Chronic nitrogen addition differentially affects gross nitrogen transformations in alpine and temperate grassland soils

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ABSTRACT

Nitrogen (N) deposition can profoundly alter soil N cycling of grassland ecosystems. Substrates and soil acidification are expected to modify soil N transformations in response to elevated N deposition. Here, we carried out ^{15}N tracing studies to test the effects of N addition rates (low: 30 kg N ha^{-1} and high: 90/120 kg N ha^{-1}) and soil acidification on gross N transformation rates using two typical Chinese grassland soils, an alpine calcareous soil and a temperate neutral soil. We found that N addition significantly increased the ratio of gross nitrification rate to gross ammonia immobilization rate (N/I) in both soils, but gross N transformation rates changed differently as a function of N addition rates and soil types. In the calcareous soil, N addition increased soil gross N transformations, largely due to mineral N substrates, SOC, TN and fungal dominance. In contrast, low N addition did not affect gross N transformation rates in the neutral soil, but high N addition significantly decreased gross N transformation rates. Although both SOC and TN were increased with N addition in the neutral soil, N-induced soil pH decline decreased gross N transformation rates. Our results indicate that the effects of N addition on grassland soil gross N transformations are highly dependent on mineral N substrates, SOC and TN. Soil acidification played a more important role than SOC and TN in gross N transformation rate changes in response to elevated N deposition. These findings suggest that the different changes of gross N transformation rates in response to N deposition and soil properties (e.g. SOC, TN and soil pH) should be integrated into biogeochemical models to better predict grassland ecosystem N cycling in the future scenarios of N deposition.

Keywords: ^{15}N tracing technology, Gross N transformations, Nitrogen deposition, Soil acidification, Grassland

1. Introduction

Nitrogen (N) is a limiting factor for the growth of organisms in grassland ecosystems (LeBauer and Treseder, 2008; Fujimaki et al., 2009). With the rapid increase in anthropogenic reactive N emissions, elevated N deposition on a global scale may mitigate N-limitation of plant growth but may also cause environmental pollution, threatening ecosystem functions and services worldwide (Galloway et al., 2008; Yang et al., 2012; Kanter and Searchinger, 2018).

Nitrogen mineralization-immobilization turnover (MIT) governs soil N availability for organisms' growth and N losses providing an important grassland ecosystem function (Ledgard et al., 1998; Huygens et al., 2007). Many N transformation processes, such as mineralization, nitrification and immobilization of NH_4^+ and NO_3^- operate simultaneously, and, thus, are jointly contributing to the fate of N in soil. Among those processes, mineralization is a major source of N supply for plant growth. Mineral N immobilization is the prime mechanism of N retention in soil. Nitrification, including autotrophic and heterotrophic nitrification, is a main mechanism to transform N in the reduced state (e.g. organic N, NH_4^+) to N in an oxidized state (e.g. NO_3^-) which is easily lost to water and air via leaching and denitrification (Schimel and Bennett, 2004; Zhang et al., 2018a; Zhang et al., 2018b). Other N transformation processes, chiefly related to N_2O production, include: denitrification, nitrifier-denitrification, coupled nitrification-denitrification and dissimilatory nitrate reduction to ammonia (DNRA).

Many previous studies have identified the impacts of N addition on N transformation rates, but the results were inconclusive (e.g. Han et al., 2011; Lu et al.,

2011; Müller et al., 2011). Some research has shown that long-term N addition significantly increased N mineralization and nitrification (Zhang et al., 2012; Wang et al., 2015a), but had no effects on gross N transformation rates in an acid forest soil (Kwak et al., 2018). Excess N addition decreased gross N transformation rates (Barraclough and Smith, 1987). As microbially mediated processes, soil N transformations are expected to be strongly regulated by soil pH. Significant soil acidification has been widely observed across forest and grassland ecosystems, partially or largely induced by N deposition (Van Breemen et al., 1982; Yang et al., 2012). In addition, N transformations are also affected by other soil properties, including soil temperature and moisture, soil organic carbon, total N, C/N ratio (Gibbs and Barraclough, 1998), and microbial community (Booth et al., 2005; Wang et al., 2015a). Hence, the impacts of N addition on N transformations are expected to be strongly related to the changes in soil physical, chemical, and biological properties (Lu et al., 2011).

In view of the variability in observed responses of soil processes to N addition, a better process-based understanding of the response of soil N transformations to N addition in grassland ecosystems is critical. Although the responses of soil N cycle to N deposition/addition have been extensively investigated, the impacts of chronic N addition on N transformations and their potential underlying mechanisms under different N addition levels and across different grassland ecosystems with various soil properties still needs clarifying.

We hypothesize that chronic N addition will increase soil gross N transformations,

but largely as a function of N addition rates and acid neutralization capacity. Soil gross N transformation rates will be enhanced with increasing N addition rates by increasing energy and substrate sources in soil (i.e. C and N). However, N-induced soil pH decline will limit or even inhibit soil gross N transformation rates by affecting soil microbial community. We tested these hypotheses by quantifying soil gross N transformation rates in response to chronic N addition (over 10 years) at low and high rates in two typical Chinese grassland soils with different properties (especially soil pH), one calcareous alpine steppe and one neutral semiarid temperate steppe, using a ^{15}N tracing approach. The results will help understanding the grassland ecosystem N cycling under elevated atmospheric N deposition (Liu et al., 2013), and predicting soil N availability and N losses in future.

2. Materials and Methods

2.1. Soils and sampling

Topsoil of 0-20 cm was sampled from two typical northern grassland sites in China in late August, 2019. The two sites, Bayinbuluk and Duolun, represent an alpine steppe and a semiarid temperate steppe, respectively.

The Bayinbuluk site (BK) is located at the Bayinbuluk Grassland Ecosystem Research Station, Chinese Academy of Sciences, Xinjiang, China (42°53′N, 83°43′E; 2,500 m a.s.l.). Mean annual precipitation is 266 mm with 78% occurring during the growing season from May to September. Mean annual temperature is -4.8 °C. Local ambient total N deposition is 7.6 kg N ha^{-1} yr^{-1} (Xu et al., 2015). The soil is a Calcic

Kas-tanozems (FAO classification), consisting of 51.2% sand, 42.2% silt, and 6.6% clay. The original topsoil (0-10 cm) before the experiment had the following physical and chemical properties: bulk density, 1.01 g cm^{-3}; soil pH (H_2O), 7.71; soil organic carbon (SOC), 32.7 g kg^{-1}; soil total nitrogen (TN), 3.1 g kg^{-1}. Dominant plant species include *Stipa purpurea*, *Festuca ovina*, *Agropyron cristatum*, *Koeleria cristata*, *Oxytropis glabra*, *Potentilla multifida* and *Potentilla bifurca*. After the fencing to exclude grazers in 2005, N addition started in 2009, including four N addition rates of 0 (N0 as control), 10 (N10), 30 (N30) and 90 (N90) kg N ha^{-1} yr^{-1} to four replicate plots of 4 m × 8 m with a 1 m wide buffer zone between adjacent plots. Three N treatments, N0, N30 and N90, were selected for this study. Nitrogen as NH_4NO_3 was uniformly applied to the plots in two equal amounts in late May and June. More information can be found in Li et al. (2015).

