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Differential responses of grasses and forbs led to marked reduction in below-ground productivity in temperate steppe following chronic N deposition

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Summary

1. Enhanced deposition of atmospheric nitrogen (N) has profound impacts on ecosystem processes such as above-ground productivity and community structure in grasslands across the globe. But how N deposition affects below-ground processes of grasslands is less well known.

2. Here, we evaluated the effects of chronic N amendment at a relatively low rate (20 kg ha^{-1} $year^{-1}$) on root traits (root productivity, root biomass, root/shoot ratio) in Inner Mongolia steppes by rhizotron and ingrowth core and soil monolith techniques at levels of individual species, functional groups and ecosystem.

3. For 8 years, N amendment suppressed above-ground net primary production (ANPP), photosynthetic rates and root biomass of forbs, but enhanced ANPP and root biomass of grasses. This led to an overall reduction in below-ground productivity of the grassland by 24–33%, while ANPP remained unchanged.

4. Nitrogen amendment acidified soil and subsequently increased extractable soil manganese (Mn) concentration. Nitrogen amendment increased foliar Mn concentrations in forb, but not grass species, leading to a significant inhibition of photosynthetic rates in forb species.

5. *Synthesis*. These findings highlight the importance of the differentiating responses of plant functional groups to long-term N deposition and the important consequences of these responses for below-ground productivity and long-term soil C sequestration.

Key-words: below-ground productivity, grasses and forbs, nitrogen deposition, photosynthetic rates, plant–soil (below-ground) interactions, root traits, soil acidification, temperate steppe

Introduction

Atmospheric N deposition has increased dramatically due to fossil fuel combustion and the use of synthetic N fertilizers in the past century (Clark & Tilman 2008; Galloway *et al.* 2008), and this increase in N deposition has been projected to reach 156% of the 2010 value globally by 2030 (Bodirsky *et al.* 2014). This elevation in N deposition should promote ecosystem productivity as productivity in most terrestrial ecosystems is often N-limited (Vitousek & Howarth 1991). Not surprisingly, numerous studies have shown that short-term N fertilization enhances the above-ground net primary productivity (ANPP) of grassland and forest ecosystems (LeBauer & Treseder 2008; Xia & Wan 2008; Lu *et al.* 2011). In addition to the enhancement of ecosystem productivity, prolonged N deposition can also have strong negative effects on terrestrial ecosystems. One such effect is a reduction in species richness (Bobbink *et al.* 2010; De Schrijver *et al.* 2011) and soil acidification, thus resulting in substantial alterations of ecosystem processes and, in extreme cases, the collapse of ecosystems (Aber *et al.* 1989, 1995; Magill *et al.* 2000; Phoenix *et al.* 2006; Bowman *et al.* 2008; Stevens, Dise & Gowing 2009). The impacts of N deposition on biodiversity and ANPP have

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been extensively investigated in different types of grasslands world-wide (Bobbink *et al.* 2010; Duprè *et al.* 2010; Isbella *et al.* 2013), but few studies have evaluated whether and how chronic N deposition affect below-ground processes and root metrics in grassland ecosystems.

Below-ground productivity is an important part of the overall net primary productivity (NPP) of grassland ecosystems, accounting for up to 80% of grassland NPP (Peek 2007). Root productivity is a major component of below-ground productivity. Unlike ANPP, accurate determination of below-ground productivity is technically challenging. Therefore, data on the responses of grassland below-ground productivity to N deposition are scarce, and root standing biomass is often used as a surrogate for root productivity in the literature (LeBauer & Treseder 2008; Xia & Wan 2008; Liu & Greaver 2010; Lu *et al.* 2011). Yet, root biomass is a static measure of root standing crop and may not accurately represent below-ground productivity because root birth and death occur simultaneously and fine roots are often lost during isolation of roots from soils in the determination of root biomass (Hendrick & Pregitzer 1992). Given that roots are a key source of soil organic C and play important roles in below-ground C storage in the control of C flux in grassland ecosystems (Farrar & Jones 2000; Loya, Johnson & Nadelhoffer 2004), elucidation of how root dynamics responds to N deposition will allow for better understanding of C cycles under elevated N deposition.

