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Multi-level nitrogen additions alter chemical composition and turnover of the labile fraction soil organic matter via effects on vegetation and microorganisms

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Key Points:

- The LF-SOM quantity increased at the lowest N additions (N10 and N20) and decreased from N40 to N160, but the decrease was weakened at the highest N addition (N160).
- Plant-derived compounds in LF-SOM were more sensitive to N addition than microbial-derived and aromatic compounds.

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- The organic compounds in LF-SOM were significantly correlated with compound degradation-related genes.

Abstract

Global nitrogen (N) deposition greatly impacts soil carbon sequestration. A two-year multiple N addition (0, 10, 20, 40, 80 and 160 kg $N \cdot ha^{-1} \cdot yr^{-1}$) experiment was conducted in alpine grassland to illustrate the mechanisms underlying the observed soil organic matter (SOM) dynamics on the Qinghai-Tibet Plateau (QTP). Labile fraction SOM (LF-SOM) fingerprints were characterized by pyrolysis-gas chromatography/tandem-mass spectrometry (Py-GC-MS/MS), and microbial functional genes (GeoChip 4.6) were analyzed in conjunction with LF-SOM fingerprints to decipher the responses of LF-SOM transformation to N additions. The significant correlations between LF-SOM and microbial biomass, between organic compounds in LF-SOM and compound degradation-related genes, as well as between LF-SOM and net ecosystem exchange (NEE) implied LF-SOM was the main fraction utilized by microorganisms and the most sensitive fraction to N additions. The LF-SOM increased at the lowest N addition levels (10 and 20 kg $N \cdot ha^{-1} \cdot yr^{-1}$) and decreased at higher N addition levels (40 to 160 kg $N \cdot ha^{-1} \cdot yr^{-1}$), but the decrease of LF-SOM was weakened at 160 kg $N \cdot ha^{-1} \cdot yr^{-1}$ addition. The non-linear response of LF-SOM to N additions was due to the mass balance between plant inputs and microbial degradation. Plant-derived compounds in LF-SOM were more sensitive to N addition than microbial-derived and aromatic compounds. It is predicted that when the N deposition rate increased by 10 kg $N \cdot ha^{-1} \cdot yr^{-1}$ on the QTP, carbon sequestration in the labile fraction may increase by nearly 170% compared with that under the current N deposition rate. These findings provide insight into future N deposition impacts on LF-SOM preservation on the QTP.

1 Introduction

In recent years, the influence of human activities, including industrial and agricultural development, as well as the combustion of fossil fuels, has induced increases in global nitrogen (N) deposition (Canfield et al., 2010; Mclauchlan et al., 2013). In 1860, the global anthropogenic active N emissions were 15 Tg N yr^{-1}, whereas the values in 2000 and 2008 reached 165 Tg N yr^{-1} (Galloway et al., 2008) and 192 Tg N yr^{-1} (Gu et al., 2013), respectively, and emissions will continue to increase in the coming decades. The high deposition of N has led to a series of environmental problems that not only affect climate change (Zaehle et al., 2010) but also influence the structure and function of ecosystems (Nadelhoffer, 2007; Manning et al., 2010), such as soil acidification, nutrient imbalances, and biodiversity loss (Gilliam, 2006; Bobbink et al., 2010). In addition, N addition exerts a significant effect on soil carbon storage by altering microbial activity (Wang et al., 2013) and also has variable effects on the release of greenhouse gases. Fang et al. (2012) found that N addition increased soil inorganic nitrogen content and promoted plant growth thereby increasing CO_2 emissions from the ecosystem. Moreover, a low amount of added N also inhibited the uptake of CH_4 in the meadow ecosystem (Fang et al., 2014).

Soil is the largest carbon pool in terrestrial ecosystems (Jobbagy & Jackson, 2000), and its organic matter degradation is significantly affected by N deposition. However, there is no

definite conclusion regarding the effect of N deposition and artificial N addition on soil organic matter (SOM) decomposition. Kuzyakov (2011) found that increased deposition of N contributed to an increase in litter input and thereby promoted the degradation of SOM. Increases in the amount of added N increased both the decomposition rate of SOM and emissions of CO_2 (Knorr et al., 2005; Bragazza et al., 2006). However, other studies showed that N deposition was conducive to carbon fixation in ecosystems. Compton et al. (2004) found that the application of N fertilizer in forest ecosystems could reduce the biomass and diversity of soil microorganisms, thus inhibiting the degradation of SOM. The discrepancies in the response of SOM to N addition may be due to different periods of N addition or different amounts of added N. Han et al. (2018) found that the effect of short periods of N addition on soil was more significant than that of long periods of N addition. Bragazza et al. (2006) showed that compared with low N deposition, high N deposition promoted increased levels of litter decomposition and CO_2 emissions. However, an excessive amount of anthropogenic N addition, usually more than 125 kg N ha^{-1} yr^{-1}, could inhibit the degradation of SOM (Knorr et al., 2005).

In addition, the responses of different soil fractions to N addition also vary (Li et al., 2017), and these variations are related to the properties of each soil fraction. The soil light fraction accounts for 1.8%-3.2% of the total soil weight, but its carbon content can reach 15%-32% of the soil total organic carbon (Wu et al., 2004). At the same time, this fraction is not closely bound to soil minerals and is usually the main soil fraction utilized by soil microorganisms (Gregorich et al., 1994). In contrast, the soil dense fraction is protected by soil minerals and its turnover time usually extend to thousands of years (Golchin, 1995). Therefore, compared to the dense fraction, the light fraction in soil is more sensitive to climate change. In addition, the response of specific compounds to N deposition has also been investigated. Sinsabaugh et al. (2002) found that N addition stimulated cellulolysis, which promoted the degradation of polysaccharides in soil. However, high concentration of N addition could lower the decomposition rate of litter by inhibiting the degradation of lignin (Sinsabaugh et al., 2002; Berg et al., 2008), because excessive added N decreased the activity of lignin-degradation enzymes (Sinsabaugh et al., 2002; Frey et al., 2004). However, SOM contains many types of organic compounds, and the responses of different compounds to N addition are different. Notably, studies of only one or two organic compounds hamper full elucidation of the response of SOM to N deposition.