The Duolun site (DL) is located at the Duolun Restoration Ecology Research Station of the Institute of Botany, Chinese Academy of Sciences, Inner Mongolia, China (42°02′N, 116°17′E; 1,324 m a.s.l.). Mean annual precipitation is 293 mm with 82% occurring during the growing season. Mean annual temperature is 3.3°C. Local ambient total N deposition is 14.7 kg N ha^{-1} yr^{-1} (Xu et al., 2015). The soil is a Haplic Calcisol (FAO classification), consisting of 62.8% sand, 20.3% silt, and 16.9% clay. The original topsoil (0-10 cm) before the experiment had the following physical and chemical properties: bulk density, 1.31 g cm^{-3}; soil pH (H_2O), 7.12; SOC, 12.3 g kg^{-1}; TN, 1.7 g kg^{-1}. Dominant plant species include *Stipa capillata*, *Agropyron cristatum*, *Cleistogenes squarrosa*, *Leymus chinensis*, *Artemisia frigida*, *Carextibetica*, *Potentilla*

acaulis, *Potentilla tanacetifolia* and *Potentilla bifurca*. The study site has been fenced since 2001 to protect it from grazing disturbance. The N addition experiment started in 2005, including six N addition rates of 0 (N0 as control), 30 (N30, since 2006), 60 (N60), 120 (N120), 240 (N240), and 480 (N480) kg N ha^{-1} yr^{-1} to five replicate plots of 5 m × 5 m with a 1 m wide buffer zone between adjacent plots. Three N treatments, i.e. N0, N30 and N120, were selected in this study. Nitrogen addition was applied as urea in 2005 and NH_4NO_3 from 2006 onward. N was uniformly applied to the plots in two equal amounts in the middle of June and July. The detailed field managements are given in Chen et al. (2019).

After manually removing the roots and impurities from the soil, collected soil samples were sieved to 2 mm and immediately stored at 4℃ until the incubation experiment began within two weeks. Parts of mixed-well soils were used for the ^{15}N dilution experiments. Another part was used to measure important soil properties, including soil pH, SOC, TN and water holding capacity (WHC). The detailed results are given in Table 1.

2.2. Laboratory ^{15}N tracing experiment

Gross N transformation rates were quantified using a ^{15}N tracing approach (Kirkham and Bartholomew, 1954; Müller et al., 2007; Zhang et al., 2012; Zhu et al., 2019). Each soil treatment was conducted in two sub-treatments, adding $^{15}NH_4NO_3$ and $NH_4{}^{15}NO_3$, respectively, with 3 replicates and 3 extraction times. Also a control (no N addition) with 3 replicates was investigated for each treatment. Sieved fresh soil,

equivalent to 20 g oven-dried soil, was added into a reagent bottle and pre-incubated under the natural water content for 24 h at 20 °C. The control soil was extracted with 2 mol L^{-1} KCl (Guaranteed Reagent) to measure soil mineral N (NH_4^+-N and NO_3^--N) concentrations (Song et al., 2011). To the other soils, 2 mL solution of $^{15}NH_4NO_3$ (^{15}N atom% excess of 9.8337) or $NH_4{}^{15}NO_3$ (^{15}N atom% excess of 9.8837) was added at a rate of 50 mg NH_4^+-N kg^{-1} or 50 mg NO_3^--N kg^{-1}, respectively (Wang et al., 2015b). The soils were adjusted to 60% WHC with high-purity water. 60% of WHC is widely used in previous studies, being an appropriate water potential to promote microbial activity under aerobic conditions (Zhang et al., 2012; Zhu et al., 2019). The bottles were sealed with parafilm® with uniformly distributed eight pinholes to allow aeration, and incubated in the dark for 48 h at 20 °C. The incubation temperature of 20 °C is close to the natural outdoor conditions in the day-time during the growing season for the two sampling sites. During the incubation, soils were extracted with 2 mol L^{-1} KCl at 0.5, 12, and 48 h after labeling to determine the concentrations and isotopic compositions of NH_4^+-N and NO_3^--N.

2.3. Analytical methods

Soil pH (H_2O) was measured with a pH meter (PHS-3C, Shanghai Yueping Scientific Instrument Co., Ltd, China) at a soil to water ratio of 1:2.5. SOC and TN were measured with an element analyzer (Vario Macro Cube, Elementar, Germany). NH_4^+-N and NO_3^--N in soils were measured with an AA3 continuous-flow analyzer (Bran + Luebbe GmbH, Norderstedt, Germany) after extraction with 2 mol L^{-1} KCl

189 solution and filtration by filter papers (Whatman, Double Ring quantitative filter paper).
190 Isotopic compositions of NH_4^+-N and NO_3^--N were determined using the modified
191 micro-diffusion method (Zhu et al., 2019) and measured using a Delta V plus isotope
192 mass spectrometer (Delta Plus XP, Thermo Finnigan, Germany). Briefly, 20 mL the
193 extract was mixed with 0.3 g MgO to release NH_3 from the NH_4^+-N pool. 0.3 g
194 Devarda's alloy was then added to the bottle to reduce NO_3^--N to NH_4^+-N, and then to
195 NH_3. Liberated NH_3 was trapped using filter paper (Whatman 41), which was acidified
196 with 1 mol L^{-1} oxalic acid. After diffusion, filters were transferred to an ammonia free
197 environment for drying, then dried filter papers were transferred to a tin capsule and
198 wrapped to enable the enrichment of ^{15}N to be analyzed.
199
200 *2.4 Soil DNA extraction, 16S/ITS rRNA sequencing and sequence analysis*
201 For each soil in BK, 0.5 g fresh mixed soil with three replicates was used to extract
202 DNA using a DNA extraction kit for soil. 1% agarose gels were used to determinate
203 DNA integrity and purity while a NanoDrop One microvolume UV-Vis
204 spectrophotometer (Thermo Scientific, USA) was used to measure DNA concentration
205 and purity (Tomaso et al., 2010). Bacterial 16S rRNA genes of V4 hypervariable regions
206 were amplified using primers 515F and 806R (Caporaso et al., 2012), and fungal ITS
207 rRNA genes of ITS1 hypervariable regions were amplified using primers ITS5-1737F
208 and ITS2-2043R with 12bp barcode (White et al., 1990; Gardes and Bruns, 1993).
209 Primers were synthesized by Invitrogen (Carlsbad, CA, USA). PCR reactions,
210 containing 25 μL 2x Premix Taq (Takara Biotechnology, Dalian Co. Ltd., China), 1 μL

