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Shifts in priming partly explain impacts of long-term nitrogen input in different chemical forms on soil organic carbon storage

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Running title**:** N chemical forms drive different priming effects

Abstract

Input of labile organic carbon can enhance decomposition of extant soil organic carbon (SOC) through priming. We hypothesized that long-term nitrogen (N) input in different chemical forms alters SOC pools by altering priming effects associated with N-mediated changes in plants and soil microbes. The hypothesis was tested by integrating field experimental data of plants, soil microbes and two incubation experiments with soils that had experienced 10 years of N enrichment with three chemical forms (ammonium, nitrate and both ammonium and nitrate) in an alpine meadow on the Tibetan Plateau. Incubations with glucose-^{13}C addition at three rates were used to quantify effects of exogenous organic carbon input on the priming of SOC. Incubations with microbial inocula extracted from soils that had experienced different long-term N treatments were conducted to detect effects of N-mediated changes in soil microbes on priming effects. We found strong evidence and a mechanistic explanation for alteration of SOC pools following 10 years of N enrichment with different chemical forms. We detected significant negative priming effects both in soils collected from ammonium-addition plots and in sterilized soils inoculated with soil microbes extracted from ammonium-addition plots. In contrast, significant positive priming effects were found both in

soils collected from nitrate-addition plots and in sterilized soils inoculated with soil microbes extracted from nitrate-addition plots. Meanwhile, the abundance and richness of graminoids were higher and the abundance of soil microbes was lower in ammonium-addition than in nitrate-addition plots. Our findings provide evidence that shifts towards higher graminoid abundance and changes in soil microbial abundance mediated by N chemical forms are key drivers for priming effects and SOC pool changes, thereby linking human interference with the N cycle to climate change.

KEYWORDS

Alpine meadow, functional groups, nitrogen chemical form, priming effect, soil microbes, soil organic carbon (SOC), Tibetan Plateau

INTRODUCTION

Anthropogenic nitrogen (N) emissions have approximately doubled atmospheric N deposition in terrestrial ecosystems since the Industrial Revolution (Galloway et al., 2008), with profound consequences for a range of ecosystem processes (Hobbie, 2000; Galloway et al., 2008; Dupre et al., 2010; Bobbink et al., 2010; Phoenix et al., 2012). There is consensus that N enrichment generally causes reductions in plant species richness and increases in plant productivity, which are commonly accompanied by a shift towards graminoid dominance and subsequent community turnover (Stevens et al., 2004; Bobbink et al., 2010; Dupre et al., 2010). In contrast, effects of N enrichment on the sequestration and decomposition of soil organic carbon (SOC) are complex and controversial (Wedin & Tilman, 1996; Nadelhoffer et al., 1999; Neff et al., 2002; Resh et al., 2002; Mack et al., 2004). N enrichment can have a positive, neutral or negative effect on SOC sequestration depending on the type of ecosystems (Nadelhoffer et al., 1999; Hobbie, 2000; Resh et al., 2002; Mack et al., 2004;

Ramirez et al., 2012). However, little is known about the mechanisms underlying the direction and extent of variation in SOC with soil N availability.

In N-limited ecosystems, N enrichment could increase fixation of atmospheric CO_2 through promoting plant photosynthesis and growth (Magnani et al., 2007; LeBauer & Treseder, 2008; Xia & Wan, 2008), an effect that is likely to enhance the carbon sink in such ecosystems. However, increasing photosynthetic carbon fixation into ecosystems could also cause changes in the direction and extent of a priming effect by new SOC on the decomposition of existing, older SOC (Kuzyakov et al., 2007; Kuzyakov, 2010). Root exudation (ranging from 5 to 33% of daily photoassimilate) and leaf litter can supply easily decomposable, labile organic carbon to soil microorganisms, which could stimulate their activity and thereby prime and accelerate decomposition of extant SOC. On the one hand, increasing availability of easily decomposable, labile organic carbon to soil microbes could greatly increase the priming of extant organic matter if the activity and growth of soil microbes are limited by nutrition, and this would reduce SOC storage (Kuzyakov et al., 2007; Cheng et al., 2014). On the other hand, the easily decomposable, labile organic matter can also be used by soil microbes to form stabilized soil organic matter. If the amount of newly formed soil organic matter is greater than the amount lost through the decomposition of the extant old organic matter, this would generate negative priming and promote SOC storage (Craine et al., 2007). Thus, N-mediated productivity increases and species dominance shifts would induce changes in the amount of labile organic carbon input. As a consequence, long-term N enrichment may alter SOC storage by affecting priming effects on SOC decomposition. So far, however, little is known about how long-term N enrichment impacts the priming of SOC through changes in the amount of labile organic carbon input.