Soil microbes, as decomposers, play an important role in the transformation of SOM. The growth of soil microorganisms is closely related to vegetation (Wan, 2008; Wang et al., 2008), soil pH (Phoenix et al., 2012) and soil nutrients; thus, N deposition can directly or indirectly affect soil microorganisms and thereby affect the degradation processes of SOM. First, the addition of N significantly affects the biomass of soil bacteria (Fierer et al., 2012) and fungi (Zhou et al., 2016). Through a seven-year experiment involving the addition of N, Bragazza et al. (2012) found that fertilization increased bacterial biomass in the topsoil and subsequently reduced the storage of carbon in the litter layer. Moreover, these researchers showed that increased N deposition increased the activity of microorganisms that affect cellulose degradation

but inhibited the microbial activity that act on the decomposition of soil protein and chitin compounds (Allison et al., 2010), resulting in differences in the degradation rates of different types of organic matter (Allison et al., 2010). In addition, the response of microbial functional genes to N addition has also been the focus of research studies. For example, Eisenlord et al. (2013) and Freedman et al. (2013) both found that the addition of N reduced the richness of microbial functional genes involved in nitrogen cycling. In addition, Compton et al. (2004) found that the amplification of *amoA* (encoding ammonia monooxygenase) and *nifH* (encoding nitrogenase reductase) was difficult in plots with high levels of added N, which suggested that N addition changed the N-fixing group. Therefore, studying the response of microorganisms to N addition is helpful for understanding the response mechanisms of SOM degradation to N deposition. However, as mentioned above, previous studies mainly focused on the response of N cycling-related genes to N addition, while few studies have examined the response of carbon degradation-related genes to N addition or the relationships between soil organic compounds and carbon degradation-related genes under N addition.

The Qinghai-Tibet Plateau (QTP) covers an area of 2.57 million square kilometres, and 1.5 million square kilometres of the QTP is covered by alpine grassland (Cui & Graf, 2009). According to records, N deposition on the QTP has increased in recent years (Wei et al., 2010; Zhao et al., 2011) and has caused a series of ecological problems, such as soil acidification, indicating that this region is more sensitive to increases in N input (Yang et al., 2012). Therefore, to study the effect of N deposition on global organic carbon pools, it is particularly important to study the response of SOM on the QTP to increases in N deposition. Hence, the soil microbial communities and labile fraction SOM (LF-SOM) in the alpine grassland of the QTP were analysed using GeoChip 4.6 and pyrolysis-gas chromatography/tandem-mass spectrometry (Py-GC-MS/MS) to explore the response mechanisms of soil microbial communities and LF-SOM to increasing amounts of deposited N.

2 Materials and Methods

2.1 Study sites and sample collection

The research site was in the Nam Co Monitoring and Research Station for Multi-sphere Interactions (Nam-MI), which is located on the central Tibetan Plateau (30°47′ N, 90°58′ E, 4730 m *a.s.l.*). The mean annual air temperature is -0.6℃, and the annual precipitation is 415 mm (Liu et al., 2013). The dominant vegetation in the research site includes *Kobresia pygmaea*, *Stipa purpurea*, *Androsace tapete* and *Leontopodium pusillum*. The data calculation of aboveground biomass (AGB) and net ecosystem exchange (NEE) was performed as described by Liu et al. (2013).

As part of the multi-level N deposition field experiment established in 2009, six N addition levels (0, 10, 20, 40, 80 and 160 kg N ha^{-1} yr^{-1}) were applied in triplicate based on the current N deposition level (less than 10 kg N ha^{-1} yr^{-1}) in this area, and the details were as

previously described (Liu et al., 2013). The soils were fertilized with NH_4NO_3 during the first week of each month from May to September in 2010 and 2011. In total, 18 soil samples (6 N level×3 replicate plots/N level) were included in the study. The soil samples were collected and pooled from three 0-15 cm soil cores and subsequently sieved to remove plant roots and stones (sieve mesh of 2 mm). The subsamples were then packed in ice and transported to the laboratory by air. Each sample was divided into two sections: one section was stored at 4℃ for soil physiochemical analyses, and the other was stored at -80℃ for microbiological analyses.

2.2 Measurement of soil properties

The soil pH was measured in a 1:2.5 mixture of soil and deionized water using a Sartorius PB-10 digital pH meter. The soil moisture was calculated on fresh 5 g subsamples that had been dried in a 105℃ oven for 12 h. The soil total organic carbon (TOC) was measured using a TOC analyser (TOC-VCPH, Shimadzu, Japan), and the soil total nitrogen (TN) was detected using the Kjeldahl method with some modifications (Bremner, 1960). The soil water-soluble organic carbon (WSOC) and nitrogen (WSON) were extracted using the methods developed by Jones and Willett (2006) and determined using a Shimadzu TOC-TN analyser. The soil NH_4^+-N, NO_3^--N and NO_2^--N were determined using an autoanalyzer (Auto Analyzer 3, Bran Luebbe, Germany) with extracts containing 0.05 M K_2SO_4 (1: 4, soil: extractant).

2.3 Sequential density fractionation

According to our previous research results, the properties of SOM with density ≤2.25 g cm^{-3} and with density >2.25 g cm^{-3} were significantly different in this studied alpine grassland, and SOM with density ≤2.25 g cm^{-3} was the main carbon source used by soil microorganisms, which was more susceptible to external climate change (Chen et al., 2019). Therefore, in this study, soil samples were divided into two fractions: labile fraction SOM (LF-SOM, $\rho \leq 2.25$ g cm^{-3}) and refractory fraction SOM (RF-SOM, $\rho > 2.25$ g cm^{-3}). In short, 10 g of air-dried soil was added to 30 mL of NaI solution ($\rho = 2.25$ g cm^{-3}), and shaken for 30 min on a shaker table. Then, the soil suspension was centrifuged at 970*g* for 20 min, and the floating material was filtered through a glass fibre filter (Whatman GF/F; 0.7-μm particle retention). The above steps were repeated three times to maximize the recovery of each soil fraction. Then, the fraction recovered on the filter and the remaining fraction were washed with deionized H_2O until the salt concentration reached <50 mS/cm, respectively. All fractions were oven-dried at 60 °C for 24 hr. Several soil samples were selected for pre-tests. The results showed that, compared to the LF-SOM, there was no significant difference in the response of the RF-SOM to different N application gradients (soil TOC and TN). Therefore, the response of LF-SOM to N addition seems more sensitive than that of the RF-SOM, and LF-SOM was selected for Py-GC-MS/MS analysis.

