

Response Letter

Michael Bahn
Co-Editor-in-Chief
Biogeosciences

Dear Dr. Bahn,

Thank you very much for offering us the chance to revise our manuscript “Initial shifts in nitrogen impact on ecosystem carbon fluxes in an alpine meadow: patterns and causes” (bg-2016-436). We have carefully considered the thoughtful and valuable comments and suggestions from you and the reviewers. The manuscript has been revised accordingly. Here are our detailed responses to the reviews. Please note that the comments from the reviewers are in *italics* followed by our responses in **bold** text.

Sincerely,

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Reply to Report #1

I have carefully read the manuscript and I think it still has the same issues I have raised in my first review of this manuscript. I still feel confused about the main story told here and the mechanisms invoked to explain main changes in NEE.

Response: Thanks for the critical comments. We are sorry about for the confusing statements. We have carefully revised the manuscript and clarified the main point and mechanisms more clearly. The specific comments are addressed below.

1) I understand that rates of NEE increase (i.e. more negative values) under mid levels of N additions 8 and 16 g N m⁻² yr⁻¹ in 2015 (second year of experiment), which seems to be the case when looking for example at Fig. 2d (this may suggest the occurrence of some sort of 'saturation' response, whose causes are far from clear).

Response: Thank the reviewer very much for the thoughtful comments. The reviewer is correct. Fig.2d showed the saturation response of NEE in 2015. Since NEE is the balance between GPP and ER, we explained the cause of NEE saturation by the response of GPP and ER. In Page10 Line21, we said that “The N saturation response of NEE in 2015 was mainly attributed to the saturation responses of ER and GEP (Fig. 2)”.

2) I am confused for example when the authors say that (page 14, line 18):”The saturation responses of NEE and ER were mainly caused by N-induced decreases in aboveground plant respiration and soil microbial respiration under high N addition rates”. However on page 13 line 21 the authors stated the opposite that: “In this study, greater plant growth and aboveground biomass under N addition enhanced aboveground plant respiration and thus stimulated ER”. In the results section (page 9 line 5), the authors also state that: “R_{above} increased with increasing N addition rates in 2014 (Fig. 4b) but got the maximum value at N16 in 2015 (Fig. 4e). If I look at Figs 4 d and e, these figures exactly show that plant respiration increased under increasing N additions. So why do the authors in the Conclusions state the opposite?

Response: We appreciate the reviewer very much for the thoughtful comments and really feel sorry for the confusion. We said the “saturation responses of NEE and ER were mainly caused by N-induced decreases in aboveground plant respiration and soil microbial respiration under high N addition rates”, because when comparing R_{above} at high N addition rate (32 gN m⁻² yr⁻¹) to that under the N saturation point (16 gN m⁻² yr⁻¹), higher N rate addition did cause decrease in R_{above} (Fig. 4e). When we compared R_{above} and ER under N addition rates of 2 - 16 gN m⁻² yr⁻¹ with those under the control, we found that “greater plant growth and aboveground biomass under N addition enhanced aboveground plant respiration and thus stimulated ER”. So, these descriptions are not really opposite. They were compared with different references. In order to avoid confusion, we have rephrased these confused sentences by emphasizing the references in the revised

MS (Page 14, Line 3).

Second, the negative trend of microbial respiration with N addition rates is not clear. If I look at Fig. 3d I don't understand why R_{mic} for example decreases around 16 g N $m^{-2} yr^{-1}$ but then almost increases again under the 32 g N $m^{-2} yr^{-1}$. Also if ER (ecosystem respiration) overall increases in 2015 and microbial respiration decreases in the same year, how could it be possible that the two data points are positively correlated in Fig. 6C? I think this graph should only show data for 2015 and not for 2014.

Response: We greatly appreciate the reviewer's thoughtful comments. If we analyzed the data year by year, we found that there was a negative relationship between ER and R_{mic} in 2015 (see Fig. R1). Based on the reviewer's suggestion, we showed the data for 2015 and not for 2014 (revised Fig. 6).

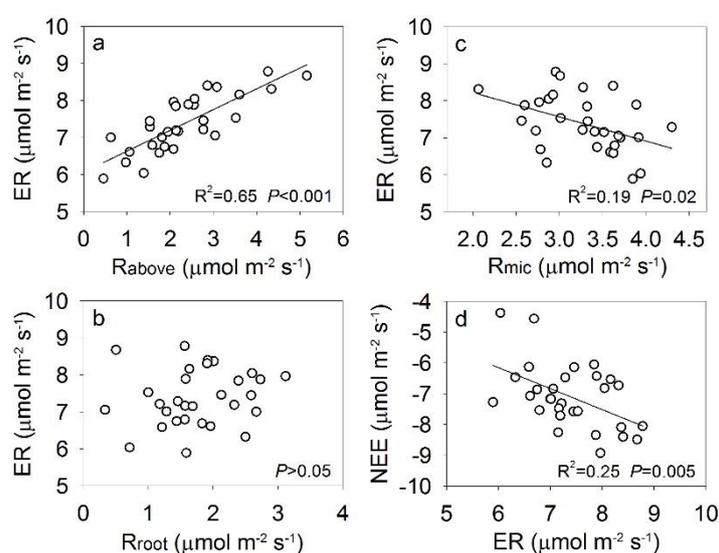


Fig. R1 Relationships between aboveground plant respiration (R_{above}), root respiration (R_{root}), soil microbial respiration (R_{mic}) and ecosystem respiration (ER) (a,b,c), ER and net ecosystem CO_2 exchange (NEE) (d) in 2015.

Actually, R_{mic} did not “increases again under the 32 gN $m^{-2} yr^{-1}$ ”. Both the mean values for R_{mic} at 16 and 32 gN $m^{-2} yr^{-1}$ were 2.97 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Please see the following column figure (Fig. R2). Thanks.

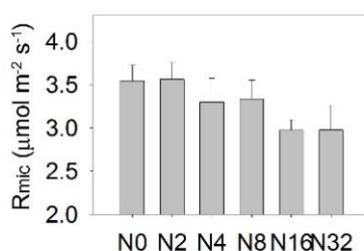


Fig. R2 Soil microbial respiration (R_{mic}) in response to the N addition gradient in 2015 (mean \pm SE, n = 5).

Overall, these contradictions and the lack of clarity in showing and linking the results with main discussion and conclusions make it very difficult to understand the potential contribution of this study to present knowledge.

Response: We have double checked the descriptions and clarified the results by emphasizing the references when make comparisons. Now we confirmed that the linkage between the results and main discussion and conclusion are correct and consistent. Hope the reviewer found our revision satisfactory. We greatly appreciate the editor for considering our manuscript!

The manuscript would need more language editing. These below are some suggestions: I would rephrase the first sentence (page 1, line 10) as:

“Increases in nitrogen (N) deposition can greatly stimulate ecosystem net carbon (C) sequestration through positive N-induced effects on plant productivity”.

Would rephrase second sentence (page 1, line 11) as: “However, how net ecosystem CO₂ exchange (NEE) and its components might respond to increases in N deposition remains unclear”.

Last sentence in abstract could be changed to: “Our findings bring evidence of short-term responses to increases in N deposition, which should be considered when predicting long-term changes in ecosystem net C sequestration”.

Response: Thank the reviewer very much for the valuable suggestions. These sentences in the abstract has been rephrased according to the suggestions (Page 1, Line 11-13; Page 2, Line 3-5).

Page 3 lines 16, change to: “It is not clear when and how ecosystem C fluxes...”

First sentence of Discussion should be changed to: “Our results show that initial ecosystem C fluxes (NEE, ER, and GEP) in 2014 suggest ecosystem N limitation, whereas in 2015 these C fluxes clearly suggest N saturation under high N addition rates”.

Lines 9-12, page 10, needs to be changed to: “These findings not only confirm the N saturation hypothesis for the response of NPP to N addition (Aber et al., 1998; Aber et al., 1989; Lovett and Goodale, 2011), but also provide comprehensive evidence of potential relationships between various ecosystem C fluxes and ecosystem N dynamics”.

Line 12, page 10: remove “most”

Line 14, page 10, change to: “Using one level of N addition only might not be enough to capture and quantify complex ecosystem responses to N addition...”.

Response: Thank the reviewer very much for the valuable suggestions. We have changed these sentences as suggested (Page 3, Line 19; Page 10, Line 11-15, 18).