To understand the responses of ecosystem C sequestration to N deposition, information on both above-ground and below-ground C cycles is required. There have been numerous studies demonstrating that N deposition can enhance ANPP (LeBauer & Treseder 2008; Xia & Wan 2008; Bai *et al.* 2010b) and reduce plant richness (Stevens *et al.* 2004; Clark & Tilman 2008; Song *et al.* 2011; Fang *et al.* 2012) across different grassland ecosystems. Moreover, species richness is closely associated with ANPP in grassland ecosystems, although the relationship between them remains controversial (Mittelbach *et al.* 2001; Shaver *et al.* 2001; Adler *et al.* 2011). Changes in species richness and ANPP resulting from enhanced N deposition in grassland ecosystems are tied to changes in below-ground productivity as root growth is closely dependent on C supply from the shoot. Different plant species and/or functional groups respond to N deposition differently, such that N deposition often favours growth of grass over forb species (Stevens *et al.* 2006; Song *et al.* 2011; Diekmann *et al.* 2014). Enhanced above-ground productivity of grasses due to elevated N input can lead to greater C allocation below-ground, while N deposition may has a less effect on C allocation below-ground in forbs. Therefore, N deposition may also have differential impacts on root processes of different species and functional groups. However, few studies have specifically investigated the impact of N amendment on root processes in grasslands at the levels of species, functional groups and ecosystem simultaneously. Simultaneous examination of root processes at different levels is necessary because ecosystem processes are emergent properties and often underlined by idiosyncratic species-specific responses.

In addition to impacts on vegetation, N deposition can lead to soil acidification in grassland ecosystems (Stevens *et al.* 2004; Bobbink *et al.* 2010; Duprè *et al.* 2010; Fang *et al.* 2012). Soil acidification in turn alters availabilities of macro- and micronutrients in soils, such that the availability of base cations (Ca^{2+}, Mg^{2+}, K^{+}) may decrease, whereas the availability of some metals (Mn^{2+}, Al^{3+}, Fe^{3+}) may increase (Aber *et al.* 1989; Roem & Berendse 2000; Bowman *et al.* 2008; Horswill *et al.* 2008; Lieb, Darrouzet-Nardi & Bowman 2011). If different plant species or functional groups differ in their responses to these changes in soil nutrient availability (Falkengren-Grerup 1998; Finzi, Canham & van Breemen 1998; Carroll *et al.* 2003), this may alter the contributions of different groups of plants to the overall ecosystem responses. Past studies have made tremendous progresses to understand how ecosystem processes such as above-ground productivity and community structure change in response to N deposition in many types of grasslands across the globe, yet how N-induced changes in soil nutrients influence responses of species and functional groups is poorly understood (Horswill *et al.* 2008; Ochoa-Hueso *et al.* 2013). Thus, our knowledge of how N-induced changes in soil properties influence below-ground productivity of different species and functional groups via changes in root traits in grasslands is still rudimentary.

The two dominant functional groups in grasslands, grasses and forbs, often differ in above-ground responses to N deposition (Stevens *et al.* 2006; Bobbink *et al.* 2010). In addition, monocot grasses and dicot forbs can vary in their acquisition systems for some mineral nutrients (Marschner 1995). Thus, the differential responses of functional groups as well as individual species to N deposition may determine the overall responses of below-ground productivity in grassland ecosystems to chronic N deposition. To test this hypothesis, we evaluated the effects of chronic N amendment at a relatively low rate (20 kg ha^{-1} $year^{-1}$ above ambient atmospheric N deposition) on root traits (root productivity, root biomass, root/shoot ratio) in an Inner Mongolia steppe by rhizotron, ingrowth core and soil monolith methods at the levels of individual species, functional group and ecosystem.

Materials and methods

STUDY SITE AND EXPERIMENTAL DESIGN

This study was conducted at the Duolun Restoration Ecology Station of the Institute of Botany, Chinese Academy of Sciences, in Duolun County (116°17′E, 42°02′N, 1324 m a.s.l.), Inner Mongolia, China. The area is located in a temperate climatic zone with a mean annual temperature of 2.1 °C and mean annual precipitation of 382 mm. Soil in the experimental site is Calcis-orthic Aridisol. Soil is composed of 62.7 ± 0.04% sand, 20.3 ± 0.01% silt and 16.9 ± 0.01% clay with mean soil bulk density of 1.3 g cm^{-3} and pH of 6.8. Vegetation in this area is a typical steppe community, and dominant species are mainly perennials, including *Stipa krylovii, Artemisia frigida, Potentilla acaulis, Potentilla tanacetifolia, Dianthus chinensis, Heteropappus altaicus, Cleistogenes squarrosa, Allium bidentatum, Leymus chinensis, Carex korshinskyi, Melilotoides ruthenica* and *Agropyron cristatum* (Fang *et al.* 2012). A comprehensive N amendment plat-