2.4 Py-GC-MS/MS analysis

LF-SOM was pyrolyzed using an EGA/PY-3030D multi-shot pyrolyzer (Frontier Lab) connected to an Agilent 7890 gas chromatograph/7000B triple quadrupole mass spectrometer (Agilent Corporation) and an Agilent gas chromatography workstation as described previously. The GC-MS/MS analysis of the chromatography conditions was performed in constant flow mode with helium as the carrier gas. The chromatographic column was HP-5 (100 m×0.32 mm×0.25 μm), and the mass spectrometry conditions were as follows: electron ionization (EI) of 70 eV, ion source temperature of 230℃, quadrupole temperature of 150℃, and a full scan scanning range of 50-550 amu. The temperature programme of the PY-3030D and the column was based on a previous study (Chen et al., 2019).

The pyrolysis compounds were identified based on an interpretation of their mass spectra using a National Institute of Standards and Technology (NIST) library or the retention times of the gas chromatography (GC) results or through a comparison with previously published literature (Kaal et al., 2008ab; Chai et al., 2008). Approximately 150 organic compounds were analysed qualitatively (Table S1) and classified as alkyl compounds (n-alkane and n-alkene), polysaccharide-derived compounds, lignin, phenols, chitin, N compounds, aromatics and polyaromatics according to their similar chemical properties. The sources of alkyl compounds are usually distinguished by their chain length. Long-chain (n>20) alkyl compounds are mainly derived from plant biopolymers (Lorenz et al., 2007), and short-chain (n≤20) alkyl compounds are derived from microbial products (Buurman et al., 2007). Polysaccharides are the main carbohydrate in soil, mostly come from plant residues (Chen et al., 2018). Lignin is a typical plant-derived compound in soil that mainly comes from the surface wax layer of plants and the protective layer of vascular plants (Almendros et al., 1996). Phenols are mostly derived from lignin and cellulose (Chen et al., 2018). Chitin in soil mainly comes from soil microorganisms, which is the main structural component of microbial cell walls. N compounds in soil include proteins, peptides and amino acids, which have several sources, such as microbial residues and their secretions or plant lignin and other phenolics (Kiersch et al., 2012). In this study, N compounds were further divided into plant-derived N compounds, including Diketodipyrrole and Indole (Barré et al., 2018), and microbial-derived N compounds, including Pyrrole, Pyridine and their derivatives (Kiersch et al., 2012). The content of each compound was calculated based on the internal standards, $C_{24}D_{50}$ and deuterated phenanthrene, which were used to quantify the alkyl compounds and the other compounds, respectively. To more intuitively observe the effect of N addition on LF-SOM, the sample quantity and the content of the internal standard were constant in each pyrolysis sample. The total ion chromatograms (TICs) obtained from the six levels of added N are shown in Figure S1. The carbon content of each pyrolytic organic compound was calculated as follows: 12 times the number of carbons of each compound multiplied by each compound's molar mass. The sum of all compounds' carbon content in the sample was used to represent the sample's carbon content.

2.5 Phospholipid fatty acid (PLFA) and GeoChip 4.6 analyses

The PLFAs of the soil microbial community were extracted using the Bligh–Dyer method with some modifications (White et al., 1979) and were analysed with an Agilent (6890/5973) GC-MS system (Agilent Technologies, Palo Alto, CA, USA) (Lei et al., 2017). Briefly, 5 g of freeze-dried soil was extracted with 19 mL of a single-phase mix of chloroform:methanol:citrate buffer solution (1:2:0.8, v/v/v, pH=7.4). After the extraction, the collected non-polar phase was fractioned into neutral lipids, glycolipids and phospholipids by sequential elution with chloroform (6 mL), acetone (6 mL) and methanol (3 mL), respectively, using pre-packed silica solid phase extraction columns (500 mg/3 mL, Cleanert™ Silica-SPE, Bonna-Agela Technologies Inc., Wilmington, DE). The phospholipid fraction was then methylated with a methanol/toluol (1:1) solution (1 ml) and 0.2 M methanolic KOH (1 ml) to produce fatty acid methyl esters (FAMEs). After the addition of fatty acid 19:0 as an internal standard, samples were analysed on an Agilent 6890A gas chromatograph (Agilent Technologies Inc., Santa Clara, CA) and identified with a microbial identification system, MIDI Sherlock 4.5 (MIDI Inc., Newark, USA). The biomass was estimated by PLFA biomarkers (Frostegård et al., 1993). In general, the biomass of bacteria was calculated by the total amount of i14:0, i15:0, a15:0, C15:1, i16:0, 16:1w9c, 16:1w7c, 16:1w5c, 16:1w3c, i17:0, a17:0, C17:1, cy17:0, C18:1, i19:0, and cy19:0 (Tunlid et al., 1989; Federle et al., 2010). Actinomycetes was indicated by 10Me16:0, 10Me17:0, and 10Me18:0 (Zelles, 1997). C18:2 and 18:1w9c were used to calculate fungi biomass (Federle et al., 2010).

GeoChip 4.6 was applied to investigate the changes in the functional structure of the soil microbial community. The genomic amplification of DNA was performed using a TempliPhi kit (GE Healthcare, Piscataway, NJ, USA), and the DNA was then labelled with the fluorescent dye Cy-5. The labelled DNA was purified with a QIAquick kit (Qiagen, Valencia, CA, USA), dried at 45℃ for 40 min and then hybridized using a MAUI hybrid workstation (BioMicro, Salt Lake City, UT, USA). After hybridization, the chip was scanned using a NimbleGen MS200 scanner (Roche, Madison, WI, USA), and the image was converted into digital information using ImaGene 6.0 software (BioDiscovery, EI Segundo, CA, USA). The original data from the gene chips were transformed into valid data after online quality control using the IEG website (http://ieg.ou.edu/microarray/). First, the low-quality points were removed. Second, the points with a signal-to-noise ratio less than 2 were removed. Third, if the point was observed only in duplicate samples, it was removed. The values were then divided by the means to standardize the data and calculate the richness of each gene, namely, the richness of each gene in each sample was divided by the average gene richness of the sample.