each primer (10 mM) and 3 μL DNA (20 ng μL^{-1}) template in a volume of 50 μL, were amplified by thermos cycling: initialization at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s; followed by final elongation at 72°C for 10 min. 3 replicates per sample and each PCR products of the same sample were mixed, the PCR instrument was BioRad S1000 (Bio-Rad Laboratory, CA). High-throughput sequencing was performed on an IlluminaHiseq2500 platform and 250 bp paired-end reads were generated.

Paired-end clean reads were merged using FLASH (V1.2.11) (Magoč and Salzberg 2011) after the removal of low-quality sequences according to the Trimmomatic (V0.33) quality controlled process (Bolger et al., 2014). After chimera detection using the UCHIME de novo algorithm, the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity by USEARCH (Edgar, 2010). Representative sequence for each OTU was screened for further annotation. For each representative sequence, the Greengenes (for 16S) and Unite (for ITS) database were used to annotate taxonomic information in Quantitative Insights Into Microbial Ecology (QIIME V1.9.1) (DeSantis et al. 2006; Abarenkov et al., 2010; Caporaso et al., 2010). The OTU and its Tags, which belong to plantae and can't be annotated to the kingdom level, were removed. Subsequent analysis of alpha diversity, including observed species, Chao1, Shannon's index, and dominance (defined as $\sum p_i^2$, where p_i is the proportion of the community represented by OTU_i), was performed with QIIME using the normalized OTU data.

233 *2.5. ^{15}N tracing analysis*

234 Gross N transformations rates were quantified via a ^{15}N tracing analysis. Measured
235 concentrations and ^{15}N enrichment values (mean ± standard deviation) of NH_4^+-N and
236 NO_3^--N from the triplicate measurements at 0.5, 12, and 48 h after labeling in the two
237 ^{15}N-added treatments were supplied to the ^{15}N tracing model $Ntrace_{Basic}$ (Müller et al.,
238 2007). Gross N transformation rates were calculated by simultaneously optimizing the
239 kinetic parameters for each individual process by minimizing the misfit between
240 modeled and observed concentrations of NH_4^+-N and NO_3^--N and their respective ^{15}N
241 enrichments. To obtain the most appropriate model, several model modifications, which
242 vary in the number of considered N pools and processes, and kinetic settings, were
243 tested (Rütting and Müller, 2008). The most appropriate model was guided by the
244 Aikaike's information criterion (AIC), selecting the minimum AIC value (Cox et al.,
245 2006; Rütting and Müller, 2007; Wang et al., 2016). In addition, the determination
246 coefficient (R^2) was also used to verify the model; if $R^2 > 0.80$, the modeled result can
247 be accepted (Quinn and Keough, 2002). The numerical optimization model based on
248 Markov Chain Monte Carlo Metropolis algorithm, can provide reliable results for a
249 large number of parameters. For more detailed information on the model development
250 and parameter optimization see Müller et al. (2007). Ten simultaneously occurring
251 transformation processes were quantified (Müller et al., 2007; McGeough et al., 2016):
252 1) M_{Nrec}, mineralization of recalcitrant organic N to NH_4^+-N; 2) $M_{Nlab,}$ mineralization
253 of labile organic N to NH_4^+-N; 3) I_{NH4_Nrec}, immobilization of NH_4^+-N to recalcitrant
254 organic N; 4) I_{NH4_Nlab}, immobilization of NH_4^+-N to labile organic N; 5) R_{NH4}, release

of adsorbed NH_4^+-N; 6) A_{NH4}, absorption of NH_4^+-N on cation exchange sites; 7) O_{NH4}, oxidation of NH_4^+-N to NO_3^--N; 8) O_{Nrec}, oxidation of recalcitrant organic N to NO_3^--N; 9) I_{NO3}, immobilization of NO_3^--N to recalcitrant organic N; and 10) D_{NO3}, dissimilatory NO_3^--N reduction to NH_4^+-N. Gross transformation rates were calculated by zero or first order kinetics. In this model, labile organic N was assumed to be the maro-organic matter fraction accounting for ca. 1% of total organic N (Rütting and Müller, 2008; Chen et al., 2015). The optimization procedure resulted in a probability density function for each parameter, from which averages and standard deviations were calculated (Müller et al., 2007). Each analysis run was carried out with three parallel sequences. Based on the kinetic setting and the final parameters, average N transformation rates were calculated over the 48 h incubation period and expressed in units of mg N kg^{-1} dry soil d^{-1} (Table 2).

2.6. Statistical analyses

One-way analysis of variance (ANOVA) with a Tukey's Honestly Significance Difference (HSD) test ($P < 0.05$) was used to test significant differences between treatments in soil pH, SOC, TN and bacterial/fungal diversity indices. Most statistical tests are inappropriate for the comparison of gross N transformation rates above, because of the large number of iterations of the ^{15}N tracing model. Parameter results based on the comparisons of standard deviations and the 95% and 99% confidence intervals were used to test significant differences between treatments in gross N transformation rates (Müller et al., 2011). If 95% confidence intervals of parameter

277 results overlap, the parameters are not significantly different. If 95% or 99% confidence
278 intervals do not overlap, the differences in results are significant or highly significant.
279 Correlation and linear or nonlinear regression analyses were used to test relationships
280 between N input and N gross transformation rates. All the statistical analyses and
281 correlation analyses were performed using IBM SPSS Version 23.0 (IBM Corp.,
282 Armonk, NY, USA).
283
284 **3. Results**
285 *3.1. Soil chemical and biological properties*
286 Compared to the control plots, long-term N addition (> 10 years) did not
287 significantly change topsoil pH in N treated plots at BK site, but significantly decreased
288 topsoil pH by 0.22 and 0.83 for N30 and N120 treatments, respectively, at DL site
289 (Table 1). Nitrogen addition also significantly increased SOC, TN and soil mineral N
290 (N_{min}, mainly NO_3^--N) at both sites, while C/N and WHC did not significantly change.
291 For soil N_{min} at both sites and soil pH at DL site, the impacts of N addition increased
292 with increasing N addition rates. At BK site, N addition significantly increased bacterial
293 observed species, bacterial Shannon's diversity index and fungal dominance, while
294 decreasing bacterial dominance and fungal Shannon's diversity index (Fig. S1).
295
296 *3.2. The concentration and ^{15}N abundance of NH_4^+-N and NO_3^--N*
297 During the incubation period, the concentrations of NH_4^+-N decreased with
298 increasing incubation time for all treatments, with low and high decline rates being

related to DL and BK (Fig. 1a). Decline rates of NH_4^+-N concentrations were similar among all three treatments at DL site, being higher in N120 treatment than other two treatments at BK site. The concentrations of NO_3^--N increased with incubation time. Net NO_3^--N production rates had a similar trend to net NH_4^+-N consumption (NH_4^+-N decline) rates among three treatments at two sites (Fig. 1b).