form to simulate a range of N deposition levels was established in 2003 after exclusion of livestock grazing. The platform contains a total of 64 plots of 15 × 10 m with an 8 × 8 Latin square experimental design. Each plot was separated by 4-m-wide buffer strips. There were eight levels of N (urea) amendment (0, 1, 2, 4, 8, 16, 32, 64 g N m^{-2} $year^{-1}$) in the platform, with eight replicates per N treatment. Urea was applied by hand spreading annually in July when maximal precipitation occurs. The rationale for the use of a low dose of N amendment (20 kg ha^{-1} $year^{-1}$) in the present study was to select an N enrichment level close to the current atmospheric N deposition level in the experimental area (16 kg ha^{-1} $year^{-1}$) (Zhang *et al.* 2008). Unlike the determination of species richness and ANPP, measuring the root traits (root productivity and root biomass) of temperate steppes is arduous and time-consuming. Therefore, it was impossible to monitor root dynamics in all the N-amended plots.

MEASUREMENTS OF ROOT TRAITS

In situ root dynamics were monitored following protocols similar to those described by Bai *et al.* (2008). Briefly, on 6 September 2009, scanners (BenQ 5000S) that were tightly sealed in Plexiglas boxes were installed in both the ambinet and the N-amended plots. The boxes were made from 0.6-cm-thickness Plexiglas, and the scanners were mounted into the boxes. A hole with a vertical soil profile of identical size to the Plexiglas box containing the scanner was dug with shovel in each plot, and the box and scanner were installed in the hole. The scanner was connected to a notebook computer by a cable sealed in a plastic bag. The observational area of the scanner was 20 cm in height and 30 cm in length, allowing for scanning of roots in the 0–20 cm soil depth. To minimize the effects of the scanner *per se* on root growth and soil regimes, a buffer time of about 8 months was maintained before observations started (Bai *et al.* 2008). Observation began on 2 May 2010 and lasted till 29 September 2011 with a sampling interval of about 15 days.

Appearance and disappearance of roots were analysed using Mapinfo Professional (5.0; Pitney Bowes Mapinfo Corporation, New York, NY, USA) as described by Bai *et al.* (2008). For the initially collected images, each root was assigned an identification number and distinguished as living or dead based on its colours as described in our previous paper (Bai *et al.* 2008). For the subsequent image sets, the tracings from the previous date were compared with the new images, thus allowing previously existing roots to be identified. Newly occurring roots were also identified and numbered. Roots that disappeared in subsequent images were assumed to be dead and decomposed. Complete records were kept for all roots, even for those classified as dead.

Root length productivity was determined using protocols described by Bai *et al.* (2010a). Briefly, root length productivity for each sampling period was measured by summing the length of all new roots and adding the extension growth of all previously existing roots. Root productivity was expressed as root length per rhizotron area observed (mm cm^{-2}).

Root biomass of individual species in the field was determined by digging a 30 × 30 × 20 cm soil blocks. Intact roots of individual species in the soil blocks were carefully collected by immersing the soil blocks in water for 24 h and washing the soil blocks with water in laboratory. The dry mass of roots was determined by oven drying at 70 °C to constant mass.

Root biomass productivity was measured using the ingrowth method. In early May of each year, we excavated two 20-cm-deep cylindrical holes using a soil auger (8 cm in diameter) in each experimental plot. The soils were refilled to the same hole after removing roots via 2-mm sieves. We collected the root ingrowth samples in late October by using a smaller soil auger (6 cm in diameter) at the centre of the original root ingrowth holes. The dry mass of root was determined by oven drying at 70 °C to constant mass.

ABOVE-GROUND VEGETATION MEASUREMENTS

Vegetation surveys were conducted in mid-August each year, the time of peak biomass. Species composition of the community in each plot was investigated in a randomly selected quadrat (1 × 1 m). Species richness was defined as the total species number per square metre (Stevens *et al.* 2004). Above-ground biomass was clipped at the ground level, and both living above-ground biomass and standing litter in the current year belonging to a same species were summered together. Plant samples were oven-dried at 70 °C for 48 h and then weighed separately to determine the biomass.