2.6 Data analyses

To investigate the effects of different levels of N addition on LF-SOM composition and microbial communities, a principal component analysis (PCA) was conducted with R software (version 2.12.1, https://www.r-project.org/). The responses of different soil organic compound

classes to N addition were analysed by linear regression (the data were the differences in the ln-transformed data of compounds between each treatment and N0) with R software, in which the slope usually represented the rate of change. A general linear model (univariate) was used to assess the differences in the slopes for these compound classes. One-way analysis of variance (ANOVA) was used to assess the differences in AGB, the molar mass of LF-SOM, the richness of microbial functional genes and environmental factors under different levels of N addition. The correlations among AGB, NEE and the molar mass of LF-SOM, as well as between LF-SOM and soil microorganisms, were determined by Pearson correlation analyses. Structural equation modelling (SEM) was used to test and quantify the effects of N additions on LF-SOM transformation. A path model was developed to relate the variables of N additions, environmental factors, vegetation and soil microorganisms to LF-SOM, based on the hypothesis that LF-SOM transformation was mainly due to the mass balance between vegetation input and microbial degradation under N additions. Adequate model fits were determined according to a non-significant χ^2 test (P>0.05), low Akaike value (AIC), high goodness of fit index (GFI>0.90), and low root mean square error of approximation (RMSEA<0.05). The above-described analyses were performed using SPSS 19.0 (IBM Corporation, Armonk, NY, USA).

3 Results

3.1 Responses of soil properties to multi-level N additions

As shown in Table S2, increases in the amount of added N had no significant effect on soil TOC, but increased soil TN (N0 to N160: 2.28 to 2.68 g kg^{-1}). The lowest levels of N addition (N10 and N20) significantly reduced the soil CN ratio, but from N40 to N160, the soil CN ratio increased with increasing N addition and reached maximum values at N80 (13.08) and N160 (12.81). The highest levels of N addition (N80 and N160) significantly reduced soil WSOC (N80, 61.05 mg kg^{-1} and N160, 68.31 mg kg^{-1}) and soil WSOC/WSON (N80, 2.60 and N160, 1.79) but significantly increased soil WSON (N80, 24.67 mg kg^{-1} and N160, 39.48 mg kg^{-1}). With the increase in N addition, the content of soil NH_4^+ showed no obvious change, but the content of soil NO_3^- and NO_2^- increased gradually (NO_3^-, 17.78 to 93.84 mg kg^{-1} and NO_2^-, 2.22 to 5.20 mg kg^{-1}) and reached maximum values at N80 and N160. The soil pH did not change significantly from N0 to N80, but decreased significantly at N160 (7.70).

3.2 Responses of LF-SOM to multi-level N additions

The PCA of LF-SOM showed that the first two principal components accounted for 62.89% (PC1 36.95%, PC2 25.94%) of the variability among the six N addition levels, and N0 was clearly separated from the others (Figure 1a). Compared with that at N0, the molar mass of LF-SOM significantly increased at N10 and N20, but from N40 to N160, LF-SOM decreased gradually, and reached the minimum value at N80 (Figure 1b). As N addition increased, AGB increased gradually and reached maximum values at N80 and N160 (43.67 and 43.46 g m^{-2} more than N0, Figure S2). There was a significant negative correlation between AGB and LF-SOM (-

0.48*, Table 1) and a significant positive correlation between NEE and LF-SOM (0.51*, Table 1).

3.3 Responses of compounds in LF-SOM to multi-level N additions

There were significant correlations between almost all compounds in LF-SOM and the N addition levels, and all compounds in LF-SOM decreased with increases in added N (Table 2, slope<0). Organic compounds in LF-SOM from plant sources, including lignin, polysaccharide-derived compounds, plant-derived N compounds, and phenols, had low slopes, -0.0173, -0.0093, -0.0082 and -0.0080, respectively. Microbial-derived and aromatic compounds, including chitin, aromatics and polyaromatics, had relatively high slopes (-0.0076, -0.0063 and -0.0061). Long-chain (n≤20) alkyl compounds (plant-derived compounds) decreased significantly with increasing N addition (slope, -0.0064, $P<0.05$), while short-chain (n>20) alkyl compounds had no significant correlation with N addition. As shown in Table S4, the slope of plant-derived compounds (including lignin, polysaccharides, plant-derived N compounds, phenols and long-chain alkyl compounds, slope=-0.0110, Table S3) was significantly smaller than those of microbial-derived compounds (including chitin, microbial-derived N compounds and short-chain alkyl compounds, slope=-0.0037, Table S3) and aromatic compounds (including aromatics and polyaromatics, slope=-0.0038, Table S3) ($P<0.05$), but there was no significant difference between the slopes of microbial-derived compounds and aromatic compounds.

3.4 Relationships between LF-SOM and soil microorganisms under multi-level N additions

The PCA of the PLFA fingerprint data showed that the first two principal components accounted for 97.72% (PC1 84.27%, PC2 13.45%) of the variability among the six N addition levels, and soil microbial community composition was significant different under the six N addition levels (Figure S3). The lowest N addition levels significantly reduced soil microbial biomass (N10 and N20), but microbial biomass continually increased after N40 (Table S5). At the same time, the biomass of bacteria, fungi and actinomycetes were all significantly correlated with the molar mass of LF-SOM (Table 3, $P<0.05$).

The PCA of the GeoChip data indicated that the microbial functional gene structure also significantly changed under N addition, and the microbial functional gene structure under N80 and N160 was significantly different from that under the other N addition levels (Figure S4a). With increasing N addition, the total richness of microbial functional genes decreased significantly (Figure S4b). As shown in Figure 2a, there was a significant positive correlation between the molar mass of LF-SOM and the total microbial functional genes richness ($P<0.05$). The *alk*, *cellulose*, *aromatic*, *polyaromatic*, *lignin*, *chitin* and *protease* genes were selected for the investigation of the relationships between different compounds in LF-SOM and microbial functional genes because these genes are related to various groups of soil organic compounds, namely, alkyl compounds, polysaccharide-derived compounds, aromatics, polyaromatics,

phenols, chitin and N compounds. The molar mass of organic compounds in LF-SOM was found to be significantly correlated with the richness of compound degradation-related genes (Figure 2b, $P<0.05$).

