Microbial Responses to Inorganic Nutrient Amendment Overridden by Warming: Consequences on Soil Carbon Stability

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Summary

Eutrophication and climate warming, induced by anthropogenic activities, are simultaneously occurring worldwide and jointly affecting soil carbon stability. Therefore, it is of great interest to examine whether and how they interactively affect soil microbial community, a major soil carbon driver. Here, we showed that climate warming, simulated by southward transferring Mollisol soil in agricultural ecosystems from the cold temperate climate zone (N) to warm temperate climate (C) and subtropical climate zone (S), decreased soil organic matter (SOM) by 6%-12%. In contrast, amendment with nitrogen, phosphorus and potassium enhanced plant biomass by 97% and SOM by 6% at the N site, thus stimulating copiotrophic taxa but reducing oligotrophic taxa in relative abundance. However, microbial responses to nutrient amendment were overridden by soil transfer in that nutrient amendment had little effect at the C site but increased recalcitrant carbon-degrading fungal *Basidiomycota* taxa by 195% and recalcitrant carbon-degrading genes by 23%-40% at the S site, implying a possible priming effect. Consequently, SOM at the S site was not increased by nutrient amendment despite increased plant biomass by 108%. Collectively, we demonstrate that soil transfer to warmer regions overrides microbial responses to nutrient amendment and weakens soil carbon sequestration.

Introduction

Anthropogenic activities, such as agricultural fertilization and fossil fuel combustion, have increased environmental nitrogen and phosphorus input, doubling nitrogen and quadrupling phosphorus terrestrial cycle turnover rates (Gruber and Galloway, 2008; Elser and Bennett, 2011). Nutrient enrichment or its deteriorated form, eutrophication, could alter the function of many ecosystems (Rockström et al., 2009; Fornara et al., 2013; Sun et al., 2016), and increase plant primary productivity and litter input to soil (Shaw et al., 2002; Liu and Greaver, 2010). Further effects on soil carbon stocks remain elusive since nutrient enrichment could increase, decrease or not alter soil carbon sequestration (Gregorich et al., 1996; Mack et al., 2004; Fornara et al., 2013; Su et al., 2015). In addition, nutrient enrichment has driven soil acidification (Guo et al., 2010), which adversely affects soil biodiversity. Given the expected increase in demand for food and energy in the next decades, nutrient enrichment in the environment is projected to continue (Gruber and Galloway, 2008).

Nutrient enrichment is concomitant with the global warming trend, which is projected to cause an increase in the mean global temperature by 1.5-4°C by Year 2100 (Greaver et al., 2016). Climate warming could stimulate plant growth (Melillo et al., 2011), further increasing fresh organic matter input to soil through litter, roots and exudates (Yin et al., 2013). Nonetheless, soil carbon input might be offset by accelerated soil microbial respiration under warmer conditions (Lu et al., 2013; Wang et al., 2014; Xue et al., 2016), which can be further complicated by the priming effect, i.e., fresh carbon input stimulates recalcitrant old soil organic carbon degradation

(Fontaine et al., 2007; Fontaine et al., 2011). As a consequence, there is high uncertainty in predicting future soil carbon fate. Since global ecosystems experience both climate change and anthropogenic nutrient enrichment, it is imperative to assess their interactive effects, which can be absent, synergetic or antagonistic (Zavaleta et al., 2003). In other words, the interactive effects could be equal to, larger, or smaller than additive effects from single-factor experiments.

There have been prior efforts to investigate the interactive effects of nitrogen amendment and climate warming. A synergetic effect of nitrogen amendment and climate warming on litter decomposition was detected, as shown by an accelerated loss in litter mass which was not detected from nitrogen supply alone (Hines et al., 2014). Some studies also detected interactive effects of nitrogen amendment and warming on Gram-positive bacterial biomass, the fungi/bacteria ratio, and the abundance of ammonia oxidizing bacteria (AOB) (Gutknecht et al., 2012; Long et al., 2012), whereas other studies found no interactive effects on microbial biomass (Shen et al., 2014; Contosta et al., 2015). However, most studies focused on nitrogen amendment, whose effect may differ from those of other nutrient additions, such as phosphorus and potassium (Hartley et al., 2010; Fornara et al., 2013). Additionally, most previous studies were conducted in forest or grassland ecosystems, there is still a lack of studies in agricultural ecosystems, where multi-nutrient amendment is very common. It is thus important to conduct an integrative, in-depth characterization of microbial responses to nutrient amendment and warming in agricultural ecosystems to achieve a mechanistic understanding of soil carbon fate.

