machado et al., 2017). The benefits of mycorrhizal inoculum can be highly context dependent (Hoeksema et al., 2010) and the use of non-native genotypes carries always the possibility of associated environmental impacts (Schwartz et al., 2006). This variability (but often not uncertainty, (Lehmann and Rillig, 2014)) in response to AM inoculation often leads to a lack of trust in its general efficiency by the agricultural community. We think AM fungal inoculum should not be used indiscriminately in general, or substitute for other AM-promoting management options. When it comes to subsoils, the evident existence of a specific AM fungal community calls for even greater caution, and at present, our knowledge is too limited to encourage the use of inoculum for the subsoil.

FUTURE RESEARCH CHALLENGES

Early research on AM fungi already observed abrupt decreases of spore abundance and colonization levels with increasing depth in agriculture (Sutton and Barron, 1972; Sutton, 1973). This could have led to a subsequent lack of interest in studying the arbuscular mycorrhizal symbiosis in deeper layers. However, outside the realm of agriculture, evidence of AM colonization was found down to 4.8 m in honey mesquite (Virginia et al., 1986) and this depth record has been recently updated to 8 m in an *Eucalyptus* and *Acacia* plantation (de Araujo Pereira et al., 2018). Very little research has been conducted on the community composition of AM fungi across different depths in agriculture, with few notable exceptions (e.g., Oehl et al., 2005; Muleta et al., 2008; Säle et al., 2015), and these spore-based studies have only recently been supported by molecular-based research (Moll et al., 2016; Wang et al., 2017; Sosa-Hernández et al., 2018a). Moreover, the only assessment on subsoil AM functionality was performed by Hafner et al. (2014), who compared root-derived C in the rhizosphere as influenced by AM fungi from two different depths in a greenhouse experiment.

Consequently, we believe that more basic, descriptive research, both spore and molecular based, needs to be performed to better understand the vertical distribution of AM fungi in agriculture and to confirm some of the already obtained knowledge across different regions and crops. We think it is particularly important to start linking agricultural management with responses in AM fungi across the entire soil profile, as exemplified by Säle et al. (2015), ideally covering aspects such as tillage, fertilization, and crop rotations. Furthermore, we also need to learn about the functioning of AM fungal communities in the subsoil, since AM fungi and roots face a very different environment than in topsoil. Rooting depth and architecture is one of the niche axes that allows plant coexistence in natural habitats (Silvertown, 2004), and roots at varying depths may forage for different resources (e. g., shallow roots acquiring P and deeper roots acquiring water). We can assume that, similarly, what the plant demands from its mycorrhizal partner might vary with soil depth, opening the possibility for specialized or even new functionality of subsoil AM phylotypes. Experiments assessing these potential differences in mycorrhizal functionality across depths are crucial and the isolation of deep soil AM fungi would go a long way toward the understanding of these communities.

Discerning the assemblage mechanisms, ecosystem role, and phylogenetic structure of AM fungi in deeper soil layers will help us answer important questions about AM fungal biogeography and diversity maintenance. Despite the three-dimensional nature of soil, to date, we have centered most of our efforts on a shallow soil layer with virtually no understanding of the ecosystem contributions of deeper AM fungi (Powell and Rillig, 2018), even if most evidence points to greater vertical than horizontal variation in fungal community composition (Bahram et al., 2015). Routinely including the vertical axis in AM studies across different biomes and in our theoretical frameworks will deepen our overall understanding of the biology of this relevant group of plant symbionts. Increasing our knowledge and expanding our perspective to include subsoil and subsoil AM fungal communities will not solve our problems on its own; however, an integrated subsoil management that takes AM fungi into account can bring us one step further in achieving sustainable and stable yields.

AUTHOR CONTRIBUTIONS

MS-H wrote the first draft of the paper; EL, RI, and MR contributed ideas and text.

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