The changes in relative abundances (%) of certain microbial functional genes with multi-level N additions were also studied. With increasing N addition, the relative abundance (%) of carbon degradation genes continually increased and reached a maximum at N80 (Figure S5). In addition, the relative abundances (%) of the functional genes involved in the degradation of plant-derived compounds (including *lignin* genes and *cellulose* genes) increased with increasing N addition, while those of the genes involved in the degradation of aromatic compounds (including *aromatic* genes and *polyaromatic* genes) decreased with the increase in N addition. The relative abundances (%) of the functional genes involved in the degradation of microbial-derived compounds (including *chitin* genes) had no significant correlations with N addition increase (Figure S6).

3.5 Relationships among LF-SOM, soil microorganisms and environmental factors under multi-level N additions

The SEM of LF-SOM, soil microorganisms and environmental factors showed that N addition was significantly negatively correlated with soil pH and the ratio of ammonia-N to nitrate-N (Figure S7, -0.76** and -0.51**). In addition, significant negative correlations were found for N addition with microbial biomass and microbial functional gene richness (Figure S7, -0.76* and -0.92**). The results indicated that soil pH significantly affected soil microbial biomass (Figure S7, -0.64*) and the ratio of ammonia-N to nitrate-N was the main factor influencing soil microbial functional gene richness (Figure S7, 0.22*). Moreover, both soil microbial biomass and microbial functional genes were significantly correlated with the molar mass of LF-SOM (Figure S7, -0.17* and 0.65**).

4 Discussion

4.1 Impacts of multi-level N additions on LF-SOM

N deposition indirectly affects SOM transformation by affecting vegetation growth, litter input, soil properties and microbial activity (Denef et al., 2009). Compared with the effects of long-term N addition, the effects of short-term N addition on vegetation and soil microbes are more obvious (Han et al., 2018). Under short-term N addition, the input of external N causes severe disturbances to soil conditions that significantly affects vegetation and soil microorganisms. However, vegetation and microorganisms will gradually adapt over longer time periods (Han et al., 2018). Therefore, studying the response of SOM to short-term N deposition is of great significance for assessing the carbon balance in ecosystems (Liu et al., 2013). In this study, N addition increased AGB (Figure S2). As the QTP is an N-limited area (Liu et al., 2013), N addition increased the content of soil inorganic N (Table S2), thus stimulating vegetation productivity (Fang et al., 2012). However, with the increase in N additions, LF-SOM decreased

significantly (Figure 1b), and there was a significant negative correlation between LF-SOM and AGB (Table 1), indicating that N addition promoted the degradation of LF-SOM in alpine grassland of the QTP. As the most active fraction with the fastest turnover rate, LF-SOM is closely related to soil microorganisms and shows the greatest sensitivity to soil physical or chemical disturbances (Chen et al., 2019). Therefore, LF-SOM plays a very important role in carbon cycling (Khanna et al., 2001) and is often used to indicate the impact of climate change on SOM degradation. In this study, NEE was positively correlated with LF-SOM (Table 1), which further proved that LF-SOM is an indicator of the response of SOM transformation to N deposition on the QTP.

To better understand the effects of N addition on LF-SOM transformation, LF-SOM was classified into several compound classes at the molecular level. Although all compounds in LF-SOM showed a trend of degradation with the increase in the N application gradient (Table 2), the sensitivity of these compound classes to N addition varied due to their different sources and characteristics (Hobbie et al., 2012). In this study, plant-derived compounds (including lignin, polysaccharide-derived compounds, long-chain alkyl compounds, plant-derived N compounds and phenols) were more sensitive to N addition than microbial-derived compounds (including chitin and microbial-derived N compounds) and aromatic compounds (including aromatics and polyaromatics). Plant-derived compounds have high carbon content and poor chemical stability, which are usually the preferred carbon source for soil microorganisms (Lu et al., 2014). When external N is added, the C/N ratio in soil decreases, which increases the preference of soil microorganisms for these high-carbon organic compounds (Lu et al., 2014). In addition, the activities of enzymes (cellulase and amylase) that degrade plant-derived compounds are mainly limited by N. The input of external N relieved the N limitation in this area and enhanced the activities of cellulase and amylase, thus promoting plant-derived compound degradation (Zhao et al., 2017). Microbial-derived compounds in the soil generally interact with soil minerals to enhance their stability to resist microbial degradation (Chen et al., 2019), and aromatic compounds in soil have strong chemical stability due to their unique chemical structures. Therefore, the responses of microbial-derived and aromatic compounds to N addition were relatively insensitive compared with those of plant-derived compounds.

4.2 Impacts of soil microorganisms on LF-SOM under multi-level N additions

The growth of soil microorganisms is closely related to plant composition, soil pH and soil nutrients (Moyer et al., 1995). The addition of N can indirectly or directly affect the growth, reproduction and activity of soil microorganisms, altering the structure and function of microbial communities (Li, 2010). The effect of N addition on fungi and bacteria, the two major microflora in soil, will directly affect the SOM degradation process. Studies have shown that the effects of external N addition on fungi and bacteria are usually related to carbon sources (Li, 2010). This phenomenon is mainly due to the physiological and ecological differences between fungi and bacteria (Han et al., 2017). Fungi can secrete many extracellular enzymes that mainly act on phenolic-containing compounds. At the same time, fungi create mycelial networks, which have

better mobility than bacteria. Therefore, fungi in soil prefer to consume plant tissue debris such as cellulose and lignin (Zhao et al., 2017). In contrast, bacteria prefer to consume easily decomposed substrates with high carbon content (Meidute et al., 2008). In this study, the increase in bacterial biomass promoted the degradation of LF-SOM, and the increase in fungal biomass further promoted the degradation of plant-derived compounds.

Furthermore, multi-level N additions also significantly changed the soil microbial functional community structure (Figure S4a), in accordance with the results from the N addition field experiments in temperate grassland and forest ecosystems (Eisenlord et al., 2013; Yao et al., 2014; Cong et al., 2015). There was a significant positive correlation between the richness of microbial functional genes and the molar mass of LF-SOM (Figure 2a). To further study their relationships under N addition, carbon degradation-related genes were selected, including the *alk*, *cellulose*, *aromatic*, *polyaromatic*, *lignin*, *chitin* and *protease* genes, which are associated with the selected compound classes in LF-SOM. As shown in Figure 2b, these compound classes in LF-SOM were all positively correlated with their corresponding functional genes ($P<0.05$).