Here we report an *in situ* field study to determine the impact of warming and multi-nutrient amendment of nitrogen, phosphorus and potassium on soil microbial community and function in agricultural ecosystems. We transferred Mollisol soil from cold temperate climate (the N site) to two warmer regions (the C and S sites, see *Experimental procedures* for details) to simulate abrupt climate warming, since the possibility of abrupt climate change events is increasing under human forcing (Alley et al., 2003). Microbial biomass was measured by phospholipid fatty acid analysis (PLFA). Bacterial and fungal communities were characterized by sequencing on an Illumina MiSeq platform, while functional structures of microbial communities were profiled by a high-throughput functional gene array (GeoChip). We hypothesize that nutrient amendment and soil transfer have interactive effects on microbial community composition and functional potentials, which in turn affect soil carbon stability. Since bacteria and fungi are fundamentally different in physiology and ecological adaptation, we also hypothesize that their responses to field manipulations are dissimilar.

Results

The *effect of soil transfer*

Environmental variables and microbial biomass are summarized in Table S1, shown by average values and significant differences between treatments tested by ANOVA. Soil transfer changed a number of soil geochemical variables (Table S2). Notably, soil

transfer caused substantial nutrient loss, since soil organic matter, total nitrogen, available phosphorus and available potassium decreased by 5% - 13% at the C site and decreased by 7% - 23% at the S site (Table S1). In addition, soil pH values were increased from 6.13 at the N site to 6.66 at the C site but decreased to 5.75 at the S site. Soil transfer increased soil bulk density but decreased soil porosity, soil moisture and water holding capacity, which might be ascribed to soil compaction during long-distance soil transportation.

Nitrification potential was increased by 43% at the C site but dramatically decreased by 70% at the S site (Table S1). CO_2 efflux was unaltered at the C site but decreased by 33% at the S site, which was similar to changes in fungal biomass at the C and S sites. In contrast, bacterial biomass was decreased by 45% at the C site and 58% at the S site, and total microbial biomass by 33% at the C site and 44% at the S site.

Soil transfer changed fungal and bacterial community composition and overall microbial functional genes ($P<0.003$, Table 1). The effect of soil transfer on abundant fungal OTUs (relative abundance > 0.002%) was obvious, which were separated into three groups according to the sites (Fig. 1A). Few fungal OTUs were abundant across all three sites, suggesting that most OTUs were affected by soil transfer. For microbial functional genes related to nitrogen cycling, there were increases in relative abundance of ammonification genes (*gdh* and *ureC)*, nitrification gene (*amoA* derived from archaea (AOA)), and nitrogen fixation gene *nifH* at the C site (Fig. S1). There were increases in ammonification gene *gdh*, nitrification gene *amoA*-AOA, and

denitrification genes (*nirS* and *nosZ)* and decreases in nitrogen fixation gene *nifH* and dissimilatory/assimilatory nitrite reduction genes (*nrfA* and *nir)* at the S site. These results suggested that soil transfer might enhance nitrification via stimulating AOA but not ammonia-oxidizing bacteria (AOB).

To exclude the possibility of microbial immigration from neighboring soil, we noted that microbial community in transferred samples shared much fewer OTUs originating from neighboring soil than from those originating from the N site (Fig. S2). Therefore, any microbial immigration from neighboring soil, if any, was minor.

The *effect of nutrient amendment*

We examined the effect of nutrient amendment at the N site. Nutrient amendment increased crop yield (seed weight) by 285% and aboveground biomass by 97% (Table S1). Accordingly, nutrient amendment increased soil organic matter by 6.03% (P=0.045), ammonium by 167%, and available phosphorus by 38%. Soil pH decreased by 0.4 units, revealing soil acidification ascribed to the use of urea and diammonium phosphate. We also found that nutrient amendment decreased bacterial biomass by 20% and fungal biomass by 43%.

Nutrient amendment affected bacterial community composition (P=0.006) and fungal community composition (P=0.001) (Table S3). A total of 44 bacterial OTUs were increased (False discovery rate (FDR)-corrected P<0.05, Table S4), among which there were many copiotrophic taxa such as genera *Sphingomonas* (increasing

from 2.6% to 5.0%), *Mizugakiibacter* (increasing from 0% to 0.9%) and *Rhodanobacter* (increasing from 0.1% to 0.7%). In contrast, 32 bacterial OTUs decreased (FDR-corrected $P<0.05$), among which 10 belonged to oligotrophic-rich *Verrucomicrobia*. For fungi, 12 OTUs were significantly (FDR-corrected $P<0.05$) changed by nutrient amendment (Table S5), including an *Exophiala spp.* of Phylum *Ascomycota* (increasing from 0.1% to 1.0%) and an unidentified *Pleosporales spp.* of Phylum *Ascomycota* (increasing from 0.2% to 2.7%).