MEASUREMENT OF CHLOROPHYLL CONCENTRATION AND PHOTOSYNTHETIC RATES

Young leaves of *A. frigida* and *S. krylovii* collected from different plants in control and N-amended plots in August 2011 were weighed and extracted in aqueous acetone (80% v/v) following the protocols described by Wang, Li & Zhang (2012). The extracted solutions were used to measure chlorophyll (Chl) with a spectrophotometer (Bio-Rad, Hercules, CA, USA) at 663 and 645 nm. Total Chl concentration was calculated as 8.02A663 + 20.21A645 and expressed as mg g^{-1} fresh weight. Photosynthetic rates of *A. frigida* and *S. krylovii* in both control and N-amended plots were measured on sunny days between 8:30 and 12:30 in August of 2011 using the LI-6400 XT portable photosynthesis system (Li-Cor, Lincoln, NE, USA). Photosynthetic rates of fully expanded leaves of *S. krylovii* and *A. frigid* were measured using the LED leaf cuvette (2 × 3 cm). Artificial illumination was applied to the leaves in the chamber from a red–blue 6400-02B LED light source attached to the sensor head with continuous light (1000 μmol m^{-2} s^{-1} photosynthetic photon flux density) and ambient CO_2 concentration of approximately 400 μmol CO_2 per mol. At least two individual *S. krylovii* and *A. fridiga* plants in each plot were selected for measuring photosynthetic rates.

DETERMINATION OF SOIL PROPERTIES

In each N-amended and control plot, three soil samples at the soil depths of 0–20 cm were taken in the August of 2011, and mineral N (NH_4^+-N, NO_3^--N) concentrations were measured using a continuous-flow ion auto-analyser (Scalar SAN, Breda, The Netherlands). A soil core (3 cm diameter) of fresh soil from 0 to 20 cm soil layer was randomly sampled in August of 2011; then, air-dried soil was passed through a 2-mm sieve for determination of soil pH and mineral elements. Soil pH was determined with a Russell RL060P portable pH meter (Thermo Electron Corporation, Waltham, MA, USA) and a water/soil ratio of 1:2.5. To measure exchangeable concentrations of Mn^{2+} and Fe^{3+} in soil, 25 grams of soil samples was extracted with 50 mL extracting agent consisting of 5 mM diethylenetriaminepentaacetic acid (DTPA), 10 mM $CaCl_2$ and 0.1 M triethanolamine (TEA) with pH 7.3 for 2 h following the protocols described in the literature (Lindsay & Norvell 1978). To extract soil exchangeable Al^{3+}, 10 grams of air-dried soil was incubated in 50 mL of the 0.1 M $BaCl_2$ (pH 5.3) for 30 min, and the filtered extraction solution was used to

determined Al^{3+}. The metal (Mn^{2+}, Fe^{3+} and Al^{3+}) concentrations of the extracted solutions were measured by ICP-OES.

POT EXPERIMENTS

Seeds of *A. frigida* and *S. krylovii* collected in the control plots were planted in pots (20 cm diameter) filled with vermiculite and irrigated with 1/8 Hoagland solution under controlled growth conditions. After growth for 5 weeks, five *A. frigida* and *S. krylovii* seedlings were left in each pot and irrigated with 1/8 Hoagland solution supplemented with and without 50 μM $MnCl_2$ for 10 days. Thereafter biomass, foliar concentrations of Mn and photosynthetic rate were determined.

STATISTICS ANALYSIS

Repeated-measures ANOVA was used to examine the effects of N treatments on root production using factorial analysis. Unpaired *t*-tests were used to evaluate the effects of 8-year N amendment on root productivity, above-ground net primary productivity, photosynthetic rate, chlorophyll concentration, leaf Fe and Mn concentration, root biomass, plant density, soil pH and soil and leaf mineral contents. All statistical analyses were conducted with SAS software (SAS Institute Inc., Cary, NC, USA).

Results

EFFECT OF N AMENDMENT ON ROOT PRODUCTION AND PRODUCTIVITY

Seasonal dynamics of root length production monitored by rhizotron peaked in May and August in 2010 and 2011, and N amendment did not alter these dynamic patterns (Fig. 1a). However, N amendment significantly reduced root length production ($P = 0.0172$, Fig. 1a). This suppression of root production led to a reduction in root length productivity by 23.9% ($P = 0.0172$) across the two growing seasons (Fig. 1b). In addition to the determination of root length productivity by rhizotron, we also measured root biomass productivity by the ingrowth core technique and found that N amendment reduced root biomass productivity by 32.7% ($P = 0.0018$) across the 2 years (Fig. 1b).