^{15}N abundances of NH_4^+-N in the $^{15}NH_4^+$ labeled treatments decreased with incubation time. The decline was steeper with increasing N addition rates especially for the BK soils while only a slight decline was observed in the DL soils (Fig. 1c). Meanwhile, ^{15}N abundances of NO_3^--N in the $^{15}NH_4^+$ labeled treatments increased at BK sites and slightly increased at DL sites. ^{15}N abundances of NH_4^+-N changed slightly in the $^{15}NO_3^-$-N labeled treatments (Fig. 1d), while ^{15}N abundances of NO_3^--N decreased with incubation time at a low and similar rates in the three DL treatments and a higher and N-dependent increase at BK sites.

3.3. Gross N transformation rates in response to N addition

Gross N transformation rates were associated with N addition rates (Fig. S2), but showed different responses at the two sites (Table 2 and Fig. 2). Many common results of N transformations were observed at two sites, i.e. autotrophic nitrification (O_{NH4}), rather than heterotrophic nitrification (O_{Nrec}). Gross rates of oxidation of recalcitrant organic N to NO_3^--N (O_{Nrec}), dissimilatory NO_3^--N reduction to NH_4^+-N (D_{NO3}), release of adsorbed NH_4^+-N (R_{NH4ads}) and absorption of NH_4^+-N on cation exchange sites (A_{NH4}), were negligible in all treatments at both sites.

There were also many different impacts of N addition on specific N transformation processes between two sites. At the DL site, gross rates of all N transformation processes slightly fluctuated in N treated plots at 30 kg N ha^{-1} yr^{-1} compared to the control plots. However, N addition of 120 kg N ha^{-1} yr^{-1} led to a significant decline of 20%, 88% and 78% for the oxidation rate of NH_4^+-N to NO_3^--N (O_{NH4}), the immobilization rates of NH_4^+-N to labile (I_{NH4_Nlab}) and recalcitrant organic N (I_{NH4_Nrec}), respectively. However, no significant difference was observed between two N treatments (i.e. N30 and N120). Further, compared to the control plots, the ratio of gross nitrification rates to gross immobilization rates of NH_4^+-N (N/I) and net NH_4^+-N production rate increased by 140% and 63%, 352% and 77% for N30 and N120 treated plots, respectively. Meanwhile, total N_{min} activity and net NO_3^--N production rate decreased by 42% and 13%, 62% and 21% for N30 and N120 treatments, respectively.

For the BK site, compared to the control plots, N addition led to a significant increase in autotrophic nitrification (O_{NH4}) of 66% and 149% for N30 and N90 treated plots, respectively. N addition of 90 kg N ha^{-1} yr^{-1} led to a significant decline of 93% in the mineralization rate of recalcitrant organic N to NH_4^+-N (M_{Nrec}), and an increase of 129% and 256% in the mineralization rate (M_{Nlab}) and the immobilization rate of NH_4^+-N to recalcitrant organic N (I_{NH4_Nrec}), respectively. Consequently, compared to the control plots, N addition increased N/I, total N_{min} activity and net NO_3^--N production rate by 37%, 31% and 80% for N30 treatments and by 50%, 81% and 166% for N90 treatments, respectively. Meanwhile, net NH_4^+-N production rates decreased by 69% and 168% for N30 and N90 treatments, respectively, compared to the control plots. In

addition, gross rates of mineralization and nitrification were negatively or positively correlated with bacterial or fungal diversity indices (dominance, Shannon's index and observed species), respectively (Fig. S3).

4. Discussion

Long-term N addition experiments have been widely conducted to accurately identify the impacts of N deposition on plant species diversity, community stability, ecosystem multifunctionality as well as biogeochemistry (Ledgard et al., 1998; Tilman et al., 2001; Isbell et al., 2013). Soils with chronic N addition, combined with quantification of gross N transformation rates, could provide reliable evidence to reveal the underlying mechanisms of soil N cycling in response to elevated N deposition concerning N availability and N losses (Zhang et al., 2012). Our results showed that chronic N addition significantly changed gross N transformation rates, but the impacts were largely dependent on N addition rates and soil properties. Generally, N mineralization, immobilization and nitrification were the dominant N transformation process while other processes were negligible, as observed in other soils (Chen et al., 2016; Zhu et al., 2019). Autotrophic nitrification dominated over heterotrophic nitrification, which is in line with previous studies (Zhang et al., 2013a; Zhu et al., 2019). However, heterotrophic nitrification may play a significant role in acid soils (Zhang et al., 2011). NH_4^+-N immobilization rather than NO_3^--N immobilization dominated N retention. NO_3^--N immobilization needs more energy than NH_4^+-N immobilization and is highly related to soil organic carbon, C/N-ratio and ATP

365 production (Bengtsson et al., 2003; Habteselassie et al., 2006) and possibly suppressed
366 by NH_4^+-N, even at relatively a low concentrations (Templer et al., 2008).
367 Consequently, the low C/N ratios (approximately 10-11) may have limited NO_3^--N
368 immobilization in this study. In addition, N addition also increased the risk of N losses
369 (N/I), especially for temperate grassland soils (DL) with an obvious shift from N
370 limitation (N/I < 1.0) to N saturation (N/I > 1.0) as N addition are increasing.