Microbial functional potentials in carbon degradation were shifted by nutrient amendment (Fig. 2A), with decreases in relative abundances of starch-degrading genes (*amyA*, *cda* and *nplT* in the range of 12% to 24%), an agar-degrading gene (*beta agarase* by 22%), a cellulose-degrading gene (*cellobiase* by 5%), a cutin-degrading gene (fungal *cutinase* by 40%), and a terpene-degrading gene (*limEH* by 44%). In contrast, there were increases in the relative abundances of pectin-degrading genes (*pme* by 8% and fungal *pme* by 21%), a hemicellulose-degrading gene (*xylA* by 17%), a cellulose-degrading gene (*endoglucanase* by 10%), and a cutin-degrading gene (bacterial *cutinase* by 7%).

Microbial functional potentials of nitrogen cycling were also shifted by nutrient amendment (Fig. 3A). There were increases in relative abundances of a number of genes (e.g. ammonification genes *gdh* and *ureC*, nitrification gene *amoA* derived from AOA, denitrification genes *narG*, *nirK*, *norB* and *nosZ*, and assimilatory nitrogen reduction gene *nir*) while only nitrogen fixation gene *nifH* was decreased, which might result in an acceleration of overall soil nitrogen cycling.

The interactive effect of soil transfer and nutrient amendment

There were interactive effects of soil transfer and nutrient amendment on environmental variables and microbial communities (Table 1 & S1). Microbial responses to nutrient amendment were completely overridden by soil transfer (multivariate regression tree (MRT) analysis, Fig. 4). For example, nutrient amendment altered soil organic matter, bacterial biomass and bacterial community composition at the N site, but did not at the C or S site (Table S1 & S3). However, fungal community composition was altered by nutrient amendment at the S site (Table S3). Closer examination showed that Class *Agaricomycetes* increased by 396% (P=0.080) and *Microbotryomycetes* increased by 1744% (P=0.001, Fig. 1B). At the species level, *Coprinellus curtus* increased from 0.002% to 1.607%, and *Rhodotorula mucilaginosa* increased from 0.53% to 10.56% (Table S5).

Microbial functional genes related to carbon and nitrogen cycling interactively responded to soil transfer and nutrient amendment (Table S6). Nutrient amendment generally increased the relative abundance of microbial carbon-degrading genes at the S site in contrast to observations at the N site, including starch-degrading genes (*cda*, *glucoamylase* and *nplT* in the range of 10% to 24%), a hyaluronic acid-degrading gene (*hyaluronidase* by 17%), a pectin-degrading gene (*rgh* by 11%), a hemicellulose-degrading gene (*mannanase* by 11%), a cellulose-degrading gene (*cellobiase* by 23%), chitin-degrading genes (*acetylglucosaminidase and chitinase* by

38% - 40%), a cutin-degrading gene (fungal *cutinase* by 37%), a lignin-degrading gene (*glx* by 21%), and a terpene-degrading gene (*limEH* by 32%) (Fig. 2C). For nitrogen-cycling genes, there were decreases in relative abundance of an ammonification gene (*gdh* by 12%), nitrification genes (*amoA*-AOA by 20% and *hao* by 13%), and denitrification genes (*narG* and *nosZ* by 3%-8%) at the S site (Fig. 3C). In contrast, assimilatory nitrogen reduction gene *nir* increased by 10%, and dissimilatory nitrogen reduction gene *nrfA* increased by 9%.

Linkages between environmental variables and microbial communities

To explain changes in microbial communities by soil transfer and nutrient amendment, we divided environmental variables into four groups (climate, plant, soil physical and soil chemical variables). Climate and soil physical variables correlated (P<0.003) with bacterial community, and climate, soil physical, and soil chemical variables correlated (P<0.002) with fungal community (simple Mantel tests; Table S7). All four groups of environmental variables correlated (P<0.016) with functional genes. To differentiate direct and indirect linkages, partial Mantel tests were performed. Climate variables correlated (P=0.001) with bacterial community and fungal community, whereas soil chemical variables (P=0.078) and plant variables (P=0.032) correlated with functional genes.

Both bacterial and fungal communities correlated with soil organic matter (P<0.041, Table S7). Therefore, we performed a multiple regression of distance

matrices (MRM) analysis, which showed that fungal community was more closely correlated with changes in soil organic matter (Table S8). The bacterial community was more aligned with nitrogen cycling, ecosystem nitrification potential correlated with *amoA* (P=0.003, Fig. S3A), which was attributed to *amoA*-AOB (P=0.020, Fig. S3B) but not *amoA*-AOA (P=0.335, Fig. S3C).