The *alk* genes are mainly involved in the degradation of soil alkyl compounds. Among them, *alkB* genes determine the first step of the oxidation-based decomposition of n-alkanes (Liu et al., 2010), which are strongly correlated with soil n-alkane degradation (Throne-Holst et al., 2006). Polysaccharide-derived compounds can be derived from plant compounds, such as *levoglucosan* and *levomannosan*, which are pyrolysis products of intact polysaccharides, such as cellulose (Poirier et al., 2005). Polysaccharides cannot be used directly, and soil microorganisms need to secrete cellulase to break long-chain polysaccharides into short-chain compounds and eventually convert them into available glucose (Liu et al., 2017). Phenols can have multiple origins, including lignin through side-chain oxidation (Van Bergen, 1998; Lobe et al., 2010). Shorter side chains and substituents on phenols derived from lignin are considered to indicate a further degree of decomposition (Zech et al., 1992; Bergen et al., 1998). Lignin-degrading enzymes, including lignin peroxidase (*LiP*), manganese peroxidase (*MnP*) and polyphenol oxidase, are thought to play key roles in the degradation of lignocellulose and phenols (Dong et al., 2014). Chitin and N compounds in soils constitute the main nitrogen storage states, including amino sugars, proteins and humus, and cannot be directly utilized by organisms without decomposition. Therefore, chitin and N compounds are usually decomposed into amino acids by chitinase and proteases, and thereby become bioavailable. Aromatics are generally considered pyrolysis products of proteins (Chiavari & Galletti, 1992), possibly from microorganisms (Schulten et al., 1991) or burned products (Kaal and Rumpel, 2009). Polyaromatics may originate from the cyclization of aliphatic compounds through pyrolysis reactions (Saiz-Jimenez, 1994) or from charred substances (Kaal & Rumpel, 2009). Polyaromatics are a class of organic compounds consisting of two or more aromatic rings and are usually degraded by *polyaromatic* genes, including *nah-like*, *phn*, and *phd* genes (Zhang et al., 2010). Yao et. al (2014) found that the change in microbial functional genes was generally consistent with that of the carbon source utilized by microorganisms. The positive correlations between the molar mass of LF-SOM and the total richness of microbial functional genes, as well as between the molar mass of compound

classes in LF-SOM and the richness of compound degradation-related genes suggested that LF-SOM was the main utilization fraction of soil microorganisms (Neff et al., 2002).

Among the microbial functional genes, carbon degradation genes play crucial roles in the degradation of SOM (Ljungdahl & Eriksson, 1985; Rubin, 2008), and the fluctuation of their relative abundance (%) usually reflects the change in SOM degradation process. In this study, N addition increased the relative abundance of carbon degradation genes, and it reached the maximum at N80 (Figure S5), which was consistent with the greatest degradation of LF-SOM at N80 (Figure 1b). Meanwhile, the relative abundance of plant-derived compound degradation genes, compared with those for microbial-derived and aromatic compound degradation genes, significantly increased with increasing N addition (Figure S6), further promoting the degradation of plant-derived compounds.

4.3 Degradation mechanisms of LF-SOM under multi-level N additions on the QTP

A schematic diagram of LF-SOM transformation under the N addition treatments on the QTP is shown in Figure 3. In this study, the response of LF-SOM to the different N addition levels varied. The lowest N addition levels (N10 and N20) contributed to the accumulation of LF-SOM. However, from N40 to N160, N addition promoted LF-SOM degradation. Although LF-SOM was still degraded at N160, the degradation degree of LF-SOM was weakened compared with that at N80 (Figure 1b). The non-linear response of LF-SOM to multi-level N additions may be due to the direct effects of N addition on vegetation and soil microorganisms. Vegetation growth is closely related to the content of soil ammonia-N and nitrate-N (Liu et al., 2013). Studies have shown that vegetation prefers to use soil nitrate-N in arid areas and soil ammonia-N in humid areas (Liu et al., 2013; Wang & Macko., 2011). As the QTP is a semi-arid area, the vegetation there preferentially utilize soil nitrate-N (Liu et al., 2013), and the increase in soil nitrate-N content under N addition can promote vegetation growth and litter input. However, there is usually a significant interaction between nutrient supply and demand in the soil. When one nutrient is added, it can aggravate the limitation of other nutrients (Lu et al., 2014), which may negatively affect soil microbial activity and SOM degradation (Lu et al., 2014; Knorr et al., 2005). At N10 and N20, N addition promoted vegetation input because of the increase in soil nitrate-N (Table, S2), but it inhibited microbial activity because of the aggravation of soil carbon (C) limitation (the decrease in soil CN ratio, Table S2), which contributed to LF-SOM accumulation. In contrast, from N40 to N160, the large increase in vegetation input could alleviate the soil C limitation caused by N addition (the increase in soil CN ratio, Table S2), which might be more favorable for the enhancement of microbial activity (the increase in microbial biomass and the relative abundance of carbon degradation genes, Table S5, Figure S5) and LF-SOM degradation. In the process of N addition from N0 to N160, the nutrient environment may change from N-limited conditions to N-sufficient conditions, and the N utilization efficiency of plants can be reduced under N-sufficient conditions, which can negatively affect vegetation growth (Liu et al., 2013). In addition, a large amount of exogenous N input can significantly increase the concentration of soil inorganic N, and the nitrification of

NH_4^+ and the leaching of NO_3^- can lead to soil acidification (Zhao et al., 2015) and reduce soil pH, which may negatively affect microbial activities. Previous studies have shown that the activity and diversity of microorganisms are lowest in acidic soil and highest in neutral soil (Fierer et al., 2006). Therefore, excessive N addition can inhibit both vegetation and microorganisms (Knorr et al., 2005; Liu et al., 2013), which may further negatively influence SOM degradation.

The results of this study show that multi-level N additions have various effects on LF-SOM transformation. Therefore, based on the pyrolysis data, it is predicted that when the current N deposition rate on the QTP increases by 10 kg N ha^{-1} yr^{-1}, the carbon sequestration of LF-SOM may increase by nearly 170% compared with the present labile fraction carbon reserves (Figure S8). These results can help us effectively predict the impacts of future N deposition on the carbon reserves of LF-SOM on the QTP.

