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LETTER

Nonlinear response of soil respiration to increasing nitrogen additions in a Tibetan alpine steppe

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Abstract

Nitrogen (N) availability is a key regulator of carbon (C) cycling in terrestrial ecosystems. Anthropogenic N input, such as N deposition and fertilization, increases N availability in soil, which has important implications for an ecosystem's C storage and loss. Soil respiration (Rs), which is the second largest C flux from terrestrial ecosystems to the atmosphere, plays an important role in terrestrial C cycles. The direction and magnitude of the responses of Rs and its components to N addition have been widely evaluated, but it remains unclear how these processes change across multiple N addition levels. Here we conducted a two-year field experiment to examine the changes of Rs and its autotrophic respiration (Ra) and heterotrophic respiration (Rh) components along a gradient of eight N levels (0, 1 2, 4, 8, 16, 24, 32 g m^{-2} yr^{-1}) in a Tibetan alpine steppe, and used structural equation modeling (SEM) to explore the relative contributions of biotic and abiotic variables and their direct and indirect pathways regulating the Ra and Rh. Our results indicated that both Rs and Ra exhibited first increasing and then subsequent decreasing trends at the threshold of 8 g N m^{-2} yr^{-1}. In contrast, the Rh declined linearly with the N addition rate continuously increasing. SEM analysis revealed that, among various environmental factors, soil temperature was the most important one modulating Rs, which not only had a direct effect on the two Rs components, but also indirectly regulated the Ra and Rh via root and microbial biomass. These findings suggest that the nonlinear response patterns of Rs should be considered for better predicting terrestrial C balance, given that anthropogenic N input to the terrestrial ecosystems is increasing continuously.

1. Introduction

Nutrient availability is recognized as a key regulator of terrestrial carbon (C) balance (Fernández-Martínez *et al* 2014, Campioli *et al* 2015, Wieder *et al* 2015). Nitrogen (N), a very important nutrient for plants and soil organisms, usually limits the productivity of most terrestrial ecosystems (LeBauer and Treseder 2008). Due to accelerating anthropogenic activities over the past few decades, reactive N entering terrestrial ecosystems has increased continuously (Galloway *et al* 2008, Bodirsky *et al* 2014), which substantially alters ecosystem C cycling processes (Janssens *et al* 2010, Liu and Greaver 2010, Zhou *et al* 2014). Numerous studies using N addition experiments have examined how changes in N availability affect diverse C processes (Janssens *et al* 2010, Liu and Greaver 2010, Yue *et al* 2015). Among those studies, most focus on the direction and magnitude of C dynamic responses to elevated N (Janssens *et al* 2010, Liu and Greaver 2010).

However, as N continues to increase, ecosystems generally experience limited to saturated N (Aber *et al* 1989, 1998), and the nonlinearity of the N-induced changes in ecosystem processes has always been observed (Arens *et al* 2008, Bradford *et al* 2008, Vivanco *et al* 2015). Thus, better understanding the nonlinear responses of various C processes to continuously increasing N inputs is of paramount importance for accurately predicting future terrestrial C balance.

Soil respiration (Rs), as the second largest C flux between terrestrial ecosystems and the atmosphere, plays an important role in terrestrial C cycles (Luo and Zhou 2006, Janssens *et al* 2010). Though some prior studies examined the nonlinear responses of above-ground C processes to multiple N loads, such as gross primary productivity (GPP) (Arens *et al* 2008) and above-ground net primary productivity (ANPP) (Vivanco *et al* 2015), how Rs, which links to many below-ground biotic and abiotic processes, responds to multiple N levels remains unexplored. Rs usually consists of two major components: autotrophic respiration (Ra) in live roots and their symbionts, and heterotrophic respiration (Rh) from soil micro-organisms during soil organic matter and litter decomposition (Boone *et al* 1998, Luo and Zhou 2006). It has been reported that root and microbial growth respond distinctly to changing soil N availability compared with above-ground, especially under high N addition levels (Wei *et al* 2013, Liu *et al* 2014). Thus, it is interesting to know how Rs and its components respond to gradually increasing N availability, particularly experiencing the N limitation to saturation scenarios.

Numerous biotic and abiotic factors are assumed to regulate Rs and its autotrophic and heterotrophic components and N addition will likely affect Rs through impacts on these drivers. For example, additional N inputs may alter soil temperature and moisture due to the changes in the interception of solar radiation and ecosystem evapotranspiration (Luo and Zhou 2006), which would substantially influence soil CO_2 flux (Davidson and Janssens 2006, Bond-Lamberty and Thomson 2010). Meanwhile, the N-induced decrease in soil pH has a great effect on microbial growth and activities (Treseder 2008, Chen *et al* 2016), usually leading to decreases in Rh (Wei *et al* 2013, Chen *et al* 2016). Besides these environmental factors, plant above and below-ground C partitioning also play an important role in regulating Rs, due to the variation in substrate supplies (Janssens *et al* 2010, Chen *et al* 2016). However, to date, we know little about how these factors directly and indirectly regulate Ra and Rh across a range of N additions.

To address the above issues, we conducted a two-year field experiment with eight N addition levels (0, 1, 2, 4, 8, 16, 24, 32 g m^{-2} yr^{-1}) in an alpine steppe on the Tibetan Plateau. This area has experienced long-term N limitation due to the low N input and slow N mineralization rate (Zhou 2001), which provides an ideal platform to simulate ecosystems experiencing

limited to saturated N scenarios. The objectives of the present study are to i) reveal the general response patterns of Rs and its components to multiple N addition levels, and ii) untangle the relative contributions of biotic and abiotic variables and their direct and indirect influences on Ra and Rh along the N addition gradient.