Discussion

As shown in the conceptual diagram (Fig. 5), nutrient amendment enhanced both aboveground biomass and soil carbon storage by regulating microbial community composition and functional potentials. Nutrient amendment is essential to maintain soil productivity or ecosystem function in agricultural soils. Therefore, it is unsurprising to observe the significant impact of nutrient amendment in this study. However, soil carbon and nitrogen storages by nutrient amendment were deteriorated when transferring to warmer regions despite that aboveground biomass was still enhanced by nutrient amendment. Therefore, changes of soil carbon and nitrogen stability at C and S sites could be mainly attributed to microbial communities, which showed fundamentally different responses to nutrient amendment compared to observations at the N site (e.g. microbial carbon and nitrogen cycling routes in Fig. 5).

The effect of soil transfer

Soil transfer decreased microbial biomass (Table S1), which was consistent with findings in other simulated warming studies (Frey et al., 2008; Flury and Gessner, 2011; Liang and Balser, 2012). Soil transfer induced soil nutrient loss (Table S1), since climate warming could accelerate soil organic matter decomposition (Melillo et al., 2002; Knorr et al., 2005). Soil transfer also increased microbial functional genes associated with ammonification, nitrification and denitrification, providing further support to previous observations that climate warming can stimulate nitrogen cycling processes (Butler et al., 2012; Zhou et al., 2012; Bai et al., 2013).

The effect of nutrient amendment

Consistent with previous findings (Treseder, 2008; Shen et al., 2014; Contosta et al., 2015), nutrient amendment decreased bacterial biomass at the N site, which might be ascribed to soil acidification (Treseder, 2008). Our results of concurrent decrease of both bacterial biomass and pH by nutrient amendment verified those observations (Table S1). Nutrient amendment altered bacterial community composition at the N site, with increase in relative abundances of copiotrophic *alpha-*, *beta-*, *gamma-Proteobacteria* and *Actinobacteria* taxa but decrease in those of oligotrophic *Verrucomicrobia* and *Acidobacteria* taxa (Table S4), unveiling a preference toward fast-growing taxa (Fierer et al., 2012; Leff et al., 2015).

Most of the microbial functional genes associated with carbon degradation were decreased by nutrient amendment at the N site (Fig. 2A), resulting in an accumulation

of soil organic carbon attributable to increased soil carbon input by aboveground plants (Table S1 & Fig. 5A). Nutrient amendment has been frequently documented to increase soil organic matter by suppressing soil organic carbon decomposition, especially for more recalcitrant soil carbon (Cusack et al., 2010; Ramirez et al., 2012; Frey et al., 2014). Nutrient amendment increased microbial functional potentials of both N_2O-producing nitrification and denitrification genes at the N site (Fig. 3A), correlating with process potentials (Fig. S3).

Interactive effects of soil transfer and nutrient amendment

In sharp contrast with recent studies showing little interactive effects of nutrient amendment and climate warming on microbial communities (Lamb et al., 2011; Li et al., 2013; Shen et al., 2014; Contosta et al., 2015), or on gram-positive bacteria and the fungal to bacterial ratio (Gutknecht et al., 2012), our hypothesis that nutrient amendment and soil transfer interactively affected microbial community was verified (Table 1), signifying differences in ecosystems, climate types and technical approaches. Unsurprisingly, nutrient amendment increased soil organic matter (Table S1), However, it was interesting to note that nutrient amendment did not alter soil organic matter when soil was transferred to warmer regions (Table S1), indicating that simulated climate warming overrides nutrient amendment effect on soil carbon dynamics. This might arise from dominant soil transfer effects over nutrient amendment on microbial communities (Fig. 4).

Nutrient amendment altered microbial community and biomass at the N site. However, nutrient amendment did not alter bacterial community composition or biomass at the C or S site (Table S1&S3). Nutrient amendment decreased fungal biomass at the C site and altered fungal community composition at the S site, with striking increases in the relative abundances of taxa from *Basidiomycota* (Fig. 1B & Table S5), verifying our second hypothesis that bacterial and fungal responses were dissimilar. It is likely that nutrient amendment stimulates fungi to degrade recalcitrant carbon via the priming effect when fresh soil carbon input from plant material is available (Fontaine et al., 2007; Fontaine et al., 2011). Fungi are the primary microbes involved in the degradation of polymeric, recalcitrant carbon (Moore-Kucera and Dick, 2008; Schneider et al., 2012). The important role of fungi was reflected in our observation of striking 396% increase in *Agaricomycetes* and 1744% increase in *Microbotryomycetes* (Fig. 1B), which were derived from *Basidiomycota*, a well-known recalcitrant litter degrading taxa group (Osono, 2007; Lundell et al., 2010). Furthermore, fungal community was more closely correlated with soil organic matter than bacteria (Table S8).