371 The impacts of N addition on some specific gross N transformation rates were
372 different between BK alpine steppe and DL temperate steppe. Enhanced N deposition
373 has been confirmed to affect soil N transformations by changing soil N availability, pH,
374 microbial biomass and microbial community composition (Liu et al., 2011). Increased
375 mineral N substrates with enhanced N deposition can promote N mineralization and
376 nitrification (Niu et al., 2016). Meanwhile, N-induced soil acidification could limit N
377 transformation processes by affecting microbial activities and N form as substrates
378 (Pietri and Brookes, 2008; Cheng et al., 2013). Although meta-analysis showed that N
379 deposition significantly increased N mineralization by 24.9% and nitrification by 153.9%
380 (Lu et al., 2011), many variable results of impacts of N deposition on soil N
381 transformations have been reported in individual studies, likely derived from the
382 differences in local climatic conditions and soil properties. Thus, some important soil
383 properties, strongly related to N transformation processes and largely affected by N
384 addition, likely explain the different impacts of N addition on N transformation rates in
385 these two grassland soils (Tables 1 and 2).

386

4.1. The comparison of N transformation rates between the calcareous and neutral soils under natural conditions

Although the two studied soils had comparable total N_{min} activities of 13.1 and 12.4 mg N kg^{-1} d^{-1} for DL and BK sites, respectively, the gross rates of some specific transformation processes were partially or completely different. Compared to DL neutral temperate grassland soil, BK calcareous alpine grassland soil had higher gross rates of mineralization (both M_{Nrec} *and* M_{Nlab}*)*, O_{NH4} and I_{NO3} and a lower gross rate of $I_{NH4\text{-}Nrec}$. High soil organic carbon (SOC) and total nitrogen (TN) in the calcareous soil can supply more energy and substrates to soil microorganism growth, favoring N transformations (Booth et al., 2005; Grosso et al., 2016). As a result of the high gross nitrification rate, soil mineral N was dominated by NO_3^--N in the calcareous soil, which was equally contributed by NH_4^+-N and NO_3^--N in the neutral soil (Table 1). Further, low NH_4^+-N in substrates and high accumulated NO_3^--N limited the immobilization of NH_4^+-N and increased the immobilization and net production of NO_3^--N in the calcareous soil. In addition, high SOC in the calcareous soil also favored NO_3^--N immobilization, needing more energy than NH_4^+-N immobilization (Bengtsson et al., 2003).

4.2. The effects of N addition on N transformations in calcareous alpine grassland soils

In BK alpine grassland soils, chronic N addition increased the gross rates of three important N transformation processes, i.e. M_{Nlab}, $I_{NH4\text{-}Nrec}$ and O_{NH4}, especially with increasing N addition rates. Increased SOC (by 9.6-12.6%) and TN (by 13.4-18.4%)

409 caused by N addition were the potential main contributors to increased N
410 transformation rates. SOC, TN and their quality (C/N ratio) have been identified to play
411 important roles in N transformations (Aber et al., 2003; Niu et al., 2016), particularly
412 N mineralization (Wang et al., 2016; Zhang et al., 2016). SOC is the energy and
413 substrate source to support microbial growth and activity (Kreitinger et al., 1985;
414 Grosso et al., 2016). Thus, close relationships between SOC and N transformations
415 were widely observed in previous studies, including microbial immobilization of
416 mineral N (Bradley, 2001; Zhang et al., 2013b), heterotrophic nitrification and
417 consequent NO_3^--N production (Hart et al., 1994; Chen et al., 2015). Increased TN also
418 favors soil N transformations by providing more N substrates (Booth et al., 2005; Zhang
419 et al., 2016). Increased N availability mitigates the limit of N on microorganism, further
420 increasing gross N mineralization rates in an N-limited ecosystem (Blaško et al., 2013;
421 Högberg et al., 2014). Studied alpine and temperate grassland ecosystems appear to be
422 N-limited (Li et al., 2015; Hao et al., 2018). Regarding C/N ratio, it is an important
423 indicator reflecting C or N limited microbial growth which in turn is controlled by the
424 mineralization-immobilization turnover (MIT). Organic substrates with high C/N ratios
425 are generally associated with turnover of recalcitrant compounds in soil organic matter,
426 which negatively influences N mineralization (Booth et al., 2005; Zhang et al., 2016).
427 Microbes immobilize mineral N to meet their N requirement when they decompose
428 organic matter with high C/N ratios (Sollins et al., 1984; Janssen, 1996; Zak et al., 2006).
429 However, C/N ratios were not changed by N addition in this study and remained around
430 10, much lower than the critical value C/N ratio of ~25 for switching between N

mineralization and immobilization processes, thus, falling into the range of C-limited microbial growth (Arunachalam et al., 1998; Zhu et al., 2015).

In addition, although soil pH plays a critical role in N transformation by affecting microbial activities and mineral N forms (especially for NH_x) (Pietri and Brookes, 2008; Cheng et al., 2013), the studied alpine grassland soils are calcareous, with a strong acid-buffering capacity. Soil pH hardly changed with N addition, being similar in the control soils even after 11 years of 90 kg N ha^{-1} yr^{-1} additions. Hence, the increased availability of SOC and TN, rather than stable C/N and soil pH, was the prime mechanism of the varying N transformation rates in response to N addition in the specific soils.

Both M_{Nlab} and $I_{NH4\text{-}Nrec}$ increased with N addition while M_{Nrec} decreased by 44-94% and total gross mineralization (M_{Nlab} + M_{Nrec}) slightly changed. Other studies showed similar results after fertilizer applications (Müller et al., 2011) or elevated CO_2 (Chen et al., 2016). Nitrogen addition may stimulate microbial growth by alleviating N limitation and/or C limitation by promoting plant growth and consequent litter and root decomposition. In the studied soil, N addition led to a slight increase in bacterial Shannon diversity and a decline in bacterial dominance. In contrast, fungal dominance was increased with N addition, suggesting some specific fungi became more prominent. Consequently, fungi-dominated immobilization into the recalcitrant organic N pool (I_{NH4_Nrec}) also significantly increased. Fungi, e.g. arbuscular mycorrhizal fungus, play an important role in the nitrogen cycle (Hodge et al., 2001; Veresoglou et al., 2012). Generally, bacteria prefer NH_4^+-N as N source (Jansson et al., 1955) but fungi may prefer NO_3^--N (Marzluf, 1997). NO_3^--N is the main form of N in soil at BK for the

persistent drought (Table 1) and high gross nitrification rate (Table 2). Those results showed that various soil organic N pools, with different turnover times, responded differently to N addition in calcareous grassland soils, i.e. feeding on labile organic N and storing as recalcitrant organic N. The observation provides compelling evidence that N addition interacted with the microbial community structure in the soil MIT and can stimulate transformations associated with SOC turnover, being an important part of the underlying mechanism of soil N retention with N addition in calcareous alpine grassland soils.