2. Materials and methods

2.1. Site description
The study was conducted on the Sanjiaocheng Sheep Breeding Farm (37°18′N, 100°15′E, 3290 m a.s.l), an alpine steppe located in Qinghai Province, China. The long-term mean annual temperature is 0.08 °C and the mean annual precipitation is 387 mm (1980–2012). The native vegetation is a typical alpine steppe community, characterized by the dominant species of *Stipa purpurea*, *Carex rigescens*, *Poa crymophila* Keng, *Agropyron cristatum*, *Heteropappus altaicus* (Willd.) Novopokr, etc. The soil type at the study site is classified as chestnut according to Chinese classification or Haplic Calcisol according to the FAO (Food and Agriculture Organization) classification, with 61.0% sand, 33.4% silt, and 4.9% clay. The chemical properties of the 0–30 cm soil layer at the beginning of the experiment were as follows: soil organic C 20.5 g kg^{-1}, total-N 2.5 g kg^{-1}, total phosphorous (P) 0.6 g kg^{-1}, pH 9.5, NO_3^--N 11.5 mg kg^{-1}, NH_4^+-N 0.1 mg kg^{-1} and available P 2.2 mg kg^{-1}.

2.2. Experimental design
The experimental site was fenced and the N enrichment experiment started in May 2013. Other than grazing, no other management had been applied on the site before the experiment. A gradient of eight N levels (0, 1, 2, 4, 8, 16, 24, and 32 g m^{-2} yr^{-1}, labeled N0, N1, N2, N4, N8, N16, N24 and N32, respectively) was established and arranged in randomized complete blocks with five replications. The plot size was 6 m × 6 m and a total of forty plots was created. The distance between any two adjacent plots within a block was 1 m and each block was separated by 2 m buffer strips. N fertilizer, in the form of ammonium nitrate, was applied at the beginning of each month during the growing season from May to September every year. At each plot, a specific amount of fertilizer was dissolved in 10 L of water and sprinkled by a backpack sprayer. Two passes were made across each plot to ensure an even distribution of the fertilizer. The control plot received the same amount of water without N.

2.3. Measurement protocols
2.3.1. Rs and its components
In May 2013, two PVC collars (20 cm in diameter) were inserted into the soil permanently at least 50 cm away from the plot edge. One collar (5 cm in height) was inserted into the soil to a depth of 3 cm for Rs

measurement, and another (62 cm in height) was inserted to a depth of 60 cm to detect Rh; these collars cut off old roots and prevented new roots from growing inside the collars. Ra was then calculated as the difference between Rs and Rh. This method has been successfully used to separate Ra and Rh in previous studies (Hasselquist *et al* 2012, Li *et al* 2013, Suseela and Dukes 2013). Soil CO_2 flux was measured using a LI-8100A Automated Soil CO_2 Flux System (Li-Cor Inc., Lincoln, NE, USA) with *ca.* every 10 d between 9:00–12:00 am (local time) from May 11 to October 22 in 2014, and from April 24 to October 13 in 2015. Living plants inside the collars were removed by hand at least one day before each measurement. During the measurements, the flux chamber was placed on the short collar (5 cm in height) first and subsequently moved to the long collar (62 cm in height) in each plot. At the same time the soil CO_2 efflux was measured, the soil temperature and volumetric moisture at a 0–10 cm soil depth were monitored adjacent to the PVC collar using a digital thermometer and a portable TDR-100 soil moisture probe (Spectrum Technologies Inc., Plainfield, IL, USA), respectively.

2.3.2. Plant and soil sampling
Plant and soil samples were collected in the middle of August in both experimental years. Within each plot, we randomly selected three 25 cm × 25 cm quadrats, and harvested green above-ground plants, which represented the current year's ANPP. After above-ground sampling, three root cores (8 cm in diameter and 10 cm in depth) were collected in the corresponding sites and then mixed as one sample. Root samples were immediately placed in a cooler and then cleaned of residual soil using a 0.5 mm sieve soaked in water. Live and dead roots were distinguished by their color, resilience and the attached fine roots (Vogt and Persson 1991, Yang *et al* 2010), and the live root biomass was presented. All plant samples were oven-dried at 65 °C until they reached a constant weight, and were then weighed to estimate dry mass. After root collection, soil cores (3 cm in diameter) were collected from the surface layer (0–10 cm) adjacent to root cores in each quadrat, and combined to gain one composite sample. After the roots and organic debris had been removed manually, soil samples were immediately sieved through a 2 mm screen in the field and then stored in a cooler. These samples were then transported to the laboratory for further analysis.