Soil organic carbon originates from microbe-derived carbon and plant-derived carbon. In recent years, microbe-derived carbon input to soil is increasingly recognized as a critical function for soil organic carbon pool (Kögel‐Knabner et al., 2008; Kindler et al., 2009), contributing terpenes, as well as a large portion of chitin, glucans, peptidoglycans, and polysaccharides as residues of cell walls (Paul, 2006; Schimel and Schaeffer, 2015). Furthermore, recalcitrant carbon compounds in soil are

mainly produced by fungi and other microbes through microbial and biochemical transformations of soil organic carbon (Prescott, 2010). Nutrient amendment decreased most of the microbial functional genes associated with carbon degradation at the N site (Fig. 2A). However, functional genes associated with both labile and recalcitrant carbon degradation were consistently increased by nutrient amendment at the S site (Fig. 2C), which was explainable by the priming effect. NS samples were nutrient poorer in comparison with N and NC samples (Table S1), which could be favorable for priming effects (Fontaine et al., 2003). Nutrient amendment increased aboveground biomass by 108% at the S site (Table S1). The stability of soil organic carbon at the S site (Table S1) depends on the balance between increased input of fresh carbon to the soil and increased recalcitrant old carbon degradation through the priming effect (Fig. 5B) (Gregorich et al., 1996; Fontaine et al., 2003). Notably, nutrient amendment has been previously shown to accelerate soil carbon loss (Hartley et al., 2010), which can offset the increased carbon input from plant biomass and litter (Mack et al., 2004). Consistently, another experiment in tropical soils showed that fertilized soil under warmer regime have higher $\Delta^{14}C$ in respired CO_2, indicating loss of aged carbon (Cusack et al., 2010). This suggests that nutrient amendment might enhance carbon loss caused by warming.

A one-year nitrogen amendment and warming experiment showed that N_2O efflux induced by nitrogen amendment could be further exacerbated by warming (Bijoor et al., 2008), as warming could enhance nitrogen cycling (Rustad et al., 2001; Dawes et al., 2016). In this study, nutrient amendment decreased microbial functional potentials

of nitrification and denitrification genes when soil was exposed at warmer climate regime at the S site (Fig. 3C), likely as a consequence of soil nitrogen loss induced by warming (Table S1). It was noted that assimilatory and dissimilatory nitrogen reduction genes were increased (Fig. 3C), suggesting that nitrite might be converted to ammonia via assimilatory and dissimilatory nitrogen reduction instead of to gaseous nitrogen via denitrification. Microbial need for soil ammonium could arise from reallocation of mineral nitrogen from soil to aboveground (Table S1) as a consequence of competition of plants over microbes (Jingguo and Bakken, 1997). Such findings provided further evidence for the priming effect at the S site, since an important mechanism of the priming effect by microorganisms is to mineralize organic matter for available nitrogen (Kuzyakov, 2010).

In this study, our analyses of microbial communities unravel molecular mechanisms for changes in soil organic matter. Most importantly, we show that increased nutrient availability might enhance carbon degradation at elevated temperature, further exacerbating carbon loss caused by climate warming. By demonstrating the complicated, interactive effects of nutrient amendment and soil transfer, our study signifies the necessity to investigate multiple co-occurring factors. In addition, our findings have important implications for predicting soil carbon storage within the context of eutrophication and climate warming. Eutrophication increased soil carbon input from aboveground plants but decreased microbial functional potentials associated with carbon degradation, resulting in increased soil carbon storage. However, climate warming could induce soil carbon loss and override

the nutrient amendment effect on microbial communities, which deteriorate soil carbon storage.