Reply to Report #2

This study assesses the effect of nitrogen addition on ecosystem CO₂ fluxes across a nitrogen addition gradient in an alpine meadow. Hence, this set up allows for testing

nonlinear effects and for saturation of responses to N addition. By inserting collars into the soil, authors also partitioned soil respiration into root and microbial respiration, addition to the process understanding of responses to N addition.

Before this manuscript can be accepted for publication, I think some aspects need to be improved.

1. Deep versus shallow collars were used to partition root from microbial respiration. This is a common technique to use for this purpose, but just like other partitioning techniques, it does have some limitations. This should be discussed in the manuscript and also its implications for the observed responses of R_{mic} and R_{root} need to be discussed. For example, changes in plant C allocation in response to N addition can have a strong effect on R_{mic} , but are excluded by the deep collar method for R_{mic} assessment. Hence, authors could argue that R_{mic} responses are not solely plant-mediated.

Response: Thank the reviewer very much for the constructive comments and suggestions. We have added some discussion about the limitations of the partitioning technique we used (Page 14, Line 15-20).

2. The pH effects are overstated. A regression for R_{mic} versus pH is the only indication for a pH effect on R_{mic} , but this relationship is not necessarily causal. If authors would have had an indicator for N availability (e.g. NH_4 and NO_3 concentrations in soil), they would have found a strong relationship with R_{mic} too. To find out if pH is really a potential driver of R_{mic} , one would have to include multiple potential drivers (like NH_4 and NO_3 concentrations) to test which of the drivers best predicts R_{mic} .

Response: The reviewer is right. Soil NH_4^+ and NO_3^- may also be drivers of R_{mic} . We re-analyzed the data and found that the correlation coefficient of the relationship between ΔR_{mic} and soil ΔpH , ΔNH_4^+ and ΔNO_3^- was 0.77, 0.68, and 0.76, respectively. So, soil pH was the most important factor driving changes in R_{mic} . Previous studies with similar N addition gradient also suggested that soil pH was the most important driver for responses of microbes under high N addition rates (Liu et al., 2014; Chen et al., 2016).

Chen D, Li J, Lan Z, Hu S, Bai Y (2016) Soil acidification exerts a greater control on soil respiration than soil nitrogen availability in grasslands subjected to long-term nitrogen enrichment. *Functional Ecology*, 30, 658–669.

Liu W, Jiang L, Hu S, Li L, Liu L, Wan S (2014) Decoupling of soil microbes and plants with increasing anthropogenic nitrogen inputs in a temperate steppe. *Soil Biology and Biochemistry*, 72, 116-122.

3. Statistical analyses can be improved. Instead of averaging values over the year to assess the treatment effect, a linear mixed model with time as a random factor would be more appropriate.

Also the use of R^2 as a criterion for selecting a linear or a quadratic function is not the best. R^2 does not penalize overfitting, and thus gives a slight advantage to the quadratic function. Using for example AIC or BIC avoids this problem.

Response: We used repeated-measures ANOVA to examine N addition effects on the ecosystem C fluxes over the growing season in each year. The statistic results show as below.

Table R1 Results (*P* values) of repeated-measures ANOVA on the effects of nitrogen addition on ecosystem C fluxes in 2014 and 2015. NEE: net ecosystem CO₂ exchange, ER: ecosystem respiration, GEP: gross ecosystem production, SR: soil respiration, R_{mic}: soil microbial respiration.

	NEE	ER	GEP	SR	R _{mic}
2014	0.020	0.033	0.002	0.209	0.246
2015	0.059	0.006	0.038	0.010	0.259

As suggested by the reviewer, we used AIC method to re-analyze data and found the same results. Specifically, quadratic function works better than linear ones for ecosystem C fluxes in 2015. While in 2014, linear function works better than quadratic ones except SR. Please see the table below.

Table R2 Comparisons of Akaike information criterion (AIC) among functions describing the relationships between NEE, ER, GEP, SR and R_{mic} (Y) and N addition rate (X). NEE: net ecosystem CO₂ exchange, ER: ecosystem respiration, GEP: gross ecosystem production, SR: soil respiration, R_{mic}: soil microbial respiration.

Functions in 2015	Linear ¹	Quadratic ²
NEE	88.69	84.82
ER	90.12	82.30
GEP	87.69	77.68
SR	79.43	78.18
R _{mic}	85.15	84.48

Functions in 2014	Linear ¹	Quadratic ²
NEE	78.39	80.26
ER	71.68	73.48
GEP	77.96	79.86
SR	87.88	87.34
R _{mic}	78.33	80.27

1) linear model: $Y = b_1 + b_2 \times X$ 2) quadratic model: $Y = b_1 + b_2 \times X + b_3 \times X^2$

Specific comments:

bottom p2 - top p3: this needs rephrasing. Authors say that NEE may respond nonlinearly to a N addition gradient because GPP and ER can respond nonlinearly, but that is not totally correct. If GPP and ER respond nonlinearly in the same way, NEE does not change at all.

Response: Thanks for the suggestion. We have rephrased the relevant sentence and stated it more clearly (Page 3, Line 2-4).

p3, 115: 'at which time' should be 'at what N level' I suppose

Response: Thanks. We have rephrased this sentence (Page 3, Line 19).

p 5, 17: was the same amount of N given each month?

Response: Yes. We have clarified this in the revised MS (Page 5, Line 12).

p6, 116: CO2 fluxes in deep collars represent a proxy for Rmic (instead of Rmic) - see also earlier comment.

p11, 114: authors state that the decline in Rmic was primarily due to the pH effect, but this statement is not well supported as no other potentially important factors were assessed. I suggest authors read for example Janssens et al (2010, Nature Geoscience) to find out about other potential reasons for Rmic to decline in response to N addition. One other possibility for reduction of Rmic in response to N addition could be a shift towards more C efficient but N demanding microbial species (Agren et al 2001, Oecologia).

Response: Thank the reviewer very much for the thoughtful suggestions. Please see the above responses in detail. We have compared the effects of potential factors of soil pH, NH₄⁺, and NO₃⁻ in driving R_{mic}.

Based on the reviewer's suggestion, we have added some discussion about the potential impact of soil microbial communities on R_{mic} (Page 11, Line 16-17). We are sorry that we did not monitor changes of microbial community in this study. Conducting a field experiment is very tough in the Tibet Plateau with the altitude of 3600m, but we will do that in the future.

p 13: authors compare their results with those of other studies. In this, they totally ignore the initial N availability as well as presence of N fixers. Both are essential though to understand differences in N effects, N saturation and the reasons behind it. I assume N availability was not measured in this experiment, or in others, but authors should recognize its importance and make readers aware that N availability needs to be assessed to further improve the understanding (differences in) responses to N addition.

Response: Thanks for the comments. N availability was measured in our study and it increased linearly with the N addition gradient. Please see the figure below (Fig. R3).

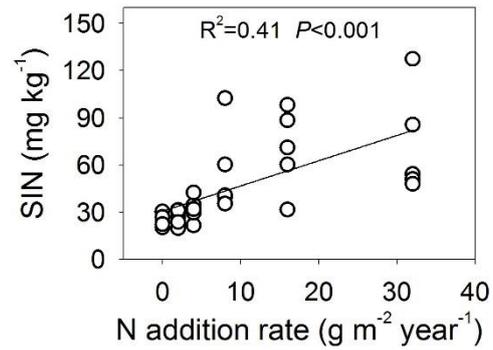


Fig. R3 Soil inorganic nitrogen (SIN, including NH₄⁺, and NO₃⁻) in response to the N addition gradient in 2015.

Last, although the manuscript reads quite well, it still contains quite a number of grammatical errors (like missing articles in several places) and weird phrasings. Thorough language editing is thus needed.

Response: Thanks for the suggestion. We have asked a native English speaker to edit the language throughout the paper.