2.3.3. Laboratory analyses
The soil's inorganic N (NO_3^--N and NH_4^+-N) was extracted with a 1 M KCl solution and analyzed using a flow injection analyzer (Autoanalyzer 3 SEAL, Bran and Luebbe, Norderstedt, Germany). The soil's microbial biomass C (MBC) was estimated using the chloroform fumigation extraction method (Vance *et al* 1987). An aliquot of 20 g of fresh soil was fumigated with chloroform for 24 h and then extracted by 0.5 M K_2SO_4. Another aliquot of 20 g unfumigated fresh soil was directly extracted by a K_2SO_4 solution. MBC was calculated from the differences between extractable C concentrations in the fumigated and unfumigated samples by a conversion coefficient of 0.45 (Joegensen and Mueller 1996, Jenkinson *et al* 2004). Soil pH was determined with a glass electrode in a 1:2.5 soil: water solution (w/v).
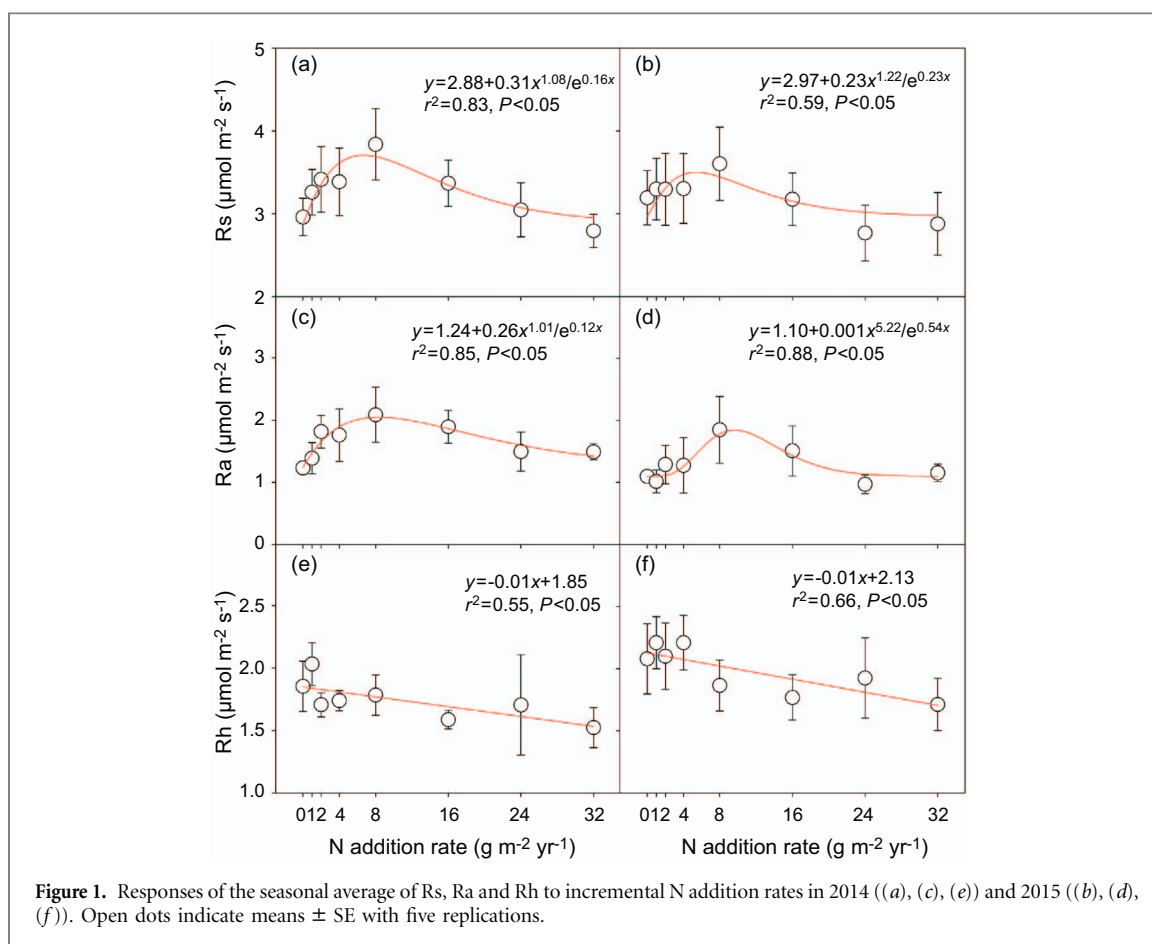
2.4. Statistical analysis
To examine the nonlinear response patterns of Rs, Ra and Rh along the N addition gradient, an empirical function ($y = a + bx^c/e^{dx}$, where y is Rs, Ra or Rh, x is the N addition rate, and a, b, c and d are the coefficients, Yang *et al* 2010) was employed to characterize the relationships of these variables with N addition rates during each growing season. One-way ANOVA was used to compare the treatment difference for Rs, Ra and Rh as well as biotic and abiotic variables. Ordinary least square regressions were conducted to explore the relationships of Rs, Ra and Rh with biotic and abiotic parameters. All these analyses were performed with SPSS statistic 16.0 (IBM SPSS, Chicago, Illinois, USA).

Structural equation modeling (SEM) was used to examine the relative importance and direct and indirect pathways of various factors regulating Ra and Rh along the N addition gradient. Before SEM analysis, we constructed an *a priori* conceptual model of hypothetical relationships (figure S1 available at stacks.iop.org/ERL/12/024018/mmedia). Theoretically, a number of biotic and abiotic factors are assumed to influence soil CO_2 flux, but our model only adopted those factors showing significant correlations with Ra or Rh along this N addition gradient, at least in 2015 (see figures 3 and 4). Therefore, we hypothesized that, soil temperature directly influenced Ra and Rh, and also indirectly influenced them through regulating root and microbial biomass. Meanwhile, soil inorganic N and pH affected Rh directly and also indirectly via MBC (figure S1). During SEM analysis, data were fitted to the model using the maximum likelihood estimation method. This procedure compared the model-implied variance–covariance matrix with the observed variance–covariance matrix, and used the chi-square goodness-of-fit statistic and its associated *P* value to test the model fit to the data. The covariance structure of the data was considered not to be significantly different from the expected model if the *P* value was larger than 0.05 (Grace 2006). SEM analyses were conducted using Amos version 21.0 (Amos Development Corporation, Chicago, IL, USA).

3. Results

3.1. Changes of Rs and its components along the N addition gradient
With the N addition rate continuously increasing, the seasonal means of Rs and Ra exhibited first increasing

Figure 1. Responses of the seasonal average of Rs, Ra and Rh to incremental N addition rates in 2014 ((*a*), (*c*), (*e*)) and 2015 ((*b*), (*d*), (*f*)). Open dots indicate means ± SE with five replications.

and subsequent decreasing trends at a threshold of $8\,\mathrm{g\,N\,m^{-2}\,yr^{-1}}$ (figures 1(*a*)–(*d*)). In contrast, a linear negative relationship was fit to Rh data under increasing N additions (figures 1(*e*) and (*f*)). During the two experimental years, except for Rh in 2014, additional N inputs significantly altered Rs and its components (table S1). The annual budgets for the three parameters were also influenced by N additions, showing similar patterns as seasonal averages along the N addition gradient (table S1).