The functional composition and activity of soil microbial communities play a key role in the dynamics of SOC decomposition (Monson et al., 2006; Lipson et al., 2009; Rousk & Bengtson, 2014). These soil microbial communities and decomposition processes can also be strongly affected by soil N availability (Nemergut et al., 2008; Ramirez et al., 2012). There

is evidence that increasing N availability can stimulate the activity of particular microbial groups, with consequences for soil biogeochemical processes (Sjöberg et al., 2004). On the other hand, long-term N enrichment can acidify soil, increase osmotic pressures, deplete soil minerals and increase aluminum toxicity, with potential negative effects on microbial processes, especially for fungal groups (Treseder, 2008; U.S. EPA, 2008). Moreover, N-mediated changes in plant productivity and shifts in plant species dominance could induce changes in soil microbial abundance and activity (Paterson et al., 2007). These direct and indirect effects of N enrichment on soil microbes can further influence the priming of SOC decomposition (Ramirez et al., 2012). The overall effect of N enrichment on soil carbon transformations will depend on the responses of different microbial functional groups (which may be specialized on specific types of SOC) to N enrichment. Therefore, long-term N enrichment may alter SOC storage by affecting priming effects on SOC decomposition through changes in soil microbial abundance and activity associated with N-mediated direct and indirect effects. To date, however, we still know little about how long-term N enrichment influences SOC decomposition through alteration in priming effects associated with changes in soil microbial composition.

In terrestrial ecosystems, N is often a limiting nutritional element to plants and available in different chemical forms (e.g. ammonium and nitrate; McKane et al., 2002; Lebauer & Treseder, 2008; Martens-Habbena et al., 2009). While soil nitrate-N concentration is higher than ammonium-N concentration in many terrestrial ecosystems, the reverse is true in some ecosystems such as alpine meadow (McKane et al., 2002; Ashton et al., 2010; Song et al., 2012). Also, atmospheric deposition enriched with certain N forms and long-term addition of agricultural fertilizers with different N forms can disproportionally increase the availability of ammonium or nitrate in soil (Fowler et al., 1998; Galloway et al., 2008; Stevens et al., 2011), and alter the original proportion of available soil N forms (Krupa, 2003). In response to changes in the availability of a N chemical form, some plant species will be confined to the form on which they are specialized, while other plant species may show plasticity in the

uptake of the N form and/or shift preference from one N chemical form to another to maintain or even increase their overall N uptake (McKane et al., 2002; Martens-Habbena et al., 2009; Song et al., 2015). Therefore, long-term enrichment of N in different chemical forms may also drive changes in plant species composition through shifts in species' abundances. Moreover, the functional composition of soil microbes could also respond to N chemical forms in different ways (Tian et al., 2014; Zheng et al., 2014). Variation in the chemical composition of N, both for background soil inorganic N forms and for external input N forms, is a largely overlooked factor that may also contribute to the inconsistent soil and vegetation responses to N enrichment in different ecosystems (McKane et al., 2002; Martens-Habbena et al., 2009). To our knowledge, no study has examined how long-term enrichment with N in different chemical forms influences SOC decomposition through alteration of priming effects.

Following the conceptual framework (Fig. 1), we hypothesized that long-term N enrichment with different chemical forms changes SOC storage by altering priming effects. To test this hypothesis, we integrated field experimental data from plants and soil microbes with two incubation experiments using soils that had experienced 10 years of N enrichment with three chemical forms (ammonium, nitrate and ammonium nitrate) in an alpine meadow on the Tibetan Plateau. In this ecosystem, low temperature restricts SOC decomposition and most soil N exists in the form of organic compounds. Thus, soil N availability (both as ammonium and as nitrate) to plants is very low, and exerts a strong limitation on plant growth and presumably on below-ground processes. This combination of factors makes for a suitable study system for investigating effects of N amount and form on soil carbon cycling. Specifically, we addressed the following questions: (1) How do the amount of labile organic carbon input mediated by productivity increase and species shifts under N addition treatments influence the priming effect on SOC decomposition? (2) What is the contribution of N-mediated changes in soil microbes to this priming effect on SOC decomposition? (3) How does long-term N enrichment with different chemical forms affect the size of the SOC pool?

MATERIALS AND METHODS

N fertilization experiment

The experiment was carried out in an alpine meadow at the Haibei Alpine Meadow Ecosystem Research Station (37°37´N, 101°12´E, 3240 m a.s.l.) located in the northeast of the Tibetan Plateau in Qinghai Province, China. The annual precipitation averaged during the past 30 years was 560 mm, 85% of which was concentrated in the growing season (from May to September). The mean annual temperature was -1.7 °C. The soil is classified as Mat Cry-gelic Cambisols (Chinese soil taxonomy research group, 1995) corresponding to Gelic Cambisol (WRB, 1998), and details of soil characteristics are shown in Table 1. The study area is dominated by the sedge *Kobresia humilis* Serg. The vegetative cover of the surface is over 95% (Song et al., 2012).