5 Conclusions

A two-year multiple N deposition (0, 10, 20, 40, 80 and 160 kg N ha^{-1} yr^{-1}: N0, N10, N20, N40, N80 and N160) experiment was conducted in alpine grassland to illustrate the response of LF-SOM transformation to N deposition on the QTP. There were significant correlations between the molar mass of LF-SOM and the microbial biomass, between the molar mass of organic compounds in LF-SOM and the richness of compound degradation-related genes (alkyl compounds with *alk* genes, polysaccharide-derived compounds with *cellulos*e genes, phenols with *lignin* genes, chitin with *chitin* genes, N compounds with *protease* genes, aromatics with *aromatic* genes and polyaromatics with *polyaromatic* genes), as well as between the molar mass of LF-SOM and NEE, proving that LF-SOM was not only the main fraction used by microorganisms but also the most sensitive fraction to N deposition. In this study, the response of LF-SOM to different N addition levels varied. LF-SOM accumulated at the lowest N addition levels (N10 and N20) and decreased from N40 to N160, but the degradation level did not increase at N160 compared with that at N80. The non-linear response of LF-SOM to multi-level N additions may be due to the effects of N additions on vegetation and soil microorganisms. Plant-derived compounds in LF-SOM were more sensitive to N addition than microbial-derived and refractory compounds because of their degradability and high carbon content. Based on the pyrolysis data, it is predicted that when the current N deposition rate on the QTP increases by 10 kg N ha^{-1} yr^{-1}, the carbon sequestration of LF-SOM will increase by nearly 170% compared with the present labile fraction carbon reserves.

Acknowledgments and Data

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Table 1. Relationships between AGB, NEE and LF-SOM.

	AGB	NEE
LF-SOM	**-0.48***	**0.51***

Note: LF-SOM, labile fraction soil organic matter ($\rho \leq 2.25$ g cm^{-3}); AGB, aboveground biomass; NEE, net ecosystem exchange. The calculated LF-SOM data were ln-transformed data. The asterisks indicate a significant correlation (*P<0.05).

Table 2. Responses of soil organic compounds in the labile fraction to nitrogen addition.

	R^2	*P*	*Slope*
Lignin	0.4031	**0.0110**	-0.0173
Polysaccharides	0.4844	**0.0040**	-0.0093
Plant-derived N compounds	0.4936	**0.0035**	-0.0082
Phenols	0.4478	**0.0064**	-0.0080
Long-chain alkyl compounds	0.3356	**0.0236**	-0.0064
Chitin	0.8609	**0.0001**	-0.0076
Microbial-derived N compounds	0.4062	**0.0106**	-0.0070
Short-chain alkyl compounds	0.1521	0.1507	-0.0059
Aromatics	0.4127	**0.0098**	-0.0063
Polyaromatics	0.3433	**0.0217**	-0.0061

Note: The data shown in the table are the R^2 values, P values and slopes obtained from linear regression analyses of the responses of different organic compounds to N addition. The calculated data are the ln-transformed data of compounds in each treatment that differ from those of the corresponding compounds in N0. The black bold font shows the P values that indicate statistical significance (P<0.05), n=15.

Table 3. Relationships between the microbial biomass and the molar mass of LF-SOM.

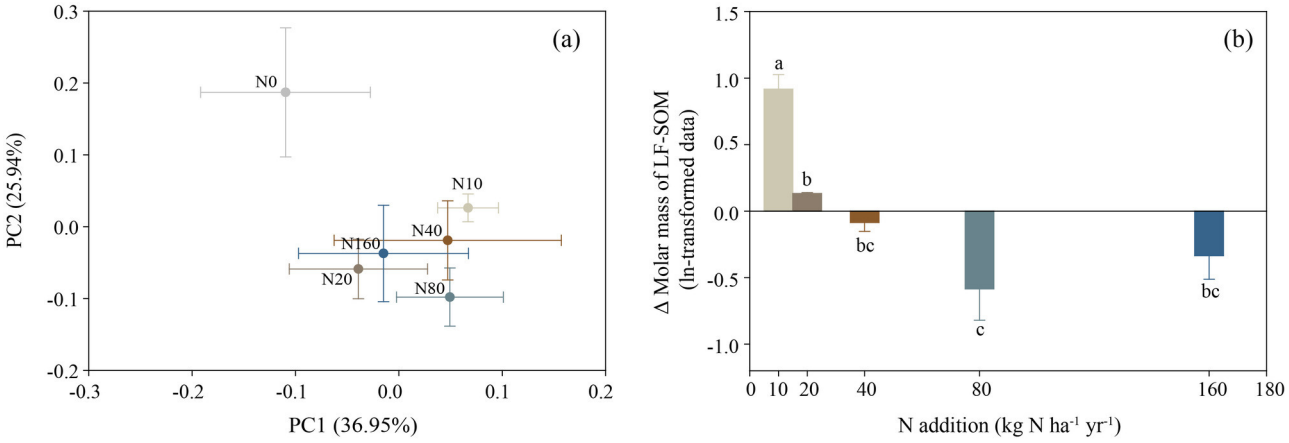
	TMB	Bacteria	Fungi	Actinomycetes	F/B
LF-SOM	**-0.69****	**-0.73****	**-0.54***	**-0.58***	-0.01

Note: LF-SOM, labile fraction soil organic matter ($\rho \leq 2.25$ g cm^{-3}); TMB, total microbial biomass; F/B, the ratio of fungal biomass to bacterial biomass. The calculated data for LF-SOM and microbial biomass were ln-transformed data. The asterisks show a significant correlation (*$P<0.05$, **$P<0.01$).

Figure 1. Principal component analysis (PCA) plot of the compositions of the labile fraction organic compounds under six levels of N addition (a) and the difference in the labile fraction molar mass between each treatment and N0 (b). The following levels of N addition were investigated: N0 (0 kg N ha^{-1} yr^{-1} added), N10 (10 kg N ha^{-1} yr^{-1} added), N20 (20 kg N ha^{-1} yr^{-1} added), N40 (40 kg N ha^{-1} yr^{-1} added), N80 (80 kg N ha^{-1} yr^{-1} added) and N160 (160 kg N ha^{-1} yr^{-1} added). Different lowercase letters (a, b and c) indicate a significant difference (P<0.05) based on ANOVA analysis.