Initial shifts in nitrogen impact on ecosystem carbon fluxes in an alpine meadow: patterns and causes

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Abstract

~~Increases in nitrogen (N) deposition can greatly stimulate ecosystem net carbon (C) sequestration through positive N-induced effects on plant productivity~~
~~The rising nitrogen (N) deposition could increase ecosystem net carbon (C) sequestration by stimulating plant productivity.~~ However, how net ecosystem CO₂ exchange (NEE) and its components ~~respond to different N addition rates remains unclear~~
~~respond dynamically to rising N deposition is far from clear.~~ Using a N addition gradient experiment (six levels: 0, 2, 4, 8, 16, 32 gN m⁻² year⁻¹) in an alpine meadow on the Qinghai-Tibetan Plateau, we explored the responses of different ecosystem C fluxes to a N addition gradient and revealed mechanisms underlying the dynamic responses. Results showed that NEE, ecosystem respiration (ER), and gross ecosystem production (GEP) all increased linearly with N addition rates in the first year of treatment, but shifted to N saturation responses in the second year with the highest NEE ($-7.77 \pm 0.48 \mu\text{mol m}^{-2} \text{s}^{-1}$) occurring under N addition rate of 8 gN m⁻² year⁻¹. The saturation responses of NEE and GEP were caused by N-induced accumulation of standing litter, which limited light availability for plant growth, under high N addition. The saturation response of ER was mainly due to ~~the N-induced saturation response of aboveground plant respiration and decreasing soil microbial respiration along the N addition gradient.~~

while decreases in soil microbial respiration under high N addition, ~~which was were~~ caused by ~~the~~-N-induced reductions in soil pH. We also found that various components of ER, including aboveground plant respiration, soil respiration, root respiration, and microbial respiration, responded differentially to the N addition gradient. These results reveal temporal dynamics of N impacts and the rapid shift of ecosystem C fluxes from N limitation to N saturation. Our findings bring evidence of short-term initial shifts of responses of ecosystem C fluxes to increases in N deposition, which should be considered when predicting long-term changes in ecosystem net C sequestration~~These findings are helpful for better understanding and model projection of future terrestrial C sequestration under rising N deposition.~~

10 **1 Introduction**

Anthropogenic reactive nitrogen (N) inputs to the terrestrial biosphere has increased more than threefold over the past century and is predicted to increase further (Lamarque et al., 2005; Galloway et al., 2008). Because of the strong coupling of ecosystem carbon (C) and N cycles, excess N deposition could have significant impacts on ecosystem C cycle (LeBauer and Treseder, 2008; Liu and Greaver, 2010; Lu et al., 15 2011). Ecosystem net C sequestration is predicted to increase or have no significant change under rising N deposition (Nadelhoffer et al., 1999; Magnani et al., 2007; Reay et al., 2008; Niu et al. 2010; Lu et al., 2011; Fernandez-Martinez et al., 2014). However, we have limited understanding on the dynamics N responses of C sequestration in terrestrial ecosystems, which is crucial for model projection of future terrestrial C cycle under rising N deposition (Reay et al., 2008).

20 Although N addition generally enhances plant growth and ecosystem net primary productivity (NPP) based on global syntheses of N addition experiments (LeBauer and Treseder, 2008; Xia and Wan, 2008;

Lu et al., 2011), the responses of ecosystem C fluxes vary with N loading rates (Liu and Greaver, 2010; Lu et al., 2011). According to N saturation hypothesis, NPP is assumed to slowly increase with N addition rates first, then get at the maximum value at N saturation point and finally decline with further increase of N input (Aber et al., 1989; Lovett and Goodale, 2011). During this process, ~~ecosystem-NPP~~ shifts from a N limited, a N intermediate, to a N saturation stage as N deposition increases. Similarly, the response of net ecosystem CO₂ exchange (NEE) ~~to rising N deposition is likely nonlinear (Tian et al., 2016), because and~~ its components of gross ecosystem production (GEP) and ecosystem respiration (ER) may ~~also~~ respond nonlinearly to increasing N loading rates (Fleischer et al., 2013; Gomez-Casanovas et al., 2016; Tian et al., 2016). In the N limited stage, low rates of N addition could stimulate ecosystem productivity (Aber et al., 1989), GEP (Fleischer et al., 2013; Gomez-Casanovas et al., 2016), and ER (Hasselquist et al., 2012; Zhu et al., 2016), while in the N saturation stage, high doses of N addition could have negative effects on GEP and ER (Treseder, 2008; Janssens et al., 2010; Maaroufi et al., 2015). The unbalanced responses of GEP and ER may lead to changes in NEE.

Moreover, ER can be divided into aboveground plant respiration, belowground plant respiration (root respiration), and soil microbial respiration. These components of ER could be affected by plant aboveground biomass, root biomass, soil organic matter and microbial biomass C, which may respond variously to N addition (Phillips and Fahey, 2007; Hasselquist et al., 2012). For example, root respiration would be enhanced or not significantly changed under N addition, while soil microbial respiration may be suppressed by N addition (Zhou et al., 2014). The different responses of various components of ER to N addition will also consequently change ~~NEE-the~~ response of NEE. Nevertheless, there is limited knowledge on how various components of NEE respond differentially to N addition gradient. In addition,

the N responses of ecosystem C fluxes may shift with time because of changes in plant community structure and other limiting factors (Niu et al., 2010). ~~It is not clear when and how ecosystem C fluxes~~
~~don't know yet at which time ecosystem C fluxes~~ get N saturated under increasing N input. The mechanisms underlying the saturation response of C fluxes are even far from clear, which hinders us from accurately predicting the C cycle in response to rising N deposition.

In this study, we explored the responses of various ecosystem C cycle processes to a N addition gradient in an alpine meadow on the Qinghai-Tibetan Plateau. The Qinghai-Tibetan Plateau has an area of 2.5 million km² with alpine meadow covering 35 % of this area, and it is sensitive to environmental change and human activities (Chen et al., 2013). The objectives of this study were to explore how different components of ecosystem C fluxes respond to increasing N loading gradient. Specifically, we addressed the following questions: (i) how do NEE and its components respond to N addition gradient in the alpine meadow? (ii) whether various C cycle processes can get N saturated? If so, at which N addition level they are saturated and how do the responses shift with time? and (iii) what are the mechanisms underlying N saturation responses of different C cycle processes?

2 Materials and methods

2.1 Study site

The study site is located in an alpine meadow in Hongyuan County, Sichuan Province, China, which is on the eastern Qinghai-Tibetan Plateau (32°48' N, 102°33' E). The altitude is ~3500 m. Long-term (1961-2013) mean annual precipitation is 747 mm with approximately 80 % occurring in May to September. Long-term mean annual temperature is 1.5 °C with monthly mean temperature ranging from -9.7 °C in

January to 11.1 °C in July. The dominant species in this alpine meadow are *Deschampsia caespitosa* (Linn.) Beauv., *Kobresia setchwanensis* Hand. -Mazz., *Carex schneideri* Nelmes, and *Anemone rivularis* Buch.-Ham.. The vegetation cover of this grassland is over 90 %. The soil in the study site is classified as Mat Cry-gelic Cambisol according to the Chinese classification, with surface soil bulk density being 0.89 g cm⁻³. The soil organic C content and total N content are 37 gC kg⁻¹ and 3.5 gN kg⁻¹, respectively. The background N deposition is ranging from 0.87 to 1.38 gN m⁻² year⁻¹ on the eastern Qinghai-Tibetan Plateau, and the natural N deposition rate in China is ranging from 0.11 to 6.35 gN m⁻² year⁻¹ (Lü and Tian, 2007).

2.2 Experimental design

We conducted a N addition experiment with six levels of N addition rate (0, 2, 4, 8, 16, 32 gN m⁻² year⁻¹) in early 2014. The six N treatments were represented by N0 (control), N2, N4, N8, N16, and N32, respectively. The treatments were randomly assigned with five replications, so there were totally 30 plots. Each plot was 8 × 8 m, and the distance between any two adjacent plots was 3 m. The N addition treatments started from May, 2014. In 2014 and 2015, N was applied by hand as NH₄NO₃ (>99 %) every month from May to September (i.e. during the growing season) before rainfall. The N amount was same in each month. In order to distribute dry NH₄NO₃ evenly in the plots, we mixed dry NH₄NO₃ with enough amounts of soil to apply.