4.3. The effects of N addition on N transformations in neutral temperate grassland soils

With long-term N addition, SOC and TN significantly increased while C/N ratios slightly changed around 11 in DL temperate grassland soils. However, N addition did not significantly increase gross rates of N transformations, even leading to a significant decrease in autotrophic nitrification (O_{NH4}) and N immobilization to both labile and recalcitrant organic N (I_{NH4_Nlab} and I_{NH4_Nrec}) at a high N addition rate of 120 kg N ha^{-1} yr^{-1}. Meanwhile, many negative relationships between N addition rates and gross N transformation rates were observed (Fig. S2). According to the key role of soil pH in soil N cycling and neutral soils are generally sensitive to acid input compared to calcareous soils (Ulrich, 1986), N-induced soil acidification was a considerable factor in the changes of N transformations in response to N addition in the specific acid-sensitive soils. A significant decline in soil pH was observed with chronic N addition in DL neutral temperate grassland soils, even at a low N addition rate of 30 kg N ha^{-1}

475 yr^{-1}, which is close to the national average N deposition (Liu et al., 2013). Although N
476 mineralization can provide pH-favorable microsites for nitrification, significant soil pH
477 decline favors soil NH_x as NH_4^+ rather than NH_3, where NH_3 is the substrate to
478 ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). Soil pH is
479 a critical factor to AOB, the abundance and activity of AOB decreased at a low soil pH
480 and consequent nitrification and net NO_3^--N production also decreased with N supply
481 promoted soil acidification (Zhang et al., 2008). Tan et al (2018), working at the same
482 site, found that N additions led to a large decline in both fungi and bacteria abundance
483 (phospholipid fatty acids (PLFAs) as the indicator) associated with a decrease in DOC
484 (dissolved organic carbon) and increasing cations (e.g. Al^{3+} and Mn^{2+}) as soil acidified.
485 However, the microbial community structures (ratio of fungal to bacterial biomass) did
486 not show any significant change (Tan et al., 2018). Further, N addition slightly changed
487 the bacterial/fungal diversity in DL soils, except bacterial Shannon's index which was
488 increased with N addition (Fig. S4). It differed considerably from BK soils with
489 significant changes in the bacterial/fungal diversity indices (especially for dominance)
490 with N addition, which could be an important contributor to the different impacts of N
491 addition on soil gross N transformations between DL and BK. Meanwhile, decreased
492 total mineral N activity and increased N/I ratio with N addition also suggested an
493 increasing potential risk of N losses (Stockdale et al., 2002). The observation indicated
494 N-induced acidification counteracted the promoting effect of increased SOC and TN
495 with N addition on the gross rates of N transformations, leading to a significant decline
496 in autotrophic nitrification and N immobilization in neutral temperate grassland soils.

5. Conclusions

Under natural N deposition, the calcareous alpine grassland soil had higher gross rates of mineralization and nitrification and a lower gross immobilization rate than the neutral temperate grassland soil, mainly contributed by the locality-specific soil properties, i.e. high soil organic carbon (SOC) and total nitrogen (TN) and the low C/N ratio. Nitrogen addition differentially affected gross N transformations in alpine and temperate grassland soils. In calcareous alpine grassland soils, N addition increased gross N transformation rates, by increasing SOC, TN, mineral N and fungal dominance. In contrast, N transformation rates in neutral temperate grassland soil did not change at a low N addition rate, but decreased at a high N addition rate caused by N-induced soil pH decline. Our results illustrated the impacts of N addition on gross N transformation rates were largely as a function of N addition rates and soil properties (e.g. SOC, TN and soil pH). Notably, N-addition induced soil pH decline (acidification) might play a more important role than SOC and TN in N transformations in response to N addition by altering soil microbial community in the acid-sensitive soils (e.g. DL neutral grassland soil). The understanding of the interactive response of the individual gross N transformation processes to N addition provides insights on how different grassland soils are likely to react to future changes in soil N availability, such as enhanced atmospheric N deposition.

Declaration of competing interest

There are no conflicts of interest associated with this publication.

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Captions of Tables and Figures

Table 1. Soil-climatic properties of six studied soils (0-20 cm) [Mean ± S.D.] at the two experimental sites Bayinbuluk (BK) and Duolun (DL).

Table 2. Gross N transformation rates calculated by ^{15}N tracing model $Ntrace_{Basic}$ (mean with S.D. in brackets, n=3).

Fig. 1. Measured (*dots*) and modeled (*lines*) concentrations of soil NH_4^+-N (a) and NO_3^--N (b), and ^{15}N enrichments of NH_4^+-N (c) and NO_3^--N (d).

Fig. 2. A schematic diagram illustrating the response of soil properties (pH, SOC, TN, and C/N) and gross N transformation rates in response to enhanced N deposition at low and high rates based on chronic N addition experiments in alpine and temperate grassland soils.

Table 1. Soil-climatic properties of six studied soils (0-20 cm) [Mean ± S.D.] at the two experimental sites Bayinbuluk (BK) and Duolun (DL).

Sampling sites	T[1] (°C)	P[2] (mm)	Climate	Treatment [3]	N input, kg N ha^{-1} yr^{-1}	Code	Soil pH (H_2O) (S:W =1:2.5)	SOC (g C kg^{-1})[4]	TN (g N kg^{-1})[5]	C/N	NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})	WHC (%)
Bayinbuluk	-4.8	266	Temperate continental	Natural	$7.6_{(dep.)}$	$BK_{Control}$	7.82 ± 0.02 a	37.4 ± 0.7 b	3.59 ± 0.13 b	10.4 ± 0.2 a	3.67 ± 0.22 b	12.23 ± 0.26 b	44.9 ± 0.1 a
				Experimental	$7.6_{(dep.)} + 30_{(fert.)}$	BK_{N30}	7.80 ± 0.04 a	41.0 ± 0.4 a	4.07 ± 0.26 a	10.1 ± 0.6 a	3.77 ± 0.13 b	16.73 ± 0.15 b	44.5 ± 0.1 a
				Experimental	$7.6_{(dep.)} + 90_{(fert.)}$	BK_{N90}	7.79 ± 0.04 a	42.1 ± 0.5 a	4.25 ± 0.12 a	9.9 ± 0.2 a	7.87 ± 0.24 a	55.81 ± 4.27 a	44.0 ± 0.7 a
Duolun	3.3	293	Temperate continental monsoon	Natural	$14.7_{(dep.)}$	$DL_{Control}$	6.58 ± 0.10 a	12.6 ± 0.9 b	1.08 ± 0.03 b	11.7 ± 0.4 a	6.77 ± 0.39 c	5.89 ± 0.42 c	28.4 ± 0.1 a
				Experimental	$14.7_{(dep.)} + 30_{(fert.)}$	DL_{N30}	6.36 ± 0.11 b	15.2 ± 0.2 a	1.34 ± 0.03 a	11.4 ± 0.2 a	7.49 ± 0.15 b	8.78 ± 0.16 b	28.4 ± 0.2 a
				Experimental	$14.7_{(dep.)} + 120_{(fert.)}$	DL_{N120}	5.75 ± 0.04 c	14.4 ± 0.7 a	1.28 ± 0.09 a	11.3 ± 0.7 a	15.13 ± 0.09 a	19.13 ± 1.31 a	28.3 ± 0.2 a