Experimental procedures

Site description and soil sampling

This study belongs to the project of the Soil Reciprocal Transplant Experiment (SRTE), which began in October 2005 at three agricultural experimental stations in China: Hailun station in the northern China (126°38′E and 47°26′N, cold temperate climate zone with soil type of Mollisol), Fengqiu station in the central China (114°24′E and 35°00′N, warm temperate climate zone with soil type of Inceptisol), and Yingtan station in the southern China (116°55′E and 28°15′N, subtropical climate zone with soil type of Ultisol). These three sites were designated as N, C, and S, respectively, according to their geographical locations in China. As previously described (Sun et al., 2013; Zhao et al., 2014; Liu et al., 2015), a total of 18 soil plots of 1.4 m × 1.2 m × 1.0 m (length × width × depth) were excavated at the N site, with vertical stratification of every 20 cm layer. Six plots served as controls by in-place mock transfers at the N site, designated as N. The other plots were transferred to the C or S site, designated as NC or NS. Since 2006, maize was annually sowed and harvested in the plots. Half plots were amended with chemical fertilizers (urea, diammounium phosphate and potassium chloride) at the level of 150 kg nitrogen, 75 kg phosphorus pentoxide and 60 kg potassium oxide per hm^2. Basal fertilizers were

added prior to cropping (all of phosphorus and potassium, and half of the total nitrogen fertilizer) while the other half of the nitrogen fertilizer was amended at the large trumpet stage of maize growth as top dressing. The suffix f in sample name indicates nutrient amendment treatment.

Soil samples were collected in August - September 2011, six years after initiating the field study. Composite soil samples from each plot were generated by collecting and thoroughly mixing ten 2-cm diameter cores from surface soil (0-20 cm). Soil samples were immediately shipped to the laboratory on ice packs, manually screened through a 2 mm mesh to remove visible roots and then divided into two subsamples. One subsample was stored at 4°C for soil geophysical and geochemical analyses, while the other subsample was stored at -80°C for microbial analyses.

Measurements of environmental variables, biogeochemical activity and microbial biomass

Climate variable data, including average temperature of 2011, total precipitation of 2011, and relative humidity of these three sites, were obtained by local meteorological observation stations as shown in Table S1. Soil temperature was measured every week from August to September with digital stick thermometers at the depth of 10 cm. Soil moisture was measured gravimetrically by oven-drying fresh soil at 105°C for 12 h. Soil moisture was measured right after sampling. To measure the soil water holding capacity (WHC), a cylinder with saturated soil sample was placed on an absorbent

membrane until water was removed by gravity, then WHC was calculated based on the weight of the water held in the sample vs. the sample dry weight. Soil bulk density was measured on soil sample collected in 100 cm^3 stainless steel ring, then dried at 105°C for 48 h in an oven. Particle density was determined by the pycnometer method. Soil porosity (p) was calculated from soil bulk density (ρ_b) and particle density (ρ_d) by equation $[1-(\rho_b/\rho_d)]\times 100\%$. Electric conductivity was measured with a conductivity meter (Thermo Fisher Scientific, EC-PH510). Soil cation exchange capacity (CEC) was determined by NH_4OAc exchange method. Briefly, 2 g air-dry soil (0.25 mm) was saturated with a 100 ml of 1 mol/L NH_4OAC (pH = 7.0), then adsorbed NH_4^+ was replaced by Na^+ before measuring NH_4^+ in the final extract. Soil pH was measured with a glass electrode in a 2.5:1 water-soil suspension. Soil organic matter was measured using the dichromate oxidation method (Walkley and Black, 1934). Total nitrogen (TN) was measured using the Kjeldahl method (Bremner et al., 1996). Nitrate (NO_3^--N) and ammonium (NH_4^+-N) were measured by an Auto Analyser 3 (Bran+Luebbe GmbH, Germany) in a 1:5 soil and 1 mol/L KCl suspension. Total phosphorus (TP) was extracted with sodium carbonate while available phosphorus (AP) was extracted with sodium bicarbonate, followed by measurements using the molybdenum blue method (Olsen, 1954). Total potassium (TK) and available potassium (AK) were measured with flame photometry (FP66400A, CANY Precision Instrument Co., Ltd., Shanghai, China) after fusing TK and AK with sodium hydroxide and extraction by ammonium acetate, respectively (Kanehiro and Sherman, 1965). Soil nitrification potential was determined by

incubating soil with ammonium sulfate ($(NH_4)_2SO_4$) (Smolders et al., 2001). Soil CO_2 efflux was measured *in situ* using a LI-6400 Portable Photosynthesis System (LI-COR Inc., Lincoln, Nebraska, USA) every week from August to September. Specifically, PVC collars (10 cm long, 10 cm inside diameter) were inserted into the inter-row soils at the depth of 5 cm at least 24 h prior to measurement. The soil respiration chamber was set on top of these collars according to the protocol recommended by the LI-6400 manual to measure undisturbed soil CO_2 efflux. The aboveground biomass and seed weight of maize were measured after harvest. Soil microbial biomass was determined using phospholipid fatty acid (PLFA) content with a modified Bligh-Dyer method (Wang et al., 2015).