2.3 Ecosystem C cycle properties and soil pH measurement

Ecosystem C fluxes were measured using a transparent static chamber (0.5 × 0.5 × 0.5 m) attached to an

infrared gas analyzer (LI-6400XT; LI-COR Environmental, Lincoln, Nebraska, USA) in the field. During each measurement, the chamber was positioned over a square steel frame, which was permanently inserted into soil and offered a flat base for the chamber. Inside the chamber, two electric fans were mounted in order to mix the chamber atmosphere. The measurements were conducted twice per month on clear, sunny days from May to September in 2014 and 2015. Nine consecutive recordings of CO₂ concentration were taken on each base at 10-second intervals. CO₂ flux rates were determined from the time-courses of the concentrations to calculate net ecosystem CO₂ exchange (NEE). After the measurement of NEE, the chamber was covered by an opaque cloth and the CO₂ measurement was repeated. As the second measurement eliminated light, the CO₂ flux value obtained represented ecosystem respiration (ER). Gross ecosystem production (GEP) was calculated as the difference between NEE and ER. Negative or positive NEE and GEP values represent net C uptake or release, respectively. The detailed methods have also been described in Niu et al. (2008) and Niu et al. (2013).

Soil respiration (SR) was assessed following the measurement of NEE and ER. It was also measured with LI-6400XT attaching a soil CO₂ flux chamber (991 cm³ in total volume; LI-6400-09; LI-COR Environmental, Lincoln, Nebraska, USA). A PVC collar (10.5 cm in diameter and 5 cm in height) was permanently installed 2-3 cm into the soil. The soil respiration chamber attached to LI-6400XT was placed on each PVC collar for 1-2 minutes to measure SR. Living plants inside the collars were removed regularly by hand to eliminate aboveground plant respiration. Soil heterotrophic respiration (i.e. soil microbial respiration, R_{mic}) was measured using the same method as soil respiration. Differently, the PVC collar was 40 cm in height and installed 36-38 cm into the soil. As >90 % of plant roots were distributing in the topsoil (0-20 cm), 40-cm-long PVC collars could cut off old plant roots and prevented new roots

from growing inside the collars. Plants in the collars were completely removed by hand to exclude C supply. The experiment was conducted in early 2014 and the measurements of CO₂ fluxes above these 40-cm-long PVC collars began in late July in 2014, leaving enough time for the remaining plant roots inside the collars to die. Thus CO₂ fluxes in those deep collars represented R_{mic}. The method was same to
5 Wan et al. (2005) and Zhou et al. (2007). Root respiration (R_{root}) was calculated by value of SR minus R_{mic}. Aboveground plant respiration (R_{above}) was calculated by ER minus SR, and ecosystem plant respiration (R_{plant}) was calculated as the difference between ER and R_{mic}. All the measurements of ecosystem C fluxes were simultaneous.

10 Soil samples were collected from the topsoil (0-10 cm) of the 30 plots on August 15, 2014 and August 14, 2015. Two soil cores (8 cm in diameter and 10 cm in depth) were taken at least 1 m from the edge in each plot, and then completely mixed to get a composite sample. The soil samples were sieved by a 2 mm mesh and then were air-dried for chemical analysis. Soil pH was determined with a glass electrode in a 1:2.5 soil:water solution (w/v).

15 **2.4 Statistical analysis**

Repeated-measures ANOVA (RMANOVA) was used to examine N addition effects on each ecosystem C flux over the growing season in 2014 and 2015. When we evaluate N addition effects on the different components of ER and their proportions, we averaged their values across the year and then used one-way ANOVA to test the differences among treatments. To test the response pattern of ecosystem C cycle
20 properties to the N addition gradient, we fitted the response parameter to linear or quadratic functions which had ~~the highest~~ higher R². Simple linear regression analyses were used to evaluate relationships of

ER with its components and NEE across the two years. ΔR_{mic} and ΔpH were calculated by data in different N addition treatments minus data in the control treatment. All data were tested for normal distribution before statistical analysis. The a posteriori comparisons were performed by DUNCAN test, and the effects were considered to be significantly different if $P < 0.05$. All statistical analyses were conducted with SAS V.8.1 software (SAS Institute Inc., Cary, North Carolina, USA).

3 Results

3.1 NEE and its components in response to N addition gradient

Net ecosystem CO₂ exchange (NEE) varied throughout the growing seasons in both 2014 and 2015. The maximum rates of net CO₂ uptake (indicated by large negative values of NEE) occurred in July in both years (Fig. 1a,d). N addition had a significant impact on NEE in 2014 ($P=0.020$) and a marginally significant effect in 2015 ($P=0.059$) (Table 1). Mean NEE across months had different responses to the N addition gradient between the two years (Fig. 1a,d). It increased linearly with N addition rates in 2014 (Fig. 2a), but shifted to a saturating response with N addition rates in 2015 (Fig. 2d). The largest NEE was $-7.77 \pm 0.48 \mu\text{mol m}^{-2} \text{s}^{-1}$ under $8 \text{ gN m}^{-2} \text{ year}^{-1}$ addition rate (N8) in 2015.

The N addition gradient had significant effects on ER ($P=0.033$ and 0.006 , respectively) and GEP ($P=0.002$ and 0.038 , respectively) in both 2014 and 2015 (Table 1). Similar to NEE, both ER and GEP showed linear responses to N addition rates in 2014 but shifted to saturation responses in 2015 (Fig. 2). On average, ER was enhanced by 0.9-16.1 % in 2014 and 7.9-23.7 % in 2015 under different N addition treatments. GEP was increased by 2.4-19.2 % in 2014 and 6.7-20.5 % in 2015 under different N addition levels, with maximal values being $-24.40 \pm 0.48 \mu\text{mol m}^{-2} \text{s}^{-1}$ under $32 \text{ gN m}^{-2} \text{ year}^{-1}$ in 2014 and -15.38

$\pm 0.72 \mu\text{mol m}^{-2} \text{s}^{-1}$ under $16 \text{ gN m}^{-2} \text{ year}^{-1}$ in 2015 (Fig. 2).

3.2 Components of ecosystem respiration in response to N addition gradient

We divided ER into aboveground plant respiration (R_{above}), soil respiration (SR), root respiration (R_{root}),
5 and microbial respiration (R_{mic}), and found that different ER components showed diverse responses to N
addition gradient. Mean SR across months was not significantly changed by N addition gradient in 2014
(Table 1; Fig. 3). However, in 2015, it ranged from $4.98 \pm 0.33 \mu\text{mol m}^{-2} \text{s}^{-1}$ to $6.23 \pm 0.23 \mu\text{mol m}^{-2} \text{s}^{-1}$
under different N addition levels, with significant reduction under high N addition levels of 16 and 32 gN
 $\text{m}^{-2} \text{ year}^{-1}$ ($P=0.010$; Fig. 3). Additionally, the relationship between SR and N addition rates was not
10 significant in 2014 (Fig. 3a), while SR leveled off under high N addition rates in 2015 (Fig. 3c).
Interestingly, R_{mic} increased linearly with N addition rates in 2014 (Fig. 3b), while it decreased with N
addition rates in 2015 (Fig. 3d). Comparing among various components of ER, only R_{mic} showed
distinctively inverse responses to N addition rates between years. ~~All other components of ER generally
showed similar response tendency between two years (Fig. 3a,3c,4).~~ R_{above} increased with increasing N
15 addition rates in 2014 (Fig. 4b) but got the maximum value at N16 in 2015 (Fig. 4e). By contrast, R_{root}
decreased with increasing N addition rates in 2014 (Fig. 4c), while it had no statistically significant
response to N addition gradient in 2015 (Fig. 4f).—

In addition, the proportions of different efflux components to ER differed in response to N addition
gradient between years (Fig. 5). The proportions of R_{above} to ER kept increasing with N addition rates in
20 2014 but got saturated at N16 in 2015 (Fig. 5a,d). The proportions of R_{root} to ER ranged from $31.90 \pm$
 6.69% in N0 plots to $11.18 \pm 1.28 \%$ in N32 plots in 2014 (Fig. 5b), but was not significantly different

among N addition levels in 2015 (Table 1; Fig. 5e). In 2014, the contributions of R_{mic} to ER did not significantly change under N addition treatments (Table 1; Fig. 5c), whereas they declined along the N addition gradient in 2015 (Fig. 5f).