[1] T is mean annual temperature;

[2] P is mean annual precipitation;

[3] 'Natural' represents the site only received N deposition without other N inputs; 'Experimental' represents the site received both natural N deposition and experimental N addition; In the subscripts of values, 'dep.' represents atmospheric N deposition, given by Xu et al., (2015) for BK and Zhang et al., (2017) for DL, and 'fert.' represents experimental N fertilizer application to simulate different levels of N deposition;

[4] 'SOC' represents soil organic carbon;

[5] 'TN' represents soil total nitrogen.

[*] The lowercases behind the values of soil properties denote the statistical difference between three treatments for each sampling site, where one-way ANOVA and

790 Tukey's HSD test were used at $P < 0.05$.

Table 2. Gross N transformation rates calculated by ^{15}N tracing model *Ntrace*$_{Basic}$ (mean with S.D. in brackets, n=3).

Gross N transformation rates (mg N kg^{-1} d^{-1})	Duolun			Bayinbuluk		
	N_0	N_{30}	N_{120}	N_0	N_{30}	N_{90}
M_{Nrec}	0.25 (0.18)	0.64 (0.55)	0.69 (0.20)	1.70 (0.31)	0.95 (0.53)	0.11** (0.04)
M_{Nlab}	0.79 (0.20)	1.07 (0.56)	0.56 (0.29)	1.41 (0.38)	2.26 (0.69)	3.24** (0.19)
$I_{NH4\text{-}Nrec}$	5.38 (0.91)	1.69 (1.20)	1.21** (0.60)	1.47 (0.89)	2.94 (1.62)	5.23* (0.86)
$I_{NH4\text{-}Nlab}$	4.22 (0.81)	1.91 (1.24)	0.51** (0.48)	2.34 (1.45)	1.68 (1.41)	1.06 (0.85)
I_{NO3}	0.01 (0.01)	0.06 (0.04)	0.04 (0.03)	0.44 (0.09)	0.07* (0.07)	0.27 (0.23)
O_{NH4}	2.43 (0.07)	2.16 (0.11)	1.95* (0.12)	5.07 (0.12)	8.40** (0.11)	12.60$^{**\#\#}$ (0.24)
O_{Nrec}	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
D_{NO3}	0.01 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.01 (0.00)
A_{NH4}	0.08 (0.03)	0.06 (0.03)	0.08 (0.04)	0.05 (0.03)	0.03 (0.02)	0.07 (0.02)
R_{NH4}	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
N/I [1]	0.25	0.60	1.13	1.33	1.82	2.00
Total N_{min} activity [2]	13.09	7.53	4.96	12.43	16.30	22.52
Net NH_4^+-N production [3]	-11.06	-4.11	-2.5	-5.82	-9.84	-15.6
Net NO_3^--N production [4]	2.41	2.1	1.91	4.63	8.33	12.32

[1] N/I is the ratio of gross nitrification rate (O_{NH4}+O_{Nrec}) to gross ammonia immobilization rate ($I_{NH4\text{-}Nrec}$ + $I_{NH4\text{-}Nlab}$);

[2] Total N_{min} activity = $M_{Nrec} + M_{Nlab} + I_{NH4\text{-}Nrec} + I_{NH4\text{-}Nlab} + I_{NO3} + O_{NH4} + O_{Nrec} + D_{NO3}$

[3] Net NH_4^+-N production = $M_{Nrec} + M_{Nlab} + R_{NH4} + D_{NO3} - I_{NH4\text{-}Nrec} - I_{NH4\text{-}Nlab} - I_{NO3} - A_{NH4} - O_{NH4}$

[4] Net NO_3^--N production = $O_{NH4} + O_{Nrec} - I_{NO3} - D_{NO3}$

Note: * and $^{\#}$ represent the parameters are significantly different at $P < 0.05$, ** and $^{\#\#}$ represent the parameters are highly significant at $P < 0.01$. * and ** represent the comparison of control plots and N treated plots at low rates (N30), $^{\#}$ and $^{\#\#}$ represent the comparison of N treated plots at low rates (N30) and high rates (N90 and N120), respectively.