The experiment was established in 2005 by fencing off an area of 80 m × 60 m and thereby excluding large grazers. Within this ungrazed area, we established 30 plots of 2 m × 2 m, arranged in three rows with ten plots each. Plots were 1 m apart. Iron sheets of 35 cm height were inserted along the four edges of the plots to a depth of 30 cm to separate the plots from the surrounding vegetation. The fertilization experiment consisted of nine N treatments and an unfertilized control, each applied to three replicate plots. In the control, no N was added. The other nine treatments were a factorial combination of three N chemical forms and three levels of N supply rates. The three N forms were ammonium N (NH_4-N), nitrate N (NO_3-N), and both NH_4-N and NO_3-N by supplying with $(NH_4)_2SO_4$, $NaNO_3$ and NH_4NO_3, respectively. The three N supply rates were equivalent to 0.375, 1.5 and 7.5 g N m^{-2} yr^{-1}, respectively. The N was supplied twice a year during the growing season (on 10-15 July and 10-15 August) from 2006, with half of the total annual N amount each. In 2005 it was added only once with the total amount on 10 July. The N was applied in aqueous solution and 5 L solution was evenly sprayed onto each plot. For each control plot, 5 L water was applied at the same time when plots received fertilizer. More detailed information about the study site

and the N fertilization experiment can be found in Song et al. (2012). The study site was performed winter graze again from November 2011 to April 2015 for removal of the excessive accumulated plant litter. The previous studies showed that, among the three N rates, addition of 0.375 and 1.5 g N m^{-2} yr^{-1} had little impact on productivity or plant species composition in this alpine meadow (Song et al., 2012). Therefore, in this study we considered only the treatments of no N addition (control) and addition of 7.5 g NH_4-N, NO_3-N, and NH_4NO_3-N m^{-2} yr^{-1}, coded as CK, Am, Ni and AN, respectively.

Plant sampling and measurement

A 1 m × 1 m quadrat was established at the center of each plot. Occurrence of each vascular species in each quadrat was recorded in the middle of August each year from 2005 to 2014. For biomass measurements, we clipped aboveground shoots within a 0.25 m × 0.25 m quadrat outside the central 1 m × 1 m quadrat but within the plot in the middle of August every year when biomass peaked. The quadrat for clipping shifted each year within the plot to avoid harvesting the same area in successive years. Shoots were clipped at ground level and sorted into plant functional groups, i.e., graminoids (grasses and sedges), legumes and forbs. All shoots were oven-dried at 60 °C for 48 h, and then weighed.

Soil sampling and preparation

In August 2014, soil samples were taken from each plot of the four N addition treatments (CK, Am, Ni, and AN). The soil sampling was combined with a ^{13}C tracing experiment in the same plots. We collected soil cores (7 cm in diameter and 20 cm in depth) from 2 sub-plots within each plot six times on August 1, 2, 6, 14, 21 and 30, respectively. Then soil samples collected from the same plot were pooled into one composite sample. After removing thick roots and stones by passing through a 2 mm mesh sieve, we stored all soil samples in iceboxes and

transferred them to the laboratory. The sieved soils were kept at 4 °C for two days before they were used for the subsequent measurements and the incubation experiment.

Soil and plant C concentration measurements

Soil moisture was measured gravimetrically. Subsamples used for C and N analysis were air-dried, sieved (2 mm mesh), and handpicked to remove fine roots, and then ground in a ball mill. The concentration of total SOC was analyzed using the modified Walkley-Black method by boiling a soil-dichromate-sulfuric acid mixture with 1:20 ratio of soil to extractant at 175 °C for 5 min (Walkley & Black, 1934; Lettens *et al.*, 2005). A conversion factor of 1.1 was applied to correct SOC measurements obtained from the modified Walkley-Black method (Lettens *et al.*, 2005). Organic matter content values were corrected for water content and expressed on a dry weight basis. Soil bulk density was determined with a soil corer with a known volume. Plant aboveground and belowground organs were dried and weighed into tin capsules and analyzed for total C (as well as N, $\delta^{13}C$, and $\delta^{15}N$) by continuous-flow isotope ratio mass spectrometry coupled with an elemental analyzer (EA 1110; CE Instruments, Milan, Italy) (EA-IRMS) and a ConFlo II device connected to IRMS (FinniganMAT 253, Bremen, Germany).

Incubation with treated whole soils

In this incubation experiment, we aimed to quantify how much of the original SOC would be released by priming effects from soils that had experienced ten years of the four N addition treatments (CK, Am, Ni, and AN). We added into soils collected from plots of each of the four N addition treatments with ^{13}C-labelled glucose at four rates: 0 (no addition), 0.3, 0.5, and 0.8 mg C g^{-1} soil. The rate of 0.5 mg C g^{-1} was based on the assumption that 10% of

photo-assimilate was input into belowground as root exudates, which is also close to half of the soil microbial biomass C (Table 1; Blagodatskaya & Kuzyakov, 2008; Chapin et al., 2012).