In general, the seasonal dynamics of Rs and its autotrophic and heterotrophic components followed a bell-like pattern in both experimental years, with higher values in the mid-growing season and lower values in the early and late seasons (figure 2). Inter-annual variations of Rs, Ra and Rh were observed across the two experimental years. The maximum values of Rs and its components appeared in mid-August in 2014 but in mid-July in 2015. In addition, N input exaggerated their inter-annual variability by strengthening the maximum values (figure 2).

3.2. Effects of increasing N additions on biotic and abiotic variables
Increasing N additions had significant influences on the biotic and abiotic factors (table 1). ANPP consistently displayed a saturation trend with increasing N additions regardless of inter-annual variation, with a saturation point occurring at $8\,\mathrm{g\,N\,m^{-2}\,yr^{-1}}$. The relationship between root biomass and the N

addition rate was characterized by a hump-shaped pattern similar to Rs and Ra, with a peak appearing in the N8 treatment. In 2014 no significant response to the N addition was observed in MBC, whereas in 2015, it increased gradually until $4\,\mathrm{g\,N\,m^{-2}\,yr^{-1}}$ and thereafter declined.

Soil temperature increased with increasing N additions up to $4\,\mathrm{g\,N\,m^{-2}\,yr^{-1}}$ and then decreased after this N level in 2014, while in 2015, no obvious change was found among the N0 to N4 treatments, but the N8 to N32 treatments significantly reduced soil temperature. In both years, soil moisture generally showed a funnel-like pattern along the N gradient, with the minimum at $8\,\mathrm{g\,N\,m^{-2}\,yr^{-1}}$. Soil inorganic N increased monotonously with increasing N additions in 2015, but the increasing trend leveled off after the N8 treatment in 2014. Soil pH was also negatively related to the N addition rate in 2015, while no obvious trends were observed along the eight N levels in 2014.

3.3. Effects of biotic and abiotic factors on Rs and its components
Across the 40 experimental plots, Rs, Ra and Rh increased linearly with increasing soil temperature in both experimental years (figures 3(*d*), (*k*) and (*r*); figures 4(*d*), (*k*) and (*r*)). Meanwhile, Rs and Ra were also positively related to root biomass (figures 3(*b*) and (*i*); figures 4(*b*) and (*i*)). In addition, Rh increased with MBC in 2015 (figure 4(*q*)). Soil inorganic N also
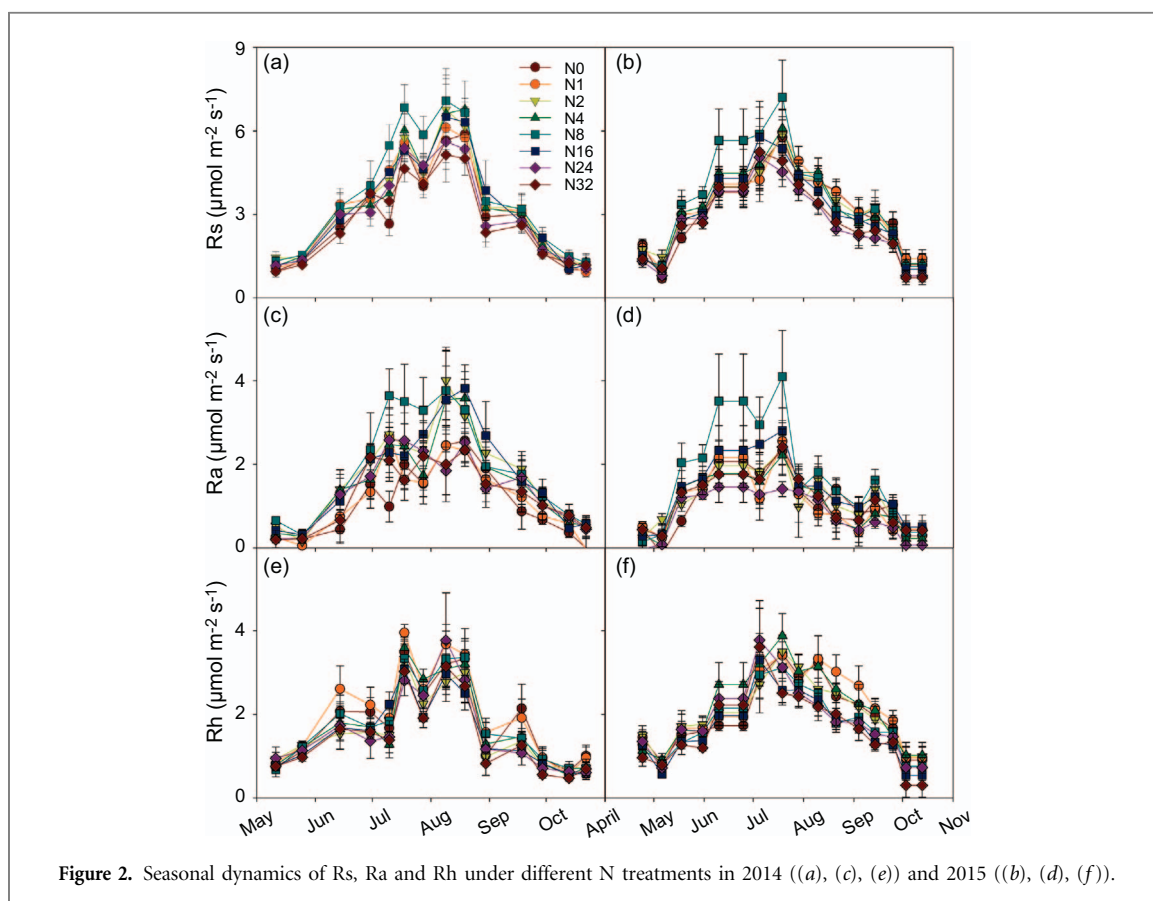
Figure 2. Seasonal dynamics of Rs, Ra and Rh under different N treatments in 2014 ((*a*), (*c*), (*e*)) and 2015 ((*b*), (*d*), (*f*)).

negatively influenced Rs and Rh (figures 4(*f*) and (*t*)), but positively regulated both variables in 2015 (figures 4(*g*) and (*u*)).