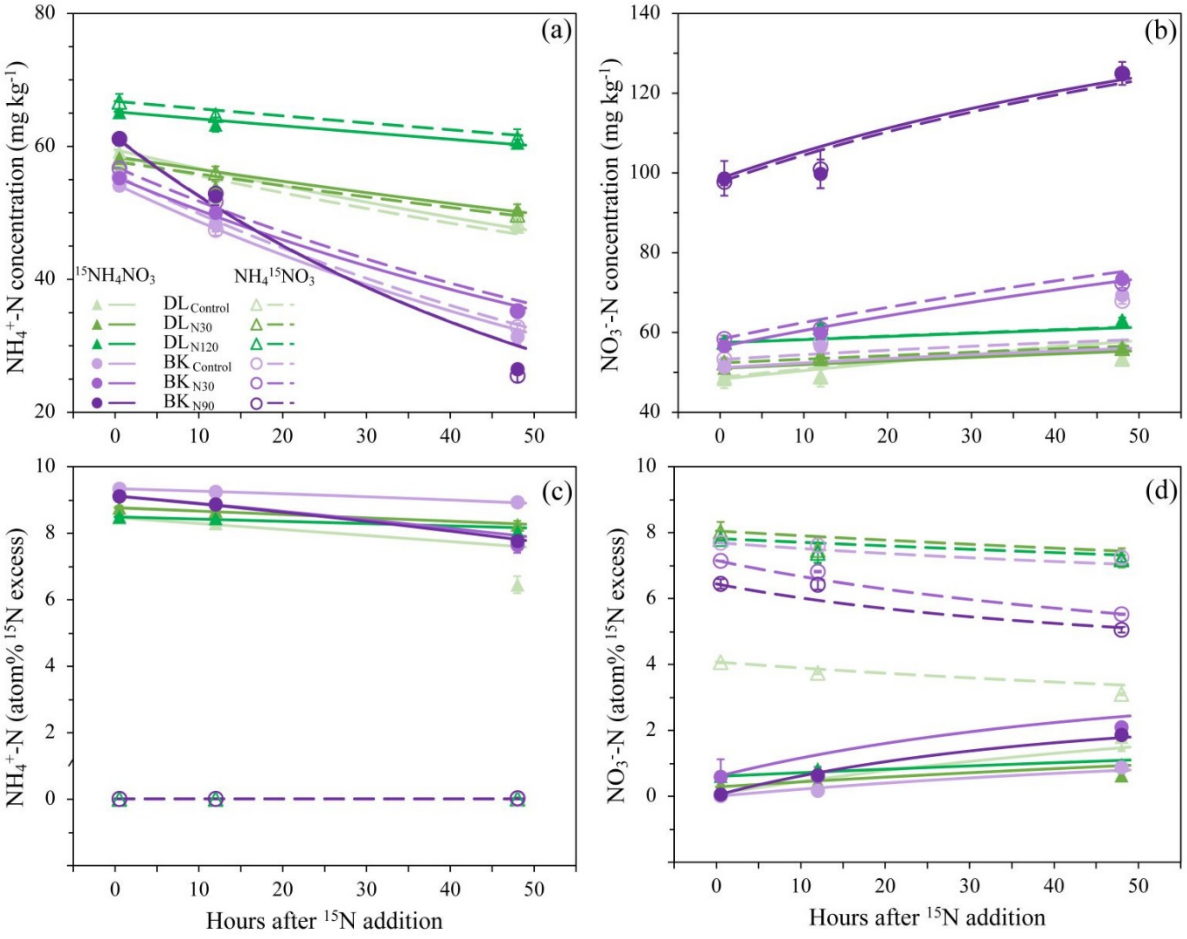

Fig. 1. Measured (*dots*) and modeled (*lines*) concentrations of soil NH_4^+-N (a) and NO_3^--N (b), and ^{15}N enrichments of NH_4^+-N (c) and NO_3^--N (d).

Note: Bars indicate S.D.; Solid lines indicate $^{15}NH_4NO_3$ added treatments; Dashed lines indicate $NH_4{}^{15}NO_3$ added treatments.

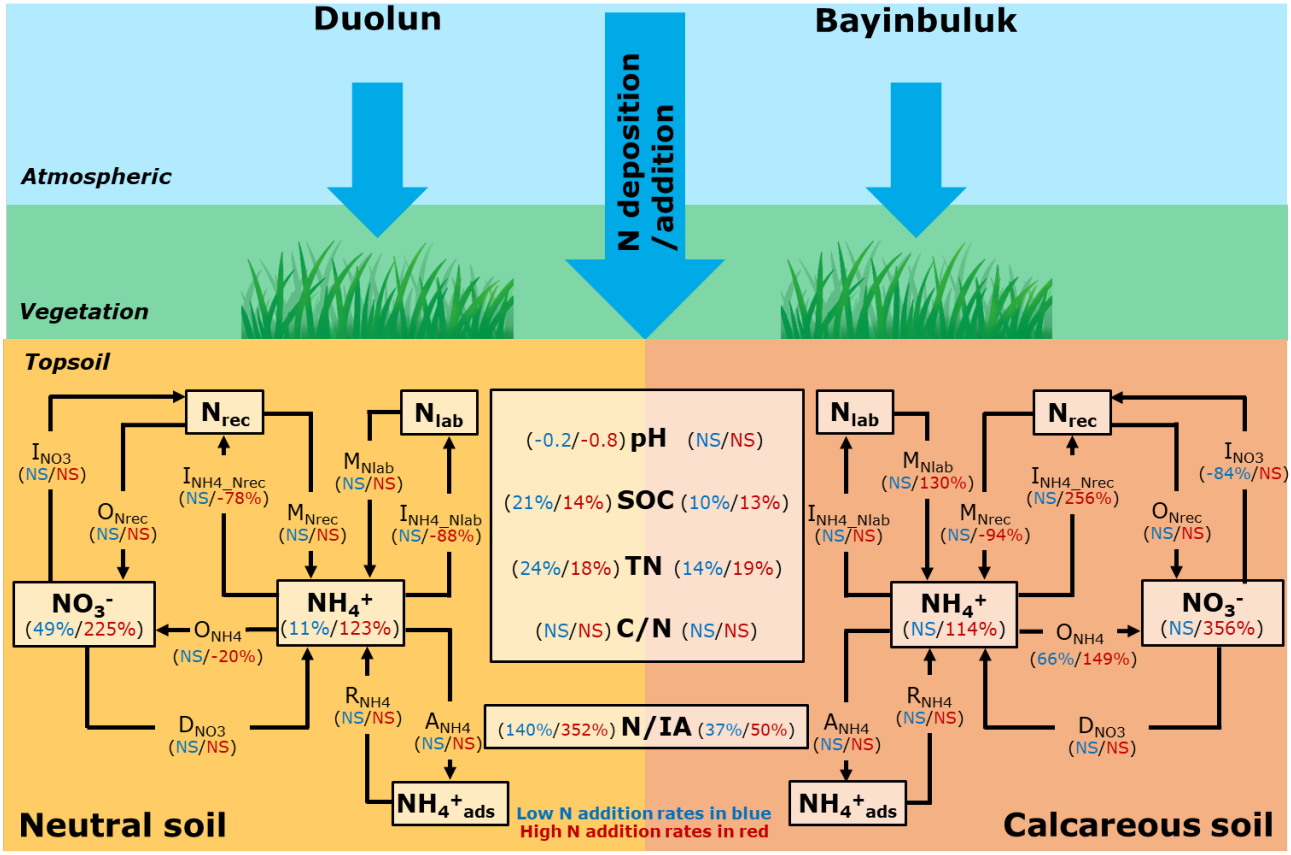

Fig. 2. A schematic diagram illustrating the response of soil properties (pH, SOC, TN, and C/N) and gross N transformation rates in response to enhanced N deposition at low and high rates based on chronic N addition experiments in alpine and temperate grassland soils.

Note: Comparing to the control plots, only N addition-induced significant changes at $P < 0.05$ (absolute difference values for soil pH and % for others) in parameters are shown in brackets, no significant difference is shown as NS. Positive values represent increase, negative values represent decrease. The values at low N addition rate were in blue (30 kg N ha^{-1} yr^{-1}) and the values at high N addition rate were in red (120 and 90 kg N ha^{-1} yr^{-1} for DL and BK, respectively).