A subsample of the sieved fresh soils was taken out and put in a shaded place to dry out. Thirty grams of the sieved, air-dried soil were added to 250-ml Schott bottles and adjusted to 60% of the water-holding capacity. All soils were pre-incubated at 15 °C for 4 days. Then water or ^{13}C-labeled glucose (5.97 atom% ^{13}C) solution was added evenly to the soil surface using a pipette to obtain a uniform distribution. After addition of ^{13}C solution, soil moisture was kept at 70% of field capacity throughout the experiment. In addition, three Schott jars without any soil were established as blanks. One gram of $CaCl_2$ was added to small cups and placed into the incubation bottles to absorb water vapor and to keep soil moisture constant during subsequent water additions. The $CaCl_2$ was replaced weekly to maintain its absorptive capacity for water (Qiao et al., 2014). All incubations were conducted at 15 ℃ and lasted 28 days. We chose the incubation temperature based on the mean daytime temperature of about 15 ℃ during July and August in the experimental site. Five mL of 1 M NaOH was placed in small cups in each incubation bottle to trap CO_2 and replaced at days 1, 3, 5, 7, 14, 21, and 28 of the experiment. CO_2 samples were trapped from each incubation bottle and analyzed for CO_2 flux rate. For $\delta^{13}C$ measurement samples at days 1, 3, 5, 14, and 28 were selected to be measured to save expenses.

To measure CO_2 absorbed in NaOH, 4 mL of 0.5 M $SrCl_2$ was added to precipitate carbonate. Unreacted NaOH was titrated with 0.2 M HCl against the phenolphthalein endpoint (Zibilske, 1994). Precipitated $SrCO_3$ was centrifuged three times at 1200 g for 10 min followed each time by rinsing with degassed water. The $SrCO_3$ was then dried at 105 °C and weighed into tin capsules to analyze for total C and $^{13}C/^{12}C$ ratios by continuous flow gas isotope ratio mass spectrometry (MAT253; Finnigan MAT, Bremen, Germany), coupled by a ConFlo III device (Finnigan MAT) to an elemental analyzer (EA 1112; CE Instruments, Milan, Italy).

Reference soil incubation with soil microbe inoculation

We conducted another experiment with soil microbe inoculation to detect how N-mediated changes in soil microbes contribute to the priming of SOC decomposition. Specifically, soil microbes were extracted from soils that had experienced ten years of the four N addition treatments (CK, Am, Ni, and AN). These extracts were then inoculated into the sterilized reference soils. The reference soils used for inoculation were collected from the same alpine meadow area directly adjacent to our field long-term N addition experiment site in July 2015. The soils were homogenized and oven-dried for 48 h, and then sterilized with high-pressure vapor for 48 h. Then 30 g of sterilized soil was transferred into each of 30 Schott bottles (250 ml each) after 24 h.

Soil microbes were extracted from soils collected at 0-20 cm depth. Soil inoculants were prepared by mixing 100 g soil from each plot with 100 mL water for four consecutive days. On each day, soil-water slurries were left to settle for 5 h after thorough mixing. Then supernatant from each soil sample out of the three replicates in each N treatment was evenly separated into two parts, and then poured into the sterilized soils in two of the 30 Schott bottles. Thus soil microbes extracted from the soils of the four N addition treatments were inoculated into the sterilized reference soil, with six replicates per treatment and 24 bottles in total. The other six bottles were used as references in the following incubation experiment. Three additional Schott bottles without any soil were established as blanks.

^{13}C-labelled glucose was added into each Schott bottle to simulate a labile organic carbon addition rate of 0.5 mg C g^{-1} soil and a treatment of 0 mg C g^{-1} soil was used as a paired reference. Water or ^{13}C-labeled glucose (5.97 atom% ^{13}C) solution was added evenly to the soil surface using a pipette to obtain uniform distribution. Subsequently, soil moisture was kept at 70% of field capacity throughout the experiment. The following steps were the same as those in the incubation experiment with treated whole soils (see above). The incubation

experiment lasted 28 days. CO_2 samples were trapped from each incubation bottle and analyzed for CO_2 flux rate. $\delta^{13}C$ signatures of gas samples were measured at days 1, 3, 5, 14, and 28.

It should be noted that sterilization could disrupt soil organic matter availability by creating necromass. However, soil microbial biomass carbon is only a very small proportion of SOC (Table 1), which would not cause a substantial and long-lasting disturbance.

Soil microbial analysis

Microbial composition in soils of the four N addition treatments (CK, Am, Ni, and AN) and in soils from the soil microbe inoculation experiment after the 28-day incubation was assessed by phospholipid fatty acid (PLFA) analysis using a modified method of Frostegård et al. (1993). Two grams of fresh soil samples were extracted using 2 ml phosphate-buffered $CHCl_3$-CH_3OH solvent. Additional chloroform and water were added to separate aqueous and organic phases. The fatty acids in the organic phase were fractionated into neutral lipids, glycolipids and phospholipids on a silica acid column. The phospholipids were transected in a mild alkaline methanolysis to convert the PLFAs into fatty acid methyl esters (FAMEs). The identity and abundance of the various FAMEs were determined by gas chromatography. All gas chromatography measurements included a blank sample with the internal standard (peak 19:0, nonadeconoate fatty acid) – one sample with a standard qualitative bacterial acid methyl esters mix (BAC mix) and one sample with a standard qualitative fatty acid methyl esters mix (FAME mix; both Sigma Aldrich Co., St. Louis, MO) for easier identification of the fatty acid peaks. The total peaks were detected per sample. The areas measured by GC-FID were used for calculating the abundance of PLFA markers in nmol g^{-1} dry weight of soil for use in further analyses.