The SEM analysis indicated that, along the gradient of N additions, root and microbial biomass were the direct controllers of Ra and Rh. Soil temperature directly influenced Ra and Rh in both years, and it also indirectly affected the Ra via root biomass and the Rh via MBC. Although the soil temperature, inorganic N and pH were all significantly correlated with Rh (figure 4), only the soil temperature entered the final model (figure 5).

4. Discussion

4.1. Partitioning of Rs components

In the current study, we used the deep-collar method to distinguish Ra and Rh, a method which has been widely applied to partition Rs in other studies (Zhou *et al* 2007, Hasselquist *et al* 2012, Li *et al* 2013, Suseela and Dukes 2013). Given that roots in the top 30 cm of soil accounted for more than 90% of the biomass of those in the 0–50 cm soil profile at our experimental site (data not shown), the 60 cm long collar was long enough to exclude most of the living roots. It has been reported that the deep-collar method generally results in a transient increase of Rs due to the decomposition of dead roots and disturbance (Hanson *et al* 2000, Zhou *et al* 2007). It has also been suggested that the transient increase of Rs lasted for about five months

after collar insertion in a prairie in Southern Great Pains, USA (Zhou *et al* 2007). Given that collars were inserted into the soil in May 2013, the effects of dead root decomposition on the Rh measured after a whole year should be minimized. More importantly, our results revealed that the contribution of Rh to Rs was 46%–63% and 52%–70% in 2014 and 2015, respectively, and these values are within the range reported in grassland ecosystems (25%–90%, Subke *et al* 2006). Therefore, despite the fact that some uncertainties may be inevitable, the root exclusion method is a feasible way to partition the two components of Rs in this alpine steppe.

4.2. Distinct responses of Ra and Rh to continuously increasing N additions
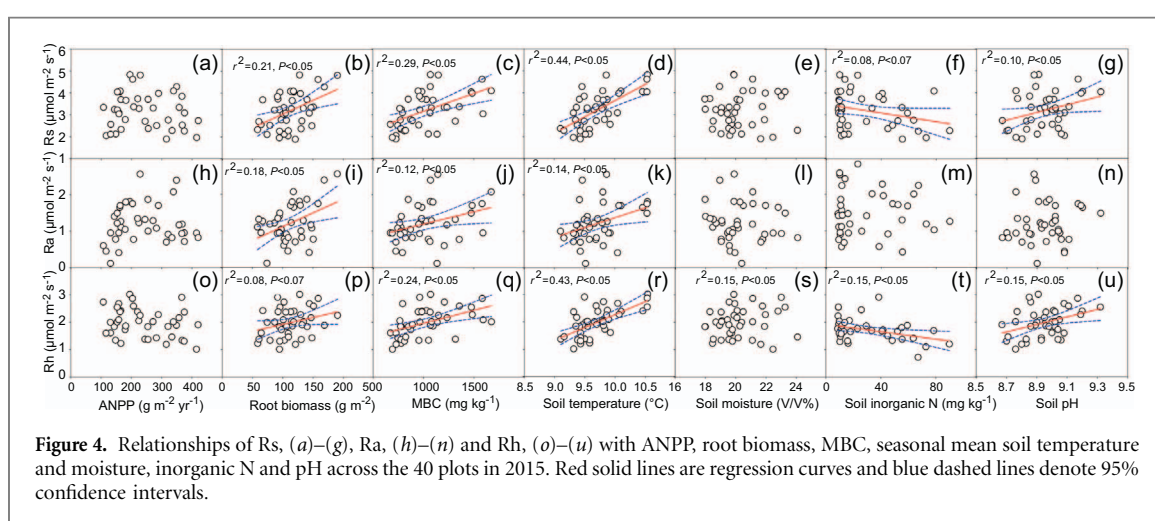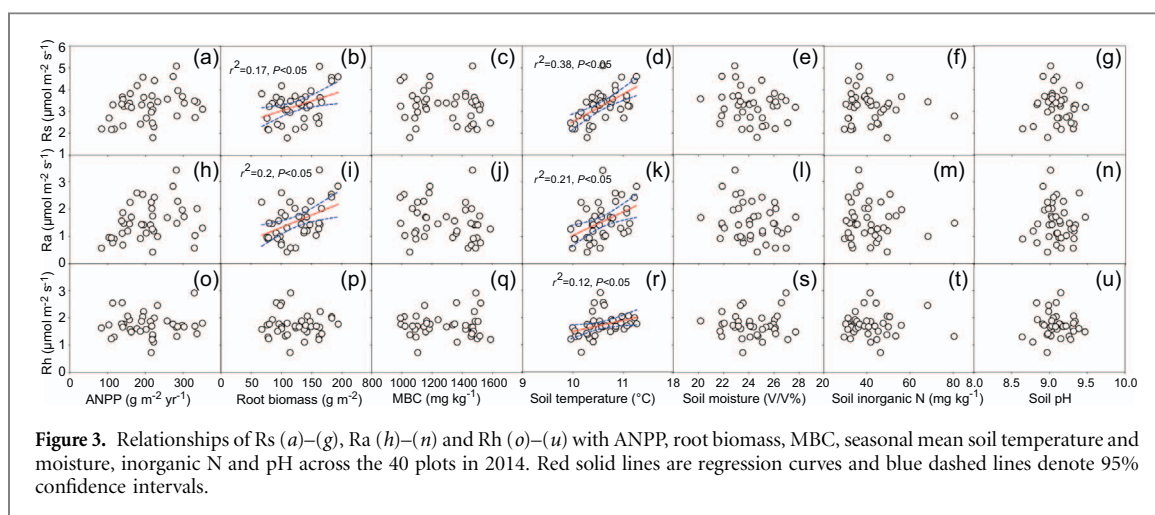
Although individual studies reported inconsistent responses of Rs and its components to N enrichment, a recent meta-analysis showed that N addition, in aggregate, increased Rs and Ra but reduced Rh in grasslands (Zhou *et al* 2014). However, to date, information is still lacking about how Rs and its autotrophic and heterotrophic components change from limited to saturated N scenarios. The present study revealed that in a N-limited alpine steppe, with the N addition rate continuously increasing, both Rs and Ra exhibited hump-shaped response patterns while Rh declined linearly (figure 1). Nevertheless, it should be noted that our study only showed the responses of soil CO_2 fluxes to short-term N additions (three years), but it remains unclear whether long-

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Table 1. Responses of biotic and abiotic variables to increasing N additions in 2014 and 2015.