5 3.3 Causes for the N saturation responses of ecosystem C fluxes

In order to examine the causes for the N saturation responses of NEE and ER in 2015, we examined the relationship between ER and its various components and also NEE. The results showed that ER had significantly positive correlation with R_{above} ~~and R_{mic}~~ (Fig. 6a,e) but not with R_{root} (Fig. 6b), and had significantly negative correlation with R_{mic} (Fig. 6c). Moreover, NEE closely correlated with ER (Fig. 6d). We further explored the causes for decreasing R_{mic} with N addition in 2015 and found that N addition significantly reduced soil pH in 2015 (Fig. 7a). N-induced reduction in soil microbial respiration (ΔR_{mic}) was positively dependent on N-induced reduction in soil pH (ΔpH) in 2015 (Fig. 7b), but not in 2014.

4 Discussion

15 4.1 Nitrogen saturation responses of ecosystem C fluxes and the causes

Our results showed that initial ecosystem C fluxes (NEE, ER, and GEP) ~~in this alpine meadow were in a N limitation stage~~ in 2014 suggested ecosystem N limitation, whereas in 2015 these C fluxes clearly suggested N saturation under high N addition rates; but in 2015, they were in the limitation stage at low N addition rates and shifted to the saturation stage at high N addition rates. These findings not only ~~confirm-extend~~ the N saturation hypothesis ~~proposed~~ for the response of NPP to N addition (Aber et al., 1998; Aber et al., 1989; Lovett and Goodale, 2011). ~~Beyond that, this study, but also~~ provides ~~more~~

comprehensive evidence ~~for of potential relationships between~~ various ecosystem C fluxes and ~~more details on the dynamic N responses ecosystem N dynamics~~. Most ~~p~~previous N addition studies used only one level of N addition and found that NEE showed a positive (Niu et al., 2010; Huff et al., 2015) or no significant response (Harpole et al., 2007; Bubier et al., 2007) to N addition. Using one level of N addition only might not be enough to capture or quantify complex ecosystem responses to N addition~~One level of N addition could not give solid assessment and quantification of ecosystem responses to N addition~~. By using a N addition gradient experiment, this study comprehensively showed the saturation responses of NEE and its components to different N loading rates.

The N saturation response of NEE in 2015 was mainly attributed to the saturation responses of ER and GEP (Fig. 2), while the N saturation response of ER was likely caused by the saturation response of aboveground plant respiration and decreasing soil microbial respiration along the N addition gradient. The decrease of aboveground plant respiration under N32 treatment was primarily due to that N addition stimulated plant growth and thus standing litter accumulation after plant senescence (Fig. S1-S2). In 2014, plant aboveground biomass (AGB) was stimulated under high N addition treatment, especially AGB of grasses (Fig. S2). In this grassland, grasses usually have higher height than other plants. The accumulation of grasses standing litter under N32 treatment limited light condition for other plants and negatively influenced plant growth in the early growing season in 2015. Therefore, GEP and NEE did not keep increasing at the highest N addition rate, leading to N saturation response. The N-induced light limitation for plant growth was also observed in other ecosystems, like temperate grassland (Niu et al., 2010; Kim and Henry, 2013). Moreover, our results showed that most components of ER had similar response patterns between the two years except soil microbial respiration that increased in 2014 but decreased in

2015 along with N addition rates. ~~The relationships between ER and soil microbial respiration (Fig. 6e) indicate that the decrease of microbial respiration contributes to the reduction of ER under high N addition rates in 2015.~~ Thus, we propose that soil microbial respiration might play a key role in mediating the N saturation effects for ER and thus NEE, which is not reported in previous studies. The decline of microbial respiration under high N addition conditions was primarily due to the N-induced reduction in soil pH (Fig. 7). Although many factors can influence soil microbial respiration, such as soil N availability and microbial community structure (Janssens et al., 2010), ~~p~~Previous ~~study~~ studies with similar N addition gradient suggested that soil pH was the most important driver for responses of microbes under high N addition rates (Liu et al., 2014; Song et al., 2014; Chen et al., 2016). N addition can lead to soil acidification and bring negative impacts on soil microbial growth and activities (Liu et al., 2014; Tian et al., 2016). In this study, the decreased soil pH may cause toxicity effects on microbial activity (Treseder, 2008; Zhou et al., 2012) and thus reduces microbial respiration after two years of N addition.

4.2 The time and N threshold for the saturation responses

Our findings demonstrate that N responses of ecosystem C fluxes shifted from linear response to saturation response over the two years of treatments (Fig. 2). A recent study revealed that ecosystem C fluxes exhibited saturating responses to N addition during two consecutive measurement years in a temperate grassland (Tian et al., 2016). However, their measurement was conducted after ten years of N addition treatments (similar N addition rates with our study), so it did not capture the early response signals of ecosystem C exchange. Results of another N addition gradient experiment carried out in three marsh ecosystems showed that aboveground plant biomass increased linearly with N addition rates after

seven months of treatment, but showed saturating responses after 14 months of N addition (Vivanco et al., 2015). Taken together with our results, it suggests that N saturation of ecosystem C fluxes might happen within couple years of N input. The different responses between years in this study are not likely due to climate differences, because temperature and precipitation were not significantly different between 2014 and 2015. We acknowledge that our findings are just based on the short-term study, while long-time experiment may capture more robust patterns on N saturation and the underlying mechanisms, but the findings of the initial shift of N responses are helpful to better understand the dynamics of ecosystem in response to external N input.

The N saturation threshold for ecosystem C fluxes of this alpine meadow is approximately $8 \text{ gN m}^{-2} \text{ year}^{-1}$. This level is much higher than that in an alpine steppe on the Qinghai-Tibetan Plateau (Liu et al., 2013). In Liu et al.'s study, biomass N concentration, soil N_2O flux, N-uptake efficiency and N-use efficiency showed saturating responses at N addition rate of $4 \text{ gN m}^{-2} \text{ year}^{-1}$. The discrepancy is probably caused by different precipitation at the two sites. The precipitation is 747 mm in our study site and is 415 mm in their study site. The lower precipitation may constrain ecosystem's response to N addition in Liu et al. (2013). Likewise, the N saturation load in our alpine meadow is higher than that in an alpine dry meadow in Colorado (Bowman et al., 2006) and is comparable with that in a temperate steppe of Eurasian grasslands which found a saturation N addition rate of approximately $10.5 \text{ gN m}^{-2} \text{ year}^{-1}$ (Bai et al., 2010). The higher saturation levels indicate that this alpine meadow is more limited by N comparing with other resources. Furthermore, the N critical load for causing changes in ecosystem C cycle processes is around $2 \text{ gN m}^{-2} \text{ year}^{-1}$ in this alpine meadow. In the first year, ecosystem C exchanges were not significantly different between N0 and N2 treatments, but C fluxes were greater in N2 plots than that in N0 plots in the

second year (Fig. 1). This threshold for triggering changes in ecosystem C fluxes is comparable to that in another alpine meadow on the mid-south of the Tibetan Plateau (Zong et al., 2016). Considering that atmospheric wet N deposition is ranging from 0.87 to 1.38 gN m⁻² year⁻¹ on the eastern Qinghai-Tibetan Plateau (Lü and Tian, 2007), our estimate on N critical load suggests that ecosystem C cycle may be largely affected under future N deposition in the alpine meadow of Qinghai-Tibetan Plateau.

4.3 Diverse responses of C flux components to N addition gradient

The components of ER showed diverse responses to the N addition gradient (Fig. 4,5). For example, in 2014, aboveground plant respiration and its proportion to ER increased, but belowground plant respiration and its proportion to ER decreased with N addition amounts (Fig. 4b,c, Fig.5a,b). ~~Microbial respiration decreased and its proportion to ER did not change with the N addition gradient.~~ To our knowledge, no previous study examined different components of ER in response to N addition gradient. Some studies conducted in alpine grassland demonstrated that N addition had no significant effects on ER (Jiang et al., 2013; Gong et al., 2014), since aboveground biomass did not respond to N addition in their studies. In this study, comparing to the control treatment (without N addition), greater plant growth and aboveground biomass under N addition—_enhanced aboveground plant respiration and thus stimulated ER. The lack of N effect on soil respiration (SR) in 2014 may be attributed to the counteractive responses of soil microbial respiration and root respiration to N addition. In the first year, N addition ameliorated the nutrient limitation for microbes, thus soil microbial activity and biomass increased in short term (Treseder, 2008) and subsequently stimulated microbial respiration (Peng et al., 2011). On the other hand, N addition could reduce belowground biomass allocation (Haynes and Gower, 1995), leading to decrease in root respiration.