To characterize the community structure, we used the terminal-branched saturated PLFA peaks i15:0, a15:0, i16:0, i17:0, a17:0 as markers for Gram-positive bacteria (G+) (Zelles,

1997). The mono-unsaturated and cyclopropyl saturated peaks 16:1ω5, 16:1ω9, 17:1ω9, cy17:0, 18:1ω11, cy19:0 were used as indicators for Gram-negative bacteria (G-), and all these together with PLFA peaks at 14:0, 15:0 and 17:0 as a measure for total bacteria (Frostegård et al., 1993; Zelles, 1997). Furthermore, 18:2ω6, 9 was used as fungal PLFA marker (Högberg, 2006). The methylic, mid-chain-branched saturated PLFA peaks 10Me 16:0, 10Me17:0, 10Me18:0 were used as indicators for actinomycetes (Frostegård et al., 1993). In total, we considered five functional groups, namely: (1) total bacteria, (2) G-, (3) G+, (4) actinomycetes, and (5) fungi.

Calculations of CO_2-C efflux

Total CO_2-C in trapped CO_2 was measured at days 1, 3, 5, 7, 14, 21, and 28 by titrating the NaOH solution against 0.2 N HCl after the addition of $SrC1_2$. NaOH solutions, incubated in Schott jars without any soils, were also analyzed as blanks. For each treatment, mean and standard deviation (SD) of CO_2 efflux were calculated based on values from the three replicates. Flux-weighted δ values were based on trapped CO_2 at days 1, 3, 5, 14, and 28 and CO_2 efflux. The end-member mixing model was used to calculate fractions of CO_2-C derived from SOC (C_{SOC}) and from added glucose ($C_{glucose}$) (Phillips & Gregg, 2001; Phillips *et al.*, 2005; http://www.epa.gov/wed/pages/models/ stableIsotopes/isosource/isosource.htm). This model allows variability from mass- spectrometric measurements to be combined with that from CO_2 efflux measurements.

Primed CO_2-C (C_{primed}) was calculated as follows using the fractions of glucose-derived CO_2-C and their SDs:

$$C_{primed} = C_{total} - C_{glucose} - C_{water\ only}$$

where C_{total} is the total CO_2-C from the glucose-treated soil (data are shown in Figure S1, S3), $C_{glucose}$ is the CO_2-C derived from added glucose (data are shown in Figure S2), and $C_{water\ only}$

is the total CO_2-C from the soil with added water only. The net C balance was calculated as the difference between C_{primed} and retention of added glucose-C (data are shown in Figure S4).

Statistical analysis

ANOVAs followed by Tukey post-hoc tests were used to test for differences in soil C pool, shoot and root C pools, the ratio of root to shoot C, 10-year averages of species richness and aboveground biomass, 3-year averages of root biomass, and PLFA abundances of soil microbes among the four N addition treatments (CK, Am, Ni, and AN). We used Tukey post-hoc tests to examine differences in total cumulative CO_2-C (C_{total}), CO_2-C released from glucose ($C_{glucose}$), and primed CO_2-C (C_{primed}) among the four glucose addition rates within each of the four N addition treatments. We also applied Tukey post-hoc tests to examine differences in C_{total}, $C_{glucose}$, and C_{primed} between soils with and without glucose addition within each of the four N addition treatments. All analyses were performed using SPSS version 16.0 (SPSS Inc. Chicago, Illinois, USA).

RESULTS

Effects of N addition on soil, shoot and root C pools

Long-term N addition in different chemical forms significantly affected the SOC pool at 0-20 cm depth (Figure 2a). Specifically, the SOC pool was 16% and 7% higher in Am than in Ni and AN, respectively (Figure 2a). Moreover, the shoot C pool was 41% higher in Am than in CK, and the root C pool was 59% and 47% higher in Am than in CK and Ni, respectively (Figure 2b, c). As a consequence, the fraction of the root C pool to the total plant C pool was 13% higher in Am than the average value for Ni, AN and control (Figure 2d).

Effect of N addition on biomass and richness of plant communities

Aboveground biomass of the community was 25% higher in AM than in CK, but it did not differ significantly between Am, Ni and AN or between CK, Ni and AN (Figure 3a). Root biomass was 25% and 40% higher in AM than in CK and Ni, respectively, but it did not differ between Ni, AN or CK (Figure 3a). Compared to CK, Am, Ni and AN treatments all significantly increased aboveground biomass of graminoids, but decreased that of legumes (Figure 3b, c). Aboveground biomass of graminoids was also significantly higher in Am than in Ni, but did not differ between Am and AN or between Ni and AN (Figure 3b). Aboveground biomass of legumes did not differ significantly among Am, Ni and AN (Figure 3c). Aboveground biomass of forbs was marginally higher in Ni than in the other three treatments ($P = 0.066$; Fig. 3d).