Illumina sequencing and raw data processing

Soil DNA was extracted using a freeze–grinding method and purified in agarose gel electrophoresis followed by phenol–chloroform–butanol extraction as previously described (Zhou et al., 1996). The V4 hypervariable region of the bacterial 16S rRNA gene was amplified by PCR primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The internal transcribed spacer II (ITS2) region of the fungal rRNA gene was amplified by PCR primers gITS7F (5'-GTGARTCATCGARTCTTTG-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') (Kostovcik et al., 2015). A nested-PCR approach was used for DNA amplification. For bacterial 16S rRNA genes, DNA was amplified for 10 cycles in triplicates to minimize stochastic variability (Schmidt et al.,

2013). The triplicate PCR products were then combined and purified using an Agencourt AMPure XP kit (Beckman Coulter, Brea, CA, USA). The purified PCR products were eluted in 50 µl water. Then a 15-µl diluted amplicon was subjected to a 20-cycle amplification with fusion primers consisting of the template primer, adapter, pad, and linker sequences. Sample-specific barcode sequence (12 mer) was added to the reverse primer. The nested PCR amplification process was conducted on a Gene Amp PCR-System 9700 (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 µl containing 2.5 µl10× PCR buffer, 0.1 µl AccuPrime™ Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA), 1 µl of each primer (10 µM) and 15 µl template DNA. PCR cycling conditions were as follows: an initial denaturation at 94°C for 1 min, followed by 10 cycles for the first step and 20 cycles for the second step at 94°C for 20 s, 53°C for 25 s and 68°C for 45 s, with a final extension at 68°C for 10 min. PCR amplification for the ITS2 region of the fungal rRNA gene was similar to that of the 16S rRNA gene except for changes in PCR conditions. ITS2 amplification required an initial denaturation at 94°C for 3 min, followed by 12 cycles for the first amplification step and 24 cycles for the second amplification step at 94°C for 30 s, 55°C for 30 s, and 68°C for 30 s, and terminated with extension at 68°C for 10 min.

The nested, triplicate PCR products were examined by 1% agarose gel electrophoresis. PCR products for each sample were subsequently combined and quantified by PicoGreen (Life Technologies, Grand Island, NY, USA) with a FLUOstar Optima (BMG Labtech, Jena, Germany). PCR products from all samples

were pooled in equimolar proportions to create an amplicon library. The pooled mixture was purified with the QIAGEN Gel Extraction Kit (QIAGEN Sciences, Germantown, MD, USA), and quantified using PicoGreen. The library was sequenced on a MiSeq (Illumina, San Diego, CA, USA) after mixing with PhiX at the Institute for Environmental Genomics (IEG), University of Oklahoma.

Raw sequencing data was processed using the Galaxy pipeline (http://zhoulab5.rccc.ou.edu)(Yue et al., 2015; Zhao et al., 2016). Low quality reads with non-assigned or over 1.5 mismatched barcodes, low quality scores (< 25), short sequence reads (< 150 bp) or more than one undetermined nucleotide (N) were discarded. Forward and reverse reads were combined using FLASH (Magoč and Salzberg, 2011). Combined sequences were trimmed to 251-253 bp for the bacterial 16S rRNA gene or 250-350 bp for the fungal ITS gene. Operational taxonomic units (OTUs) were generated by UPARSE at the 97% sequence similarity level (Edgar, 2013). Taxonomy assignment utilized the RDP classifier based on 16S rRNA gene training set and UNITE fungal ITS training set (Wang et al., 2007; Kõljalg et al., 2013). The 16S and ITS OTU matrices were rarefied to 10,884 and 13,286 sequences per sample, respectively.

Experiments with GeoChip and raw data analyses

Soil DNA was labeled with the fluorescent nucleic acid dye Cy5. After purification with QIAGEN DNA Purification Kit (QIAGEN Sciences, Germantown, MD, USA), 1 µg of DNA was hybridized with GeoChip 4.6 microarrays (Ding et al., 2015). After

washing away unbound DNA, the slides were scanned with a NimbleGen MS 200 Microarray Scanner (Roche, Basel, Switzerland). The signal intensity of each probe was quantified with ImaGene 6.0 (Biodiscovery, EI Segundo, CA, USA).

Raw data were processed by removing probes with signal-to-noise ratio (SNR) <2.0 and those detected only once among triplicates (Yang et al., 2009). The relative abundance of each probe was calculated by dividing total signal intensities from each sample and multiplying by a constant. Natural logarithmic transformation was performed prior to statistical analysis.