Variable	Year	N treatment							
		N0	N1	N2	N4	N8	N16	N24	N32
ANPP	2014	126.9 ± 10d[a]	142.2 ± 17.8d	152.4 ± 13.9dc	186.7 ± 13.9c	256.6 ± 14.3ab	241.2 ± 21.4b	271.7 ± 26.1ab	284.9 ± 27.6a
(g m^{-2} yr^{-1})	2015	146.0 ± 11.6c	146.8 ± 8.3c	175.7 ± 14.7c	180.4 ± 13.6c	265.1 ± 23.5b	287.5 ± 21b	350.4 ± 13.3a	373.0 ± 18.6a
RB	2014	83.8 ± 4.3c	124.5 ± 13.4ab	113.5 ± 21.5bc	130.9 ± 15.3ab	153.0 ± 15.6a	122.4 ± 10.3ab	127.9 ± 10.5ab	139.3 ± 9.6ab
(g m^{-2})	2015	82.1 ± 11.9b	111.1 ± 9ab	106.4 ± 7ab	119.4 ± 15.9a	132.0 ± 24.2a	122.6 ± 11.1a	103.6 ± 5.1ab	120.0 ± 9a
MBC	2014	1237 ± 93a	1296 ± 83a	1289 ± 89a	1241 ± 99a	1354 ± 76a	1326 ± 91a	1255 ± 92a	1287 ± 95a
(mg kg^{-1})	2015	934 ± 94b	945 ± 137ab	1088 ± 137ab	1208 ± 121a	1052 ± 94ab	1028 ± 125ab	868 ± 63b	1062 ± 177ab
ST	2014	10.5 ± 0.2b	10.6 ± 0.2ab	10.6 ± 0.1ab	10.8 ± 0.2a	10.6 ± 0.2ab	10.6 ± 0.2ab	10.5 ± 0.2b	10.5 ± 0.2b
(°C)	2015	9.9 ± 0.1a	9.9 ± 0.2a	9.9 ± 0.2a	9.9 ± 0.2a	9.6 ± 0.1b	9.5 ± 0.2b	9.4 ± 0b	9.5 ± 0.1b
SM	2014	24.5 ± 0.6ab	25.3 ± 0.9a	24.8 ± 0.9ab	24.6 ± 0.4ab	22.6 ± 0.3b	23.3 ± 0.5ab	24.3 ± 1.2ab	24.5 ± 0.8ab
(V/V, %)	2015	20.1 ± 0.7ab	20.3 ± 0.9ab	19.2 ± 0.2b	20.7 ± 0.3ab	19.2 ± 0.4b	19.9 ± 0.7ab	21.1 ± 0.7a	21.5 ± 0.8a
Inorganic N	2014	34.3 ± 2.6c	37.8 ± 3.1bc	35.9 ± 3.7bc	33.1 ± 0.9c	52.6 ± 9.1a	46.8 ± 3.4ab	43.5 ± 1.8abc	45.5 ± 1.4ab
(mg kg^{-1})	2015	9.5 ± 0.3e	11.4 ± 1.5e	11.8 ± 1.1e	11.9 ± 0.7e	22.4 ± 2.5d	41.8 ± 2.5c	56.7 ± 5b	69.6 ± 7.1a
pH	2014	8.9 ± 0.10b	9.2 ± 0.06a	9.2 ± 0.08a	9.1 ± 0.07ab	9.1 ± 0.08ab	9.1 ± 0.07ab	9 ± 0.03ab	9.1 ± 0.10ab
—	2015	9.0 ± 0.07bc	9.1 ± 0.06a	9.0 ± 0.03ab	9.0 ± 0.05ab	8.9 ± 0.03cd	9.0 ± 0.04ab	8.8 ± 0.04d	8.8 ± 0.05d

[a] Means in rows in each year followed by a different letter represent a significant difference among N treatments ($P < 0.05$).

Abbreviations. ANPP: above-ground net primary productivity; RB: root biomass; MBC: microbial biomass C; ST: soil temperature; SM: soil moisture.

Figure 3. Relationships of Rs (*a*)–(*g*), Ra (*h*)–(*n*) and Rh (*o*)–(*u*) with ANPP, root biomass, MBC, seasonal mean soil temperature and moisture, inorganic N and pH across the 40 plots in 2014. Red solid lines are regression curves and blue dashed lines denote 95% confidence intervals.

Figure 4. Relationships of Rs, (*a*)–(*g*), Ra, (*h*)–(*n*) and Rh, (*o*)–(*u*) with ANPP, root biomass, MBC, seasonal mean soil temperature and moisture, inorganic N and pH across the 40 plots in 2015. Red solid lines are regression curves and blue dashed lines denote 95% confidence intervals.

term N enrichment would cause some differences. For example, the N-induced shifts in plant community composition and structure exert great influences on ecosystem C cycling (Niu *et al* 2010, Chen *et al* 2016), but we know little about how these changes will affect Rs after a long time period, particularly under multiple N loads. Thus, experiments with long duration and multiple N loads (at least more than five N addition levels) are called for to better depict the Rs/Ra/Rh–N rate relationships in future studies.