Species richness of both the community and forbs was significantly higher in CK than in Ni and AN, but did not differ between CK and Am or between Am, Ni andure (Figure 3e, h). Species richness of graminoids was higher in Am than in Ni, but did not differ between CK, Am and AN or between Am and AN (Figure 3f). Richness of legumes was significantly higher in CK than in Am, Ni and AN, but did not differ among Am, Ni and AN (Figure 3g).

Effects of N addition on soil microbe abundance

Nitrogen addition did not affect the PLFA abundance of gram-positive bacteria (Figure 4a). The PLFA abundance of gram-negative bacteria was significantly greater in Ni than in Am (Figure 4b). The PLFA abundance of both actinomycetes and total bacteria was significantly higher in Ni than in CK, Am and AN (Figure 4c, d), and the PLFA abundance of fungi was significantly lower in Am than in CK and AN (Figure 4e).

Effects of glucose rate on primed CO_2-C in soils experienced N addition treatments

Soils that had experienced N-addition treatments had a marked effect on the priming of SOC (Figure 5). For the soil from Am, primed CO_2-C (C_{primed}) was negative at all three rates of glucose addition (0.3, 0.5, and 0.8 mg glucose-C g^{-1} soil) during the entire incubation (Figure 5b), but for the soil from Ni and AN, C_{primed} was always positive (Figure 5c, d). With regard to the soil from CK, at the highest rate of glucose addition (0.8 mg glucose-C g^{-1} soil) C_{primed} was positive and increased rapidly during the entire incubation, but at the two lower rates (0.3 and 0.5 mg glucose-C g^{-1} soil) it was slightly negative at days 1 to 3 and then increased slowly to become positive during the rest of the incubation (Figure 5a).

The glucose addition rate significantly affected C_{primed} and this effect also varied with the soils used (Figure 5). C_{primed} increased with increasing rate of glucose addition for the soils from CK and AN (Figure 5a, d), and was much higher at the highest rate of glucose addition than at the two lower rates for the soil from AN (Figure 5d). For the soil from Am, C_{primed} was the highest at 0.5 mg glucose-C g^{-1} soil, lowest at 0.3 mg and intermediate at 0.8 mg at days 1 to 5, but it was the highest at 0.3 mg glucose-C g^{-1} soil, lowest at 0.8 mg and intermediate at 0.5 mg during the rest of the incubation; overall, the effect of the glucose addition rate was smaller for the soil from Am than for soils from the other three treatments (Figure 5b). For the soil from Ni, C_{primed} was higher at the low glucose addition rate, but it increased rapidly at all rates of glucose addition during the incubation (Figure 5c).

Effects of N-mediated changes in soil microbes on primed CO_2-C

N-mediated changes in soil microbial abundance and composition had a great effect on the priming of SOC (Figure 6). C_{primed} was always negative and also decreased with the incubation time in the reference soils inoculated with microbes extracted from the soils from the long-term CK and Am treatments (Figure 6). In the reference soil inoculated with microbes extracted from the soil from Ni, C_{primed} was positive and kept stable during the

incubation with the exception of a negative value on the first day (Figure 6). In the reference soil inoculated with microbes extracted from the soil from AN, C_{primed} was initially negative, but it increased rapidly and turned to positive later during the incubation (Figure 6).

DISCUSSION

Ten years of N addition in different chemical forms had clear effects on the soil organic carbon (SOC) sequestration in this alpine grassland ecosystem. Interestingly, opposite directions and different extents of SOC variation were observed through priming effects in soils that had experienced N addition in different chemical forms. By considering the effects of N-form-mediated variation in microbial composition and activity on the priming of SOC decomposition, we also provided evidence for the relative importance of microbe-driven mechanisms underpinning the long-term effect of N addition on SOC.

Input of labile organic carbon through root exudation and litter decomposition can greatly enhance native SOC decomposition via positive priming effects (Kuzyakov et al., 2007; Fontaine et al., 2007; Blagodatskaya & Kuzyakov, 2008). Interestingly, we observed both positive and negative priming effects in the whole soils with different legacies from long-term addition of N in different chemical forms, and these effects corresponded to the directions of the changes in SOC pools in the field plots. Negative priming effect in the whole soil with a legacy of ammonium addition (Figure 5b) indicate that some added glucose carbon had accumulated into the SOC pool (Figure S4). In contrast, positive priming effects were observed in the whole soil with a legacy of nitrate addition (Figure 5c), suggesting that labile organic carbon input stimulated decomposition of extant SOC. These results are consistent with the finding that the SOC pool was higher in the plots with ammonium addition than in the plots with nitrate addition (Figure 2a), suggesting that differences in the direction and the extent of priming effects can partly explain the effect of long-term N addition with different chemical forms on SOC dynamics in the alpine meadow.