Statistical analyses

Significant differences of environmental variables and microbial biomass between treatments were tested by one-way Analysis of Variance (ANOVA) and followed by Duncan *post-hoc* test. The significance of nutrient amendment and soil transfer effect on environmental variables and microbial biomass was tested by two-way ANOVA, and on community matrix was tested by two-way *adonis*, a permutational multivariate analysis of variance (PermANOVA) using the R *vegan* package. The significance of nutrient amendment and soil transfer effect on individual OTU was examined by Wald tests on log2 fold change using the *DESeq2* package (Love et al., 2014). To examine significance of nutrient amendment effect on functional gene with different probes, two-way ANOVA of nutrient amendment and probe was performed. *P* values were adjusted using the method of false discovery rate (FDR) when conducting multiple comparisons. To evaluate the relative importance of soil transfer and nutrient

amendment on microbial communities, multivariate regression trees (MRT) analysis was performed with the R *mvpart* package (De'Ath, 2002). Mantel tests were performed to investigate linkages between microbial communities and environmental variables. The importance of bacterial community and fungal community for soil organic matter was estimated using multiple regression of distance matrices (MRM) with the R *ecodist* package. Abundant fungal OTUs (relative abundance > 0.002%) were logarithmically transformed prior to generating a heatmap, which was performed using the function ‘aheatmap’ in the R *NMF* package.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability

The sequencing and GeoChip data is available online (http://www.ncbi.nlm.nih.gov/geo/). The accession number of sequencing data is SRP069263 and the accession number of GeoChip data is GSE77546.

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Table 1. Effects of nutrient amendment and soil transfer on microbial biomass and taxonomic and functional community compositions, indicated by R^2 (*P* value).

Effect	T[c]	N	T×N
Total biomass[a]	**0.556 (0.002)**[d]	0.058 (0.156)	0.085 (0.224)
Bacterial biomass	**0.816 (0.001)**	0.018 (0.165)	**0.070 (0.037)**
Fungal biomass	0.034 (0.557)	**0.398 (0.003)**	**0.233 (0.042)**
Bacterial community[b]	**0.179 (0.003)**	**0.070 (0.040)**	**0.361 (0.001)**
Fungal community	**0.187 (0.001)**	0.040 (0.211)	**0.371 (0.001)**
Functional genes	**0.291 (0.001)**	**0.110 (0.003)**	**0.282 (0.001)**

[a]Effects on biomass were examined by two-way ANOVA.

[b]Effects on microbial community matrices were examined by two-way *adonis*.

[c]Abbreviations: T – soil transfer; N – nutrient amendment.

[d]Significant effects ($P<0.05$) are marked in bold.

Figure legends

Figure 1. (A) Hierarchical clustering analysis of abundant ITS OTUs (relative abundance >0.002%). These OTUs are divided into three groups. OTUs in Group 1 were abundant at the N site, OTUs in Group 2 were abundant at the C site and OTUs in Group 3 were abundant at the S site. (B) OTU composition of three groups at the class level. Significance was indicated by "*" when $0.05<P<0.1$, "**" when $0.001<P<0.05$, and "***" when $P<0.001$. N: samples at the N site; NC: samples transferred from the N site to the C site; NS: samples transferred from the N site to the S site. The postfix f represents samples with fertilizer amendment.

Figure 2. Percent change in relative abundance of functional genes associated with carbon degradation by nutrient amendment at the (A) N site, (B) C site, and (C) S site. Only significantly (FDR-corrected $P<0.05$) changed functional genes are shown.

Figure 3. Percent change in relative abundance of nitrogen cycling genes by nutrient amendment at the (A) N site, (B) C site, and (C) S site. Significance was indicated by '*' for FDR-corrected $P<0.1$, '**' for $P<0.05$, "***" for $P<0.001$. The grey genes were detected by GeoChip 4.6.

Figure 4. Multivariate regression trees (MRT) analysis to evaluate the relative importance of soil transfer and nutrient amendment in affecting (A) bacterial community composition, (B) fungal community composition, and (C) functional genes.

Figure 5. Conceptual diagrams of nutrient amendment effects on ecosystems and

microbial responses to nutrient amendment at the (A) N site and (B) S site. Material pools are represented by yellow rectangles, gases by blue rectangles, microbial processes by pink parallelograms, plant processes by green parallelograms, and priming effect by purple wave rectangle. Black arrows indicate material flows, red arrows depict microbial effects, and dashed arrows show priming effects on carbon decomposition. Labels of '+', '-', and '~' in circles indicate positive effect, negative effect, and no effect, respectively.