The increase of soil microbial respiration partly offsets the decrease of root respiration. As a result, SR had no significant difference among N treatments in the first year. However, in the second year, soil microbial respiration declined under high N addition levels, in combination with the low root respiration, resulting in decreases of SR under N16 and N32 treatments. This decrease in SR was also observed in other ecosystems under long-term or high levels of N addition (Yan et al., 2010; Zhou and Zhang, 2014; Maaroufi et al., 2015). ~~In summary, these results indicate that ER and its components could respond to N addition gradient in different ways.~~ We are fully aware that there are some limitations for the partitioning technique, by which we used deep versus shallow collars to partition root from microbial respiration. This approach excludes effects of changes in plant C allocation on microbial respiration. However, microbial respiration responses are not solely plant-mediated (Janssens et al., 2010). The method we used is still a common and useful technique to partition the components of ER, which is widely used in previous studies (Wan et al., 2005; Zhou et al., 2007).

5 Conclusions

Based on a field N addition gradient experiment, this study tested N saturation theory against multiple C cycle processes ~~and~~. We found that ecosystem C fluxes of NEE, GEP, and ER shifted from linear responses to saturation responses over two years of N addition. The saturation responses of NEE and ER were mainly caused by the N-induced saturation response of aboveground plant respiration and decreasing soil microbial respiration along the N addition gradient. ~~decreases in aboveground plant respiration and soil microbial respiration under high N addition rates.~~ Furthermore, N-induced reduction in soil pH was the main mechanism underlying declines in microbial respiration under high N addition.

~~The N critical load for causing ecosystem C fluxes changes and the N saturation threshold in this alpine meadow were 2 and 8 gN m⁻² year⁻¹, respectively.~~ We also revealed that various components of ER, including aboveground plant respiration, soil respiration, root respiration, and microbial respiration, responded differentially to N addition gradient. The findings suggest that the C cycle processes have differential responses to N addition between aboveground and belowground plant parts, and between plants and microbes. Our findings provide experimental evidences for the dynamic N responses of ecosystem C cycle, which is helpful for parameterizing biogeochemical models and guiding ecosystem management in light of future increasing N deposition.

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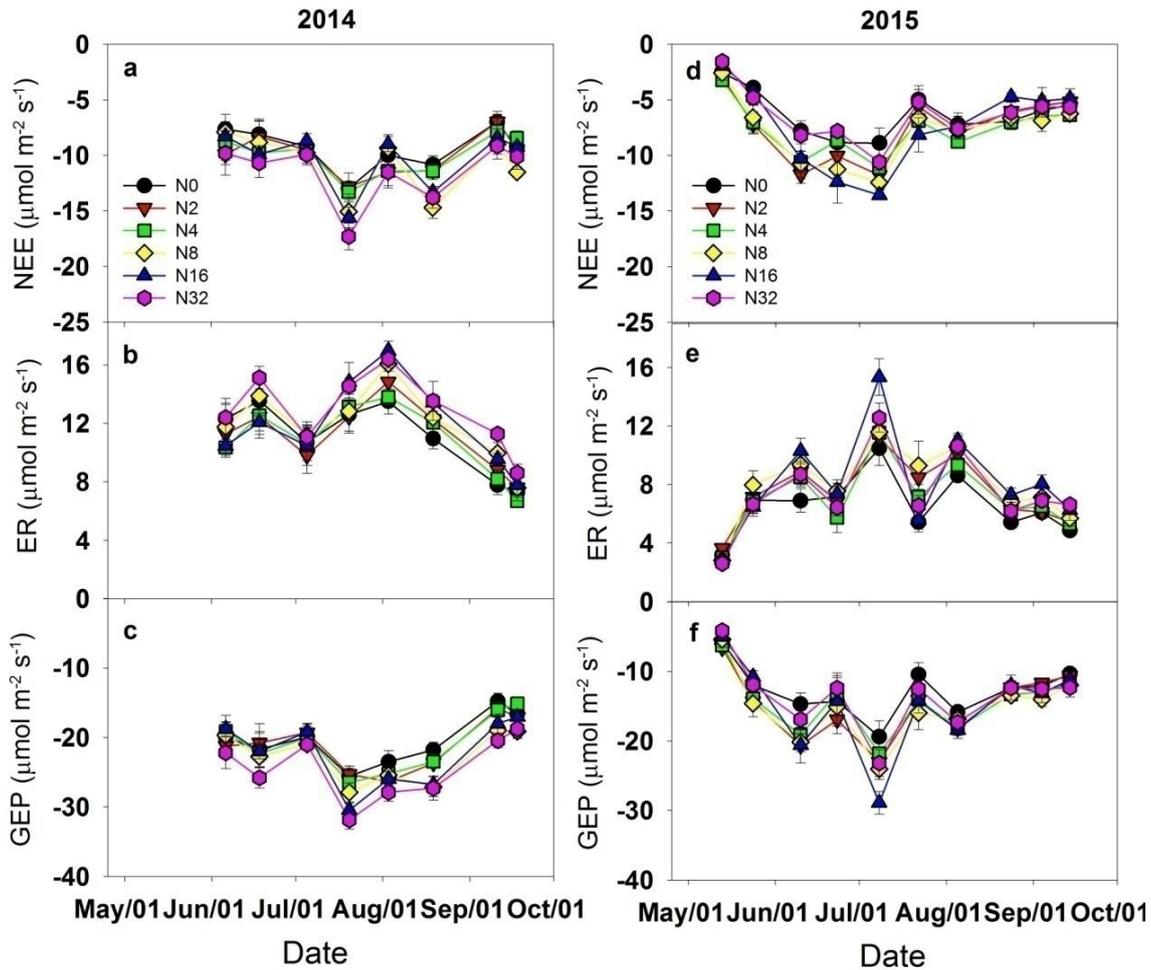


Figure 1. Seasonal dynamics of net ecosystem CO₂ exchange (NEE) (a, d), ecosystem respiration (ER) (b, e), and gross ecosystem production (GEP) (c, f) in 2014 and 2015. N0, N2, N4, N8, N16, N32 represent N addition rate of 0, 2, 4, 8, 16, 32 gN m⁻² year⁻¹, respectively.

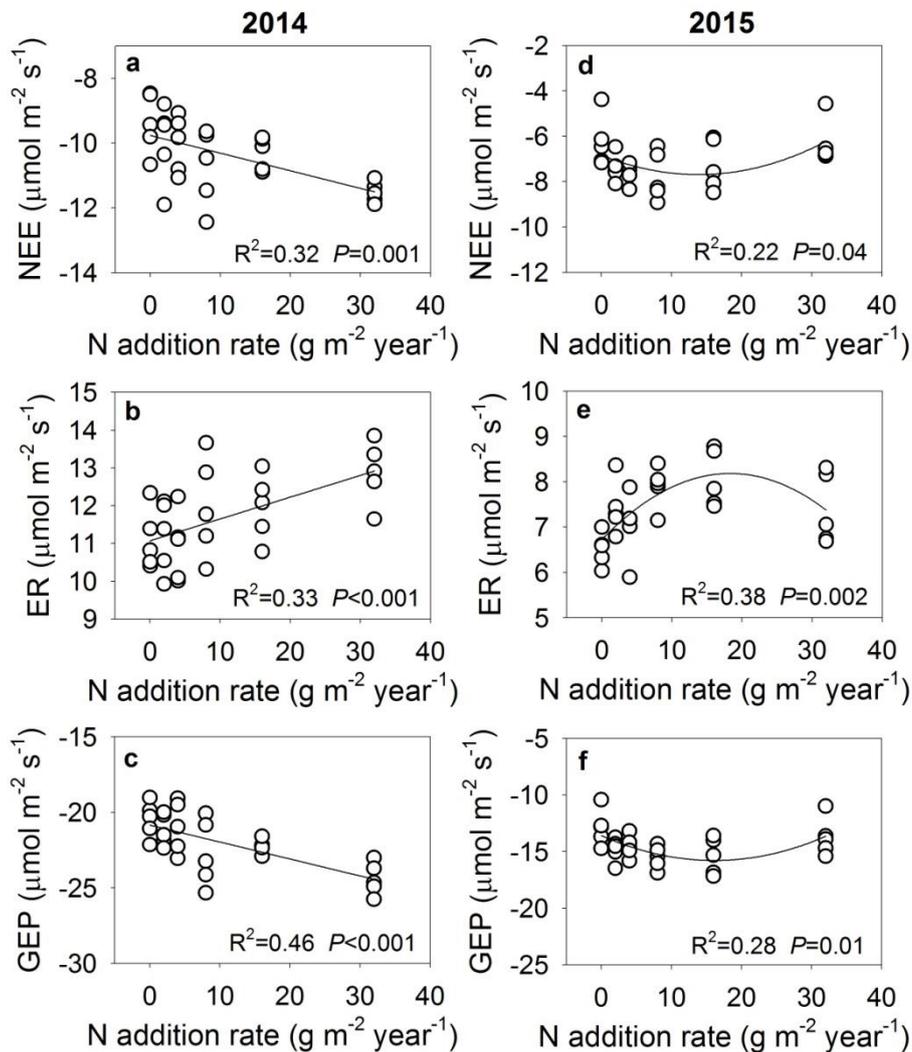


Figure 2. Relationships between N addition rate and net ecosystem CO₂ exchange (NEE) (a, d), ecosystem respiration (ER) (b, e), and gross ecosystem production (GEP) (c, f) in 2014 and 2015.