The direction and the extent of the priming effects are proportional to the amount of labile organic carbon that plants return to soil as substrates (Chapin 2003; Lavorel et al., 2007; Paterson & Sim, 2013; Qiao et al., 2014). The abundance and species richness of graminoids were significantly greater in plots with ammonium addition than in plots with nitrate addition (Figure 3b, f), and also graminoids contributed to the larger (74%) aboveground biomass of the whole plant community (Figure 3b; Figure S6). It is reasonable to assume that the amount of labile organic carbon input into the soil that was associated with the N-mediated increase in the graminoid abundance, may partly contribute to the enhancement of SOC storage in this alpine meadow (Figure S1 and S2; Hafner et al., 2012). Thus, one explanation is that the N-form-mediated change in the direction and extent of the priming of SOC is related to the N-form-mediated change in plant species composition, especially the change towards the dominance of graminoids (Cornelissen et al., 2007; Cornwell et al., 2008). These results suggest that N-form-mediated shifts in plant species abundance could be one of the important mechanisms underlying the opposite effects of N addition on the decomposition of SOC through priming. One caveat is that we only tested the quantity of labile organic carbon input, but did not consider the quality of the plant carbon source, which could also have an impact on the priming of SOC. Therefore, the next critical step is to examine impacts of the quality of labile organic carbon on the priming of SOC.

Soil microbes are another driver of SOC dynamics (Allison et al*.,* 2008; Campbell et al., 2010; Ramirez et al., 2012), and the direction and the extent of the priming of SOC may also be closely related to soil microbial composition and abundance (Blagodatskaya & Kuzyakov, 2008; Kuzyakov, 2010). Because long-term N addition may greatly alter the abundance and composition of soil microbes due to e.g. soil acidification (Treseder, 2008; Campbell et al., 2010), the N-form-mediated differences in the priming of SOC detected in the whole-soil incubation experiment could also be due to N-form-mediated differences in soil microbial abundance and composition. The results from our reference-soil incubation experiment showed that the reference soil inoculated with microbes extracted from the soil from the

ammonium addition treatment generated a negative priming effect on SOC decomposition, whereas the reference soil inoculated with microbes extracted from the soil from the nitrate addition treatment produced a positive priming effect (Figure 6; Figure S3). Meanwhile, we observed that N forms could alter soil microbial abundance and composition (Figure 4; Figure S5). In particular, the abundances of gram-negative bacteria, total bacteria and actinomycetes were significantly lower and that of fungi apparently lower in the ammonium addition treatment than in the nitrate addition treatment (Figure 4b-d). Changes in soil microbial community composition may partly be due to the change in soil acidity caused by long-term N addition rate, i.e. ammonium and nitrate reduced pH (7.01 and 7.63 in ammonium and nitrate addition soil, respectively) as compared with the pH of the control (8.10) in our study. Such N-mediated changes in soil microbial abundance and composition can lead to alterations in functional properties of soil microbes and thus changes in their capacity of decomposing different fractions of SOC, as certain classes of enzymes are produced by specific groups of soil microbes (Cotrufo et al., 2013; Koranda et al., 2013). Bacteria and fungi play different roles in nutrient cycling due to their different inherent stoichiometries, especially tissue C:N (De Deyn et al., 2008). An increase of fungal dominance in soils is typically associated with a high organic matter content and low substrate quality (high C:N) (Bardgett et al., 2005; van der Heijden et al., 2008). A decrease in soil fungal abundance may be one important reason for the increase in SOC sequestration in the ammonium addition treatment. In contrast, an increase in bacterial abundance is often associated with relatively fast nutrient cycling (De Deyn et al., 2008). We found consistent positive and significantly higher $\delta^{15}N$ in plant aboveground tissues in the nitrate addition treatment (Figure S7), indicating that long-term nitrate addition might accelerate nutrient cycling and soil C and N release, i.e. resulting in more “open” soil C and N cycles.

Soil microbial activity could also influence the extent of the priming of SOC. As an important energy source for soil microbes, increasing labile organic carbon supply usually stimulates soil microbial activity and thereby enhances the priming effect and accelerates the

decomposition of extant SOC (Kuzyakov, 2010; Cheng et al., 2014). However, a very high abundance of easily decomposable, labile organic matter would generate a negative priming effect and promote SOC storage when the activity and growth of soil microbes are not limited by nutrition (Craine et al., 2007). In our study, although increasing glucose addition rates generally increased CO_2-C released from glucose, the cumulative CO_2-C release and the CO_2-C release from glucose from soils in the nitrate, ammonium and ammonium nitrate addition treatments were lower than or similar to those in the control (Figure S1, S2, and S4). We presume that soil microbial activity could be inhibited by certain N forms, such as that in the ammonium addition treatment, either through N-mediated declines in soil microbial abundance or through N-mediated shifts in the composition of soil microbial functional groups. For instance, an incubation experiment on 28 different types of soils showed that N addition depressed soil microbial activity by shifting the metabolic capacities of soil bacteria, resulting in communities that were less capable of decomposing more recalcitrant soil organic carbon and thus leading to a potential increase in soil carbon sequestration rates (Ramirez et al., 2012). However, we lack knowledge on how activities of different microbial functional groups respond to the increased availability of N with different chemical forms. More attention should be paid to N-mediated changes in both composition and activity of soil microbes in future studies. Moreover, we are not sure whether the N effect on the microbial community was indirectly mediated by plant community composition. Furthermore, we still know little about the mechanisms underlying the role of soil microbes in regulating the priming of SOC. Ideally experimental testing by manipulating the abundance of N-sensitive species would be needed to properly evaluate their effects.