The seasonal dynamics of Ra and Rh displayed temporal variability across the two experimental years (figure 1). Inter-annual variation of Rs, Ra and Rh have been frequently reported in grasslands and other ecosystems (Liu *et al* 2002, Concilio *et al* 2009), which may result from temporal variations in various biotic and abiotic factors (Luo and Zhou 2006). First, the precipitation distribution varied significantly during the two growing seasons (figure S2). In particular, for the peak plant growth in August, the precipitation amount in 2015 (79 mm) was significantly lower than in 2014 (186 mm). Low soil moisture in 2015 (11%) compared with 2014 (30%) may have repressed root and microbial activities (Luo and Zhou 2006), thus

leading to reduced Rs during this period (figure 2). Second, the soil's inorganic N concentration increased linearly with increasing N additions in 2015, but the increasing trend leveled off after the N8 treatment in 2014 (table 1). The distinct response patterns are possibly due to the high NO_3^--N leaching to deeper soils under larger precipitation amounts in 2014. This is partly supported by the greater soil NO_3^--N concentration in the 30–50 cm soil layers measured in mid-August (figure S3). The high inorganic N accumulated in 0–10 cm of soil may be toxic to microbial growth and activities (Aber *et al* 1989), thus resulting in significant negative correlations of Rs and Rh with this variable in 2015 (figures 4(*f*), (*t*)). Third, the root and microbial biomass in 2014 was greater than those in 2015 (table 1). Larger root biomass indicates more C was allocated below ground (Peng and Yang 2016), which is usually associated with higher root respiration (Bai *et al* 2010). Meanwhile, increased microbial biomass was generally observed to have higher Rh (Zhou *et al* 2014, Chen *et al* 2016). These differences between the two experimental years may lead to greater soil CO_2 fluxes during the peak growth stage in 2014 (figure 2).
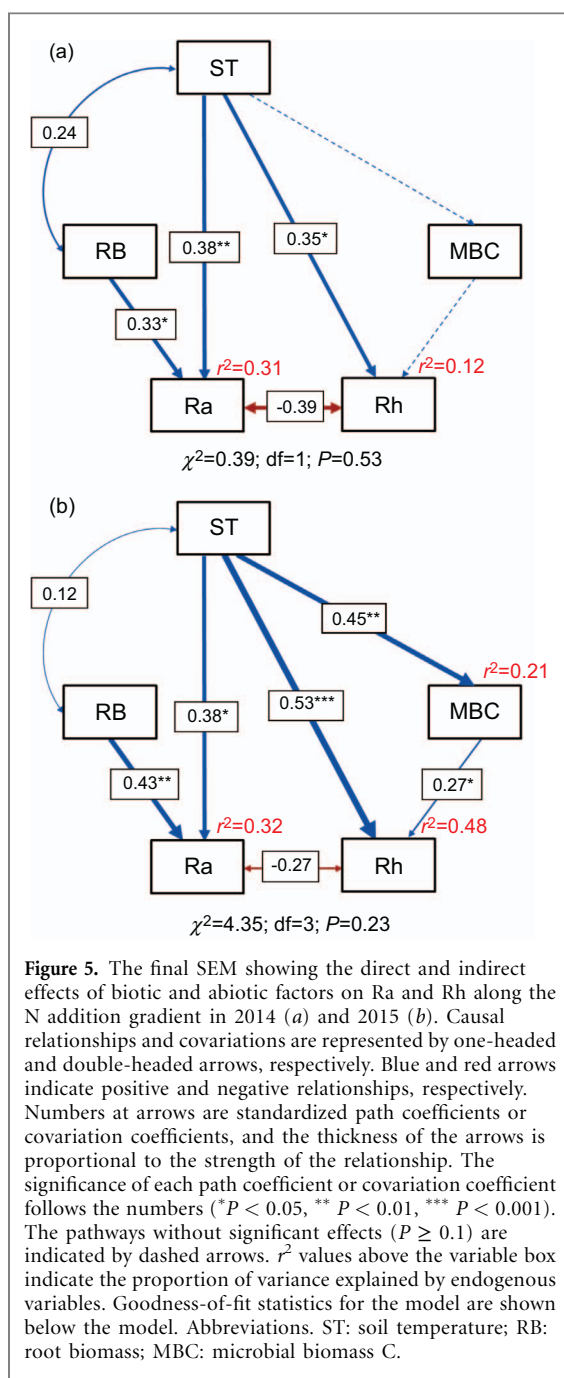
Figure 5. The final SEM showing the direct and indirect effects of biotic and abiotic factors on Ra and Rh along the N addition gradient in 2014 (*a*) and 2015 (*b*). Causal relationships and covariations are represented by one-headed and double-headed arrows, respectively. Blue and red arrows indicate positive and negative relationships, respectively. Numbers at arrows are standardized path coefficients or covariation coefficients, and the thickness of the arrows is proportional to the strength of the relationship. The significance of each path coefficient or covariation coefficient follows the numbers (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). The pathways without significant effects ($P \geq 0.1$) are indicated by dashed arrows. r^2 values above the variable box indicate the proportion of variance explained by endogenous variables. Goodness-of-fit statistics for the model are shown below the model. Abbreviations. ST: soil temperature; RB: root biomass; MBC: microbial biomass C.