Figure 2. Relationship between the molar mass of LF-SOM and the microbial functional gene richness (a) and relationships between the molar mass of organic compounds in labile fraction and the richness of microbial functional genes (b). The solid line indicates that the relationship is significant (P<0.05) based on a linear regression estimated using ordinary least squares. The shaded region represents the 95% confidence limits on the regression estimates. The asterisks indicate a significant correlation (P<0.05). Alkyl-*alk*, alkyl compounds and *alk* genes; Ps-*cell*, polysaccharide compounds and *cellulous* genes; Ar-*ar*, aromatic compounds and *aromatic* genes; Pa-*pa*, polyaromatic compounds and *polyaromatic* genes; Ph-*lg*, phenol compounds and *lignin* genes; Chi-*chi*, chitin and chitin genes; N-*pro*, N compounds and *protease* genes.

Figure 3. A schematic diagram illustrating observed changes in LF-SOM under N additions. The black up arrows represent accumulation (the thicker up arrow, the greater accumulation) and the black down arrows represent degradation (the thicker down arrow, the greater degradation). The green arrow represents the effect of N additions on vegetation (the thicker up arrow, the greater increase), and the red arrow represents the effect of N additions on soil microorganisms (the thicker up arrow, the greater increase and the thicker down arrow, the greater decrease).



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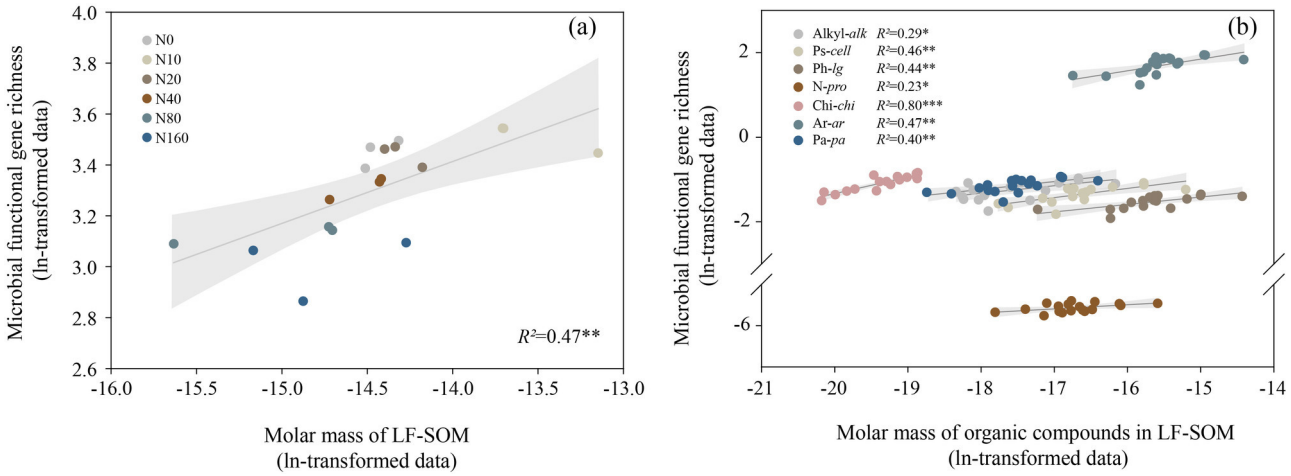

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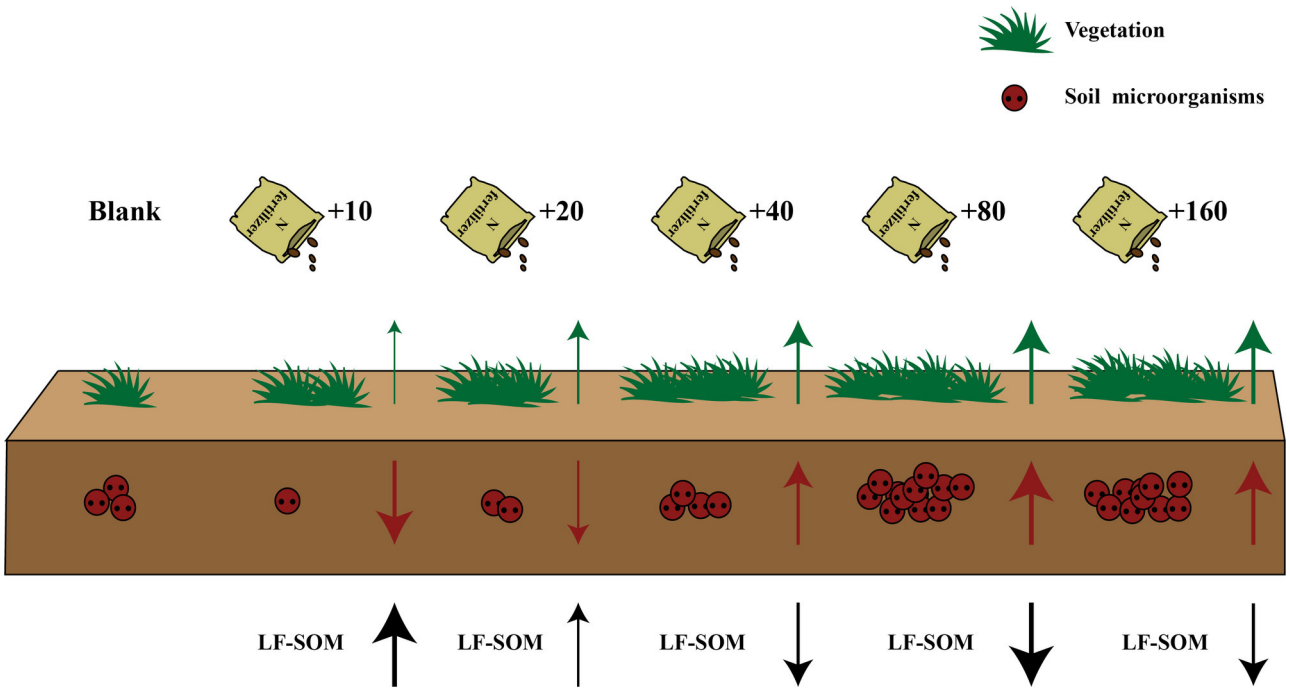

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