While we have provided likely mechanisms underlying N-mediated priming effects on SOC sequestration associated with changes in plant and soil microbial composition, some other mechanisms could also play important roles. For instance, soil acidification caused by N addition may also influence DOC production (Monteith et al., 2007; Evans et al. 2008), although we did not observe a significant difference in DOC concentrations among

treatments (ranging from 0.42 mg g^{-1} in the nitrate addition treatment to 0.48 mg g^{-1} in the control). In addition, N form-mediated plant carbon input could have contributed to the differences in SOC storage. For example, shoot and root biomass in the ammonium addition treatment were higher than those in the control or the nitrate addition treatment (Figure 3a). The higher root biomass, especially that of graminoids, might have contributed greatly to the increase in the soil C storage in the ammonium addition treatment.

In conclusion, ten years of addition of different N chemical forms resulted in differences in the accumulation of SOC, and the significant negative and positive priming effects provide a mechanical explanation for the SOC dynamics. The N-form-mediated changes in the abundances of graminoids and soil microbes could be important drivers regulating the direction and extent of the priming effects on SOC decomposition. Our long-term study provides one case, from an alpine meadow biome, to explain how alteration of vegetation composition will affect soil carbon sequestration under global changes. More relevant studies from other biomes across the world are necessary in this field, with a special focus on different direct and indirect drivers of SOC priming, SOC formation and stability under different nutrient availability regimes in terms of both N amount and N chemical form.

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TABLE 1 Characteristics of the upper 10 cm of soils in the experimental site in the alpine meadow on the Tibetan Plateau. Data (means ± SE) are shown (n = 6 - 8). Data were collected in 2004 (Song *et al.*, 2007).

pH	8.00 ± 0.10
Bulk density (g cm^{-3})	0.70 ± 0.05
C:N ratio	19.60 ± 0.30
SOC (kg m^{-2})	11.80 ± 0.30
Total soil N (kg m^{-2})	0.60 ± 0.04
Microbial biomass N (g m^{-2})	6.50 ± 0.30
DON (g m^{-2})	1.80 ± 0.10
NH_4-N (g m^{-2})	2.71 ± 0.23
NO_3-N (g m^{-2})	0.50 ± 0.02

LEGENDS

FIGURE 1 Conceptual framework describing one of the mechanisms underlying responses of soil organic carbon (SOC) pool to N enrichment. N-mediated increases in productivity and shifts in species composition influence priming effects on SOC decomposition via the amount of labile organic carbon input. Such changes in carbon source associated with N-mediated changes in soil microbial composition alter the direction and extent of the priming effects. The grey arrow indicates the potential effect of plant species composition on soil microbial composition.

FIGURE 2 Effects of long-term N addition in different chemical forms on (a) soil C pool, (b) root C pool, (c) shoot C pool, and (d) fraction of root C pool to total plant C pool. Means ± SE are given. Bars sharing the same letters are not different at $P = 0.05$ (by Tukey test). CK, Am, Ni and AN represent no N addition and addition of 7.5 g NH_4-N, NO_3-N, and NH_4NO_3-N m^{-2} yr^{-1}, respectively.

FIGURE 3 Effects of long-term N addition in different chemical forms on 10-year average of shoot biomass (a-d) and species richness (e-h) of the community and of each of the three functional groups. Root biomass (panel a) are averaged values in three years of 2007, 2012, and 2014 during the N addition treatment. Means ± SE are given. Bars sharing the same letters are not different at $P = 0.05$ (by Tukey test). CK, Am, Ni and AN represent no N addition and addition of 7.5 g NH_4-N, NO_3-N, and NH_4NO_3-N m^{-2} yr^{-1}, respectively.

FIGURE 4 Effects of long-term N addition in different chemical forms on PLFA abundances of soil microbial groups. Means ± SE are given. Bars sharing the same letters are not different at $P = 0.05$ (by Tukey test). CK, Am, Ni and AN represent no N addition and addition of 7.5 g NH_4-N, NO_3-N, and NH_4NO_3-N m^{-2} yr^{-1}, respectively.

FIGURE 5 Primed cumulative CO_2-C efflux traced by ^{13}C-glucose during the 28-day incubation from soils that had experienced 10-year N addition treatments. Control, Ammonium-N, Nitrate-N and Ammonium-Nitrate-N represent no N addition and addition of 7.5 g NH_4-N, NO_3-N, and NH_4NO_3-N m^{-2} yr^{-1}, respectively.

FIGURE 6 Primed CO_2-C efflux during the 28-day incubation traced by glucose-^{13}C in sterilized alpine meadow soils inoculated with soil microbes extracted from soils that had experienced 10-year N addition treatments. Control, Ammonium-N, Nitrate-N and Ammonium-Nitrate-N represent no N addition and addition of 7.5 g NH_4-N, NO_3-N, and NH_4NO_3-N m^{-2} yr^{-1}, respectively.