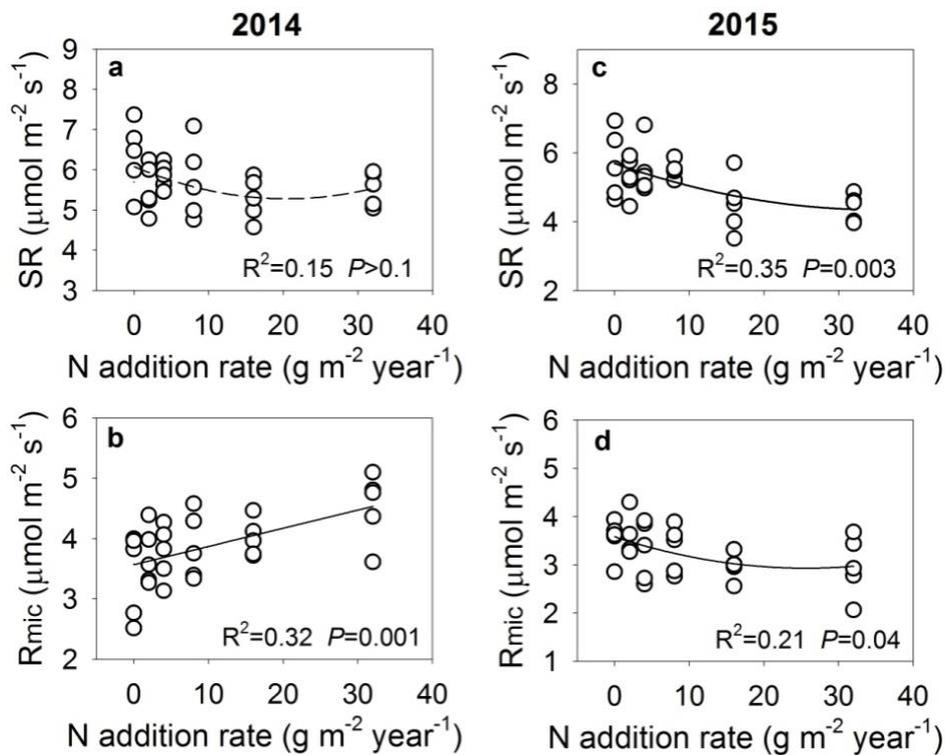


Figure 3. Relationships between N addition rate and soil respiration (SR) (a, c), and soil microbial respiration (R_{mic}) (b, d) in 2014 and 2015.

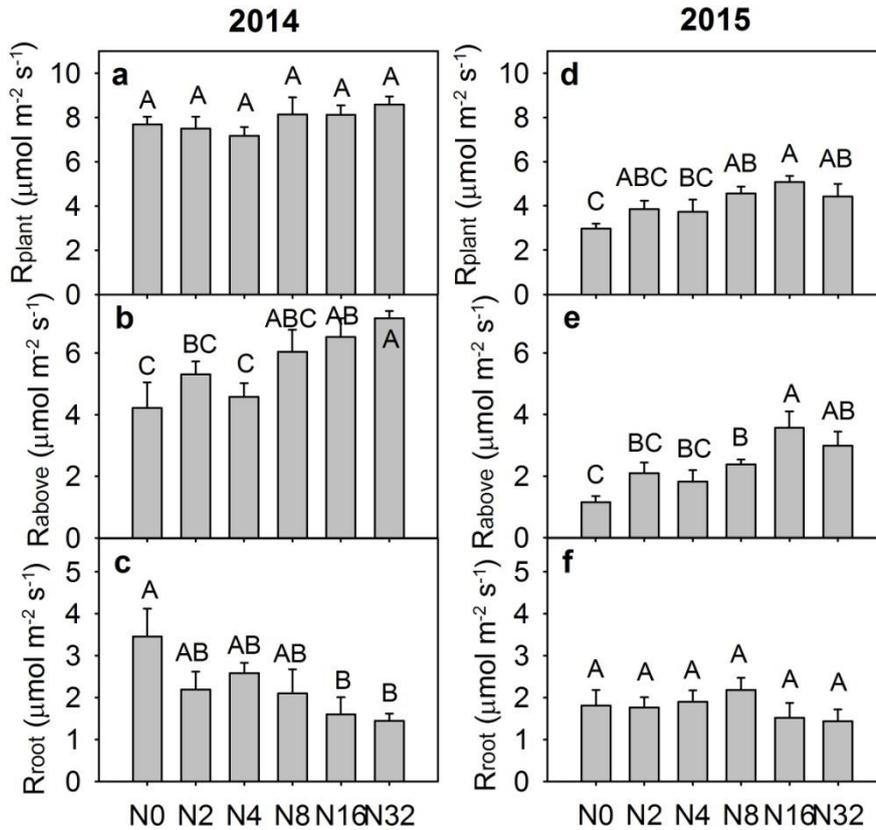


Figure 4. Plant respiration and its components in response to the N addition gradient in 2014 and 2015 (mean \pm SE, n = 5). R_{plant} : plant respiration (a, d), R_{above} : aboveground plant respiration (b, e), R_{root} : plant root respiration (c, f). N0, N2, N4, N8, N16, N32 represent N addition rate of 0, 2, 4, 8, 16, 32 gN m⁻² year⁻¹, respectively.