EFFECT OF N AMENDMENT ON ABOVE-GROUND NET PRIMARY PRODUCTIVITY (ANPP)

In contrast to root productivity, ANPP of the community in the N-amended plots was not significantly different from that in control plots (ambient N) across the 2 years ($P = 0.8076$) (Fig. 2a). The community was mainly composed of forbs and grasses. Our results showed that N amendment had contrasting effects on ANPP of forbs and ANPP of grasses, such that N amendment reduced ANPP of forbs by 27.9% ($P = 0.0001$), while the same N amendment led to an increase in ANPP of grasses by 31.7% ($P = 0.0011$) (Fig. 2b). Forb *A. frigida* and grass *S. krylovii* are two dominant species, which can account for up to 80% of ANPP of the community. Like ANPP of forbs and grasses, ANPP of

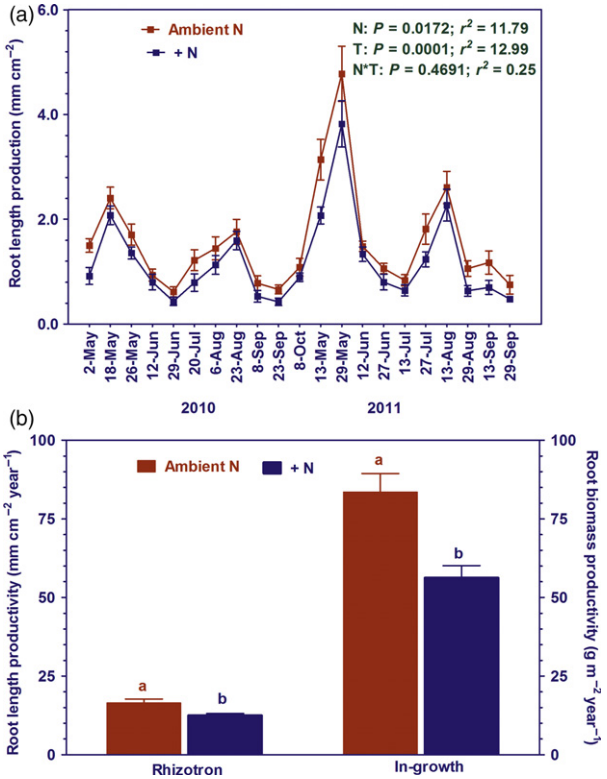

Fig. 1. Seasonal dynamics of root length production in 2010 and 2011 (a) and annual root length and biomass productivity (b) determined by rhizotron and ingrowth methods under ambient and long-term N amendment at the rates of 20 kg ha^{-1} $year^{-1}$ (+N) across the 2 years. Data are means ± SE ($n = 8$). Different letters above the bars mean significant difference between ambient N and N amendment at $P < 0.05$ determined by *t*-tests.

the two dominant species also displayed contrasting responses to the N amendment, that is N amendment reduced ANPP of *A. frigida* by 22.8% ($P = 0.0007$), while it enhanced ANPP of *S. krylovii* by 28.7% ($P = 0.0006$) (Fig. 2c).

EFFECT OF N AMENDMENT ON ROOT BIOMASS

To test whether the suppression of ecosystem root productivity by N amendment affects root standing crop, we monitored the effect of N amendment on root biomass at levels of ecosystem, functional group and individual dominant species in 2010 and 2011. N amendment had no impact on the overall root biomass of the community across the 2 years (Fig. 3a). In contrast, N amendment had contrasting impacts on root biomass of forb and grass species. For example, N amendment significantly reduced root biomass of forbs by 58.9% ($P = 0.0001$), while the same treatment enhanced root biomass of grasses by 47.3% ($P = 0.0001$) (Fig. 3b). Moreover, root biomass of the forb *A. frigida* and the grass *S. krylovii* also responded to N amendment differently, such that N amendment reduced root biomass of *A. frigida* by 50.1% ($P = 0.0001$), while it increased root biomass of *S. krylovii* by 26.2% ($P = 0.0032$) (Fig. 3c).

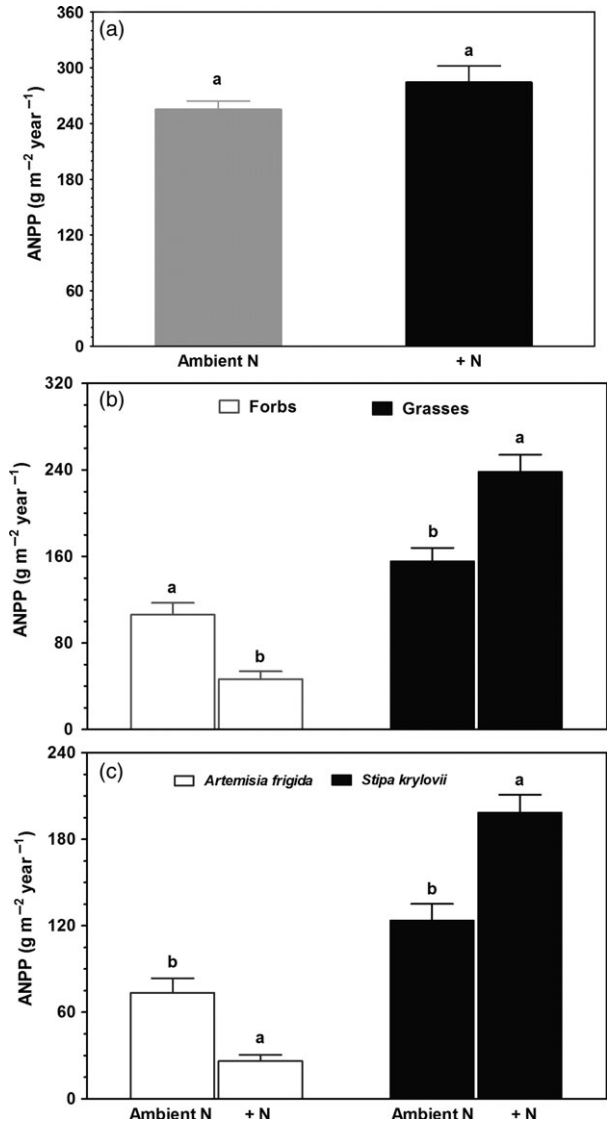

Fig. 2. Effect of chronic N amendment (20 kg ha^{-1} year^{-1}) on total community above-ground net primary productivity (ANPP) (a), above-ground net primary productivity of grass and forbs (b) and *Artemisia frigida* and *Stipa krylovii* (c) across the 2010 and 2011. Data are means ± SE(n = 8). Different letters above the bars mean significant difference between ambient and N amendment at $P < 0.05$ determined by t-tests.

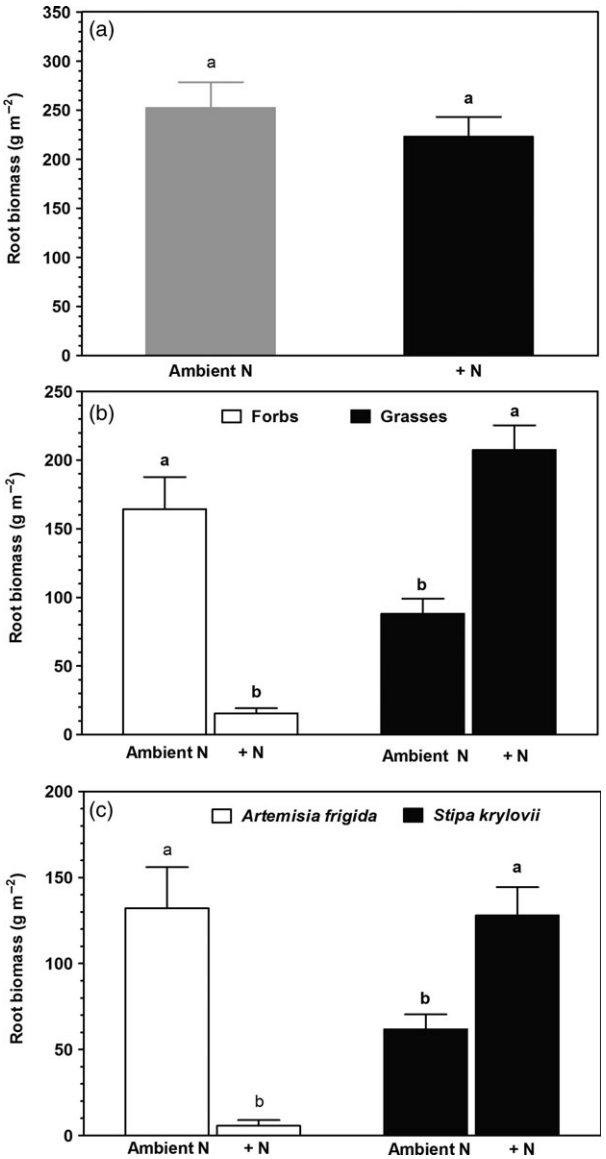

Fig. 3. Effect of chronic N amendment (20 kg ha^{-1} year^{-1}) on total community root biomass (a), root biomass of grass and forbs (b) and *Artemisia frigida* and *Stipa krylovii* (c) across 2010 and 2011. Data are means ± SE (n = 8). Different letters above the bars mean significant differences between ambient and N amendment at $P < 0.05$ determined by t-tests.

EFFECT OF N AMENDMENT ON PHOTOSYNTHETIC RATES AND FOLIAR MN/FE RATIO

To further explore the mechanism by which chronic N amendment differentially affected ANPP of forb and grass species, we measured photosynthetic rates (Pn) and foliar chlorophyll concentrations in *A. frigid* and *S. krylovii* in both N-amended and control (ambient N) plots in August 2011. As shown in Fig. 4, N amendment reduced Pn and chlorophyll (Chl) concentrations in *A. frigida* by 53.75% (P = 0.0001) and 22.14% (P = 0.03), respectively. By contrast, N amendment had no impact on Pn of *S. krylovii*, and it increased foliar Chl concentrations in *S. krylovii* by 29.26% (P = 0.0001). Iron (Fe) and manganese (Mn) play important roles in chloroplast development and photosynthetic processes (Marschner 1995). To test whether the differential responses of Pn and Chl to the N amendment in the two species were caused by differential accumulation of Mn and Fe, we investigated the effect of N amendment on foliar Fe and Mn concentrations of the two species. Our results showed that chronic N amendment reduced foliar Fe concentration by 43.96% (P = 0.0081) and increased foliar Mn concentration by 38.38% (P = 0.0235), leading to an increase in foliar Mn/Fe ratio by 150.88% in *A. frigida* (P = 0.0052) (Fig. 5). In contrast to *A. frigida*, foliar Fe and Mn concentrations (P = 0.3245 and P = 0.8047) as well as Mn/Fe ratio (P = 0.7177) in *S. krylovii* were not significantly changed by the chronic N amendment (Fig. 5).

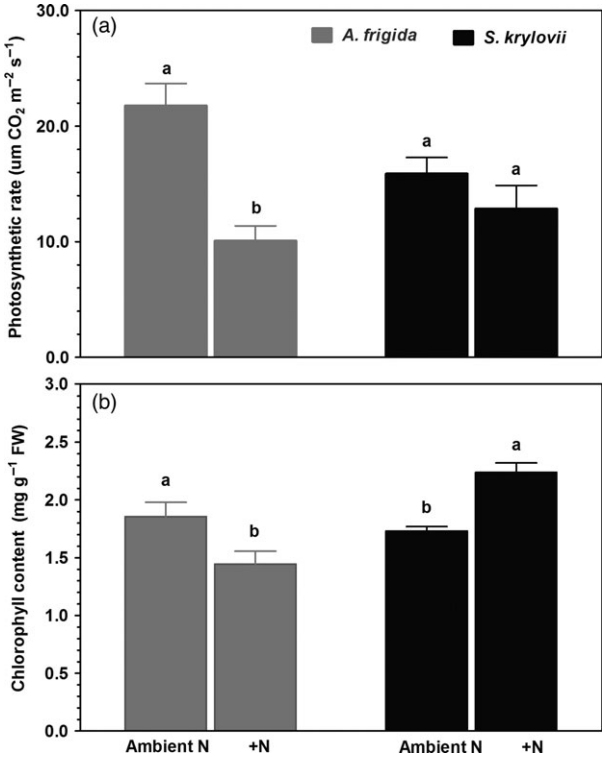

Fig. 4. Effect of 8-year N amendment on photosynthetic rate (a) and chlorophyll content (b) of *Artemisia frigida* and *Stipa krylovii*. Data are means ± SE (n = 8). Different letters above the bars