4.3. Regulations of biotic and abiotic factors in Ra and Rh

Several biotic and abiotic factors regulate the change of Ra and Rh along this N addition gradient. The SEM analysis indicated that root biomass and MBC were direct controllers of Ra and Rh, and further, that soil temperature also directly and indirectly explained their variations (figure 4). Root biomass had a direct effect on Ra, because larger root biomass means greater root surface area for respiration (Zhou *et al* 2014). Consistent with this point, root biomass had a similar hump-shape response pattern to Ra along this N gradient (table 1). Before the N saturation (8 g N m^{-2} yr^{-1}), root biomass increased in both years (table 1). This is because during this time, albeit with the N

addition reducing growth limitation to a certain degree, plants are still under the limited-N condition. Thus, they need to allocate a portion of C to roots for additional N uptake to maintain plant growth (Peng and Yang 2016). Meanwhile after the saturation point, root biomass decreased; this was likely due to 1) decreased root production. In cold regions, such as the tundra or alpine steppe, experimental N addition generally decreased C input to roots (Peng *et al* 2017), especially under high levels of N addition (Sullivan *et al* 2007). This is likely because temperature is usually the major growth-limiting factor in these sites, and plants cannot absorb too much N due to the low growth rate even under N addition. Meanwhile, high N accumulation in mineral soils may be harmful for root growth (Marschner 2011), thus resulting in low production of roots. 2) increased root turnover rate, or in other words, reduced root life span. For instance, Bai *et al* (2008) reported that an addition of 32 g N m^{-2} yr^{-1} significantly reduced root life span from 81 d to 64 d in a 0–30 cm soil depth in a temperate steppe on the Inner Mongolian Plateau. Taken together, the decreased root production and increased turnover rate may cause reduced root standing biomass at high N addition levels.

Microbial biomass directly explained the variance in Rh along the gradient of N additions in 2015 (figure 4). Low N addition levels (from 1 to 4 g N m^{-2} yr^{-1}) gradually increased microbial biomass, but high levels of N addition suppressed it (table 1). The stimulation of microbes by low N may be due to the reduced C and N limitation after increased litter input from the first two years; furthermore, the decrease in the microbial biomass under the high N supply is potentially because the high dose of N addition reduced the C transfer to the rhizosphere, and induced shifts in the microbial community and decomposing enzymes; this would enhance the formation of recalcitrant compounds, thus causing decreased microbial respiration (DeForest *et al* 2004, Janssens *et al* 2010, de Vries *et al* 2014). However, under low N levels, the changes in the microbial biomass did not match well with the changes in Rh, and the underlining mechanisms need further investigation.

Soil temperature was the only environmental factor entering the final SEM (figure 5), likely indicating that it is the most important abiotic factor regulating Rh, while the effects of pH and inorganic N are relatively weaker along this N-addition gradient. Soil temperature not only directly explained the variations of Ra and Rh by regulating respiration enzymes (Atkin and Tjoelker 2003), but also indirectly influenced them via affecting root and microbial growth (Janssens *et al* 2010). We observed that soil temperature displayed a decreasing trend with increasing N additions, especially for those plots receiving a N rate of more than 8 g m^{-2} yr^{-1} (table 1). This is likely because N addition increased above-ground plant growth and the more closed canopy

prevented the incoming solar radiation to the soil surface (Luo and Zhou 2006). Consistent with this deduction, we observed a negative relationship between soil temperature and light interception in 2015 (figure S4). Thus, the CO_2 respired from roots and microbes could be reduced at high-level N input due to the decreased soil temperature, especially in cold regions where temperature is usually considered a limiting factor for living beings (Lloyd and Taylor 1994).

In grassland ecosystems, water availability is usually observed to constrain CO_2 flux with or without N additions (Harpole *et al* 2007, Xia *et al* 2009, Yan *et al* 2010). However, in our present experiment, we did not find any significant correlations of Rs, Ra and Rh with soil moisture along the N addition gradient (figures 3(*e*), (*i*), (*s*); figures 4(*e*), (*i*), (*s*)). This is likely because of the large moisture variation during both growing seasons (figure S5). Previous studies indicated that there might be a threshold of soil moisture in grasslands (8%–14% V/V%, Zhou *et al* 2007, Yan *et al* 2010), above which water is not the constraint of soil C process responses to climate change. Due to this point, it may be that, in aggregate, there were no detectable influences of the N-induced soil moisture changes on soil CO_2 fluxes since the soil moisture was above the critical level at most of our measurement times (figure S5). Nevertheless, as we mentioned above, precipitation-associated water changes are still very important in this alpine steppe because they regulate the seasonal dynamics and inter-annual variation of Rs and its components (figure 2).

5. Conclusions and implications

The present study revealed that, Rs and its autotrophic component displayed a nonlinear (hump-shaped) response to increasing N additions, while Rh declined linearly along the N levels. Soil temperature as well as root and microbial biomass explained the variations in Rs and its components. These findings have two important implications for understanding C-climate feedback in terrestrial ecosystems: 1) Many studies predicting the future terrestrial C balance have assumed a linear relationship between ecosystem C fluxes and N additions (e.g. Janssens *et al* 2010, Zhou *et al* 2014, Greaver *et al* 2016). However, the results observed in the current study, together with the nonlinear GPP and ANPP responses to increasing N inputs reported in previous studies (Arens *et al* 2008, Vivanco *et al* 2015), indicate that the linear relationship of the above and below-ground C fluxes with increasing N inputs assumed by previous studies may be inaccurate. Furthermore, nonlinear responses of various ecosystem components to increasing N additions are suggested when modeling the global C cycling, given that anthropogenic N input to the

terrestrial ecosystems is continuously increasing (Penuelas *et al* 2013). Meanwhile, the hump-shaped response pattern of Rs to continuous N addition are different from GPP and ANPP, which generally increased first and then plateaued after saturation level (see Arens *et al* 2008, Vivanco *et al* 2015). Therefore, Earth System Models also need to consider the different response patterns in C fluxes from various ecosystem components. 2) Since significant climate warming has been widely reported on the Tibetan Plateau during recent decades (Wang *et al* 2008), the negative N effect on soil temperature as revealed in our study highlights that increased N input might mitigate the positive C feedback, not only via the enhanced plant C accumulation but also through reduced warming effects on Rs. Overall, the nonlinear responses of Rs and its components to multiple N loads merit further attention in future experimental and modeling analyses.

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