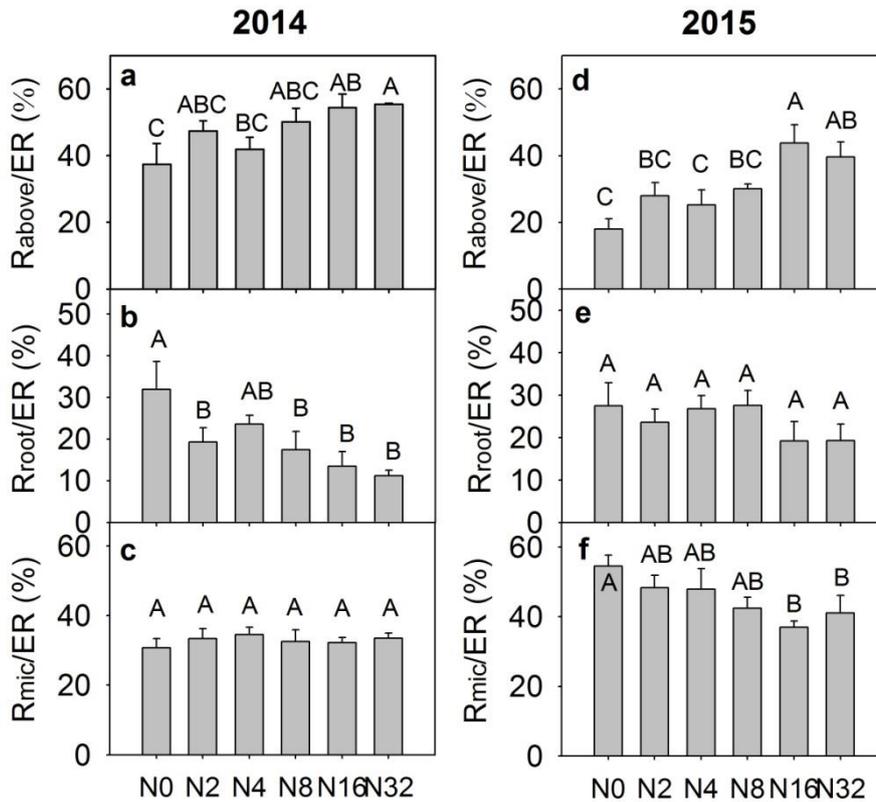
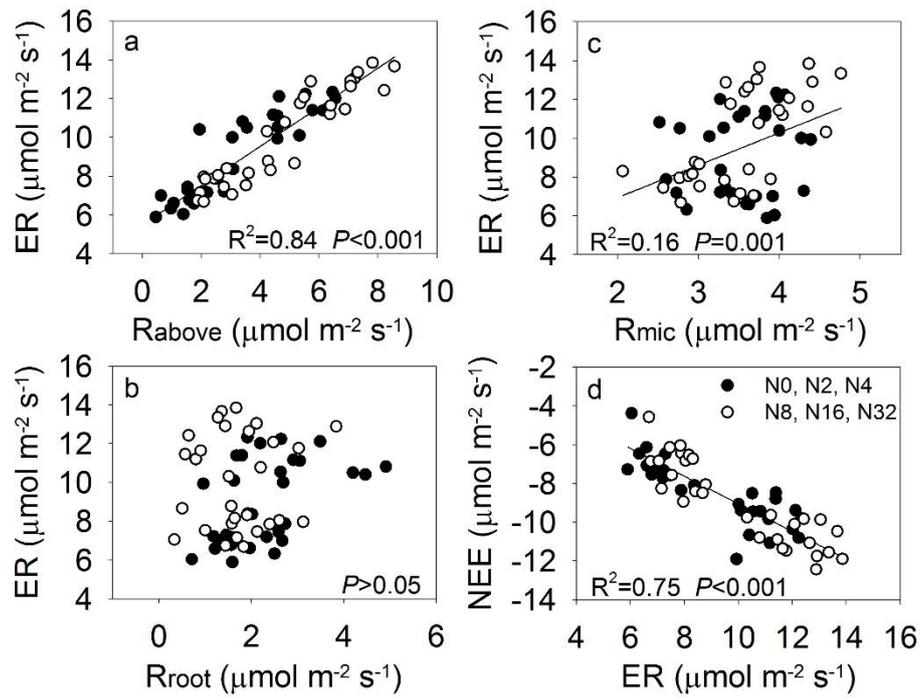


Figure 5. The contributions of different source components to ecosystem respiration (ER) in response to the N addition gradient in 2014 and 2015 (mean \pm SE, n = 5). **R_{above}**: aboveground plant respiration, **R_{root}**: plant root respiration, **R_{mic}**: soil microbial respiration. N0, N2, N4, N8, N16, N32 represent N addition rate of 0, 2, 4, 8, 16, 32 gN m⁻² year⁻¹, respectively.



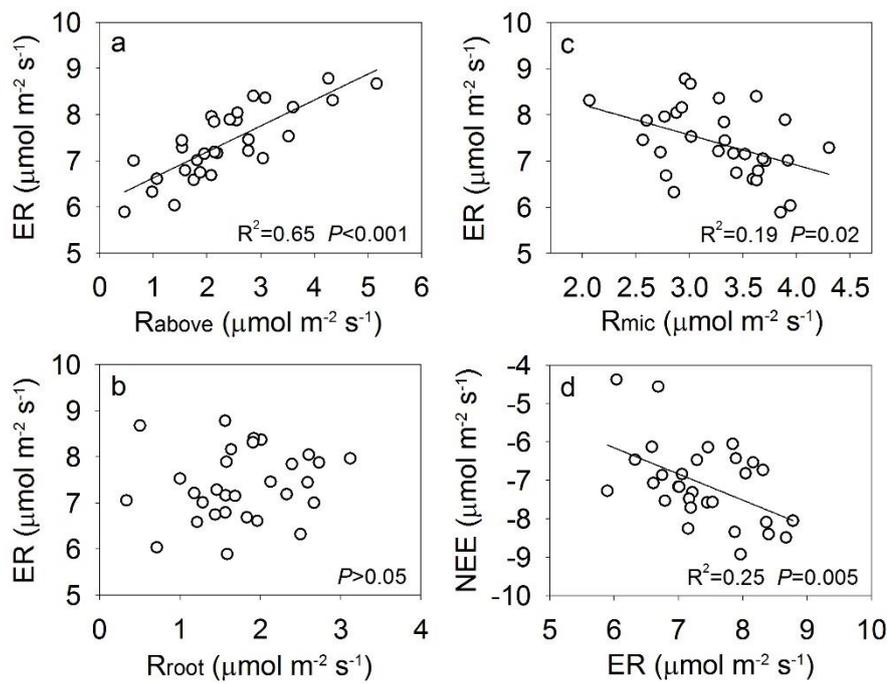


Figure 6. Relationships between aboveground plant respiration (R_{above}), root respiration (R_{root}), soil microbial respiration (R_{mic}) and ecosystem respiration (ER) (a,b,c), ER and net ecosystem CO₂ exchange (NEE) (d) across all plots in 2014 and 2015.

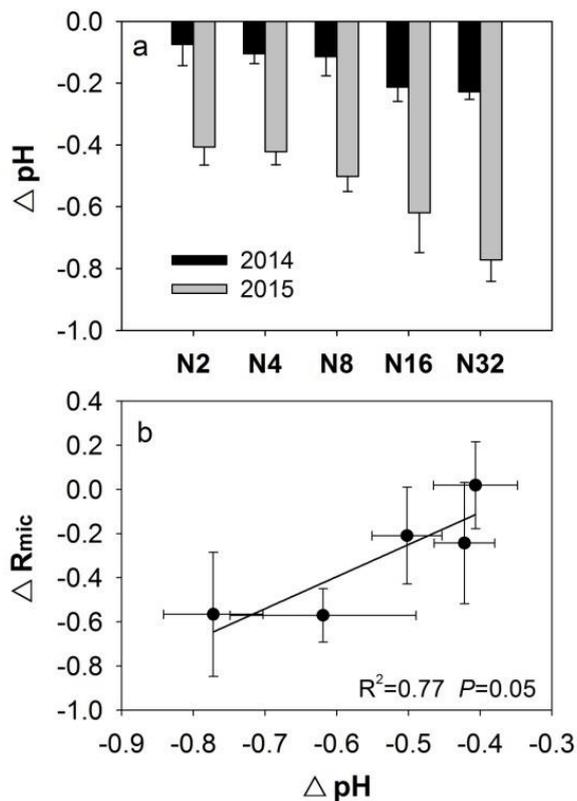


Figure 7. N-induced changes in soil pH (Δ pH) (a) (mean \pm SE, n = 5) and the dependence of N-induced changes in soil microbial respiration (Δ R_{mic}) on N-induced changes in soil pH (Δ pH) in 2015 (b). N2, N4, N8, N16, N32 represent N addition rate of 2, 4, 8, 16, 32 gN m⁻² year⁻¹, respectively.

Table 1. Results (F and P values) of one-way ANOVA on the effects of nitrogen addition on ecosystem C fluxes in 2014 and 2015. NEE: net ecosystem CO₂ exchange, ER: ecosystem respiration, GEP: gross ecosystem production, SR: soil respiration, R_{mic}: soil microbial respiration, R_{plant}: plant respiration, R_{above}: aboveground plant respiration, R_{root}: plant root respiration.

df	NEE		ER		GEP		SR		R _{mic}		
	F	P	F	P	F	P	F	P	F	P	
2014	5	3.35	0.020	2.95	0.033	5.37	0.002	1.56	0.209	1.49	0.246
2015	5	2.50	0.059	4.35	0.006	2.83	0.038	3.94	0.010	1.40	0.259

df	R _{plant}		R _{above}		R _{root}		R _{above} /ER		R _{root} /ER		R _{mic} /ER		
	F	P	F	P	F	P	F	F	F	P	F	P	
2014	5	1.06	0.409	3.84	0.011	2.64	0.049	3.08	0.027	3.56	0.015	0.28	0.919
2015	5	3.25	0.022	5.38	0.002	0.78	0.573	5.54	0.002	0.97	0.456	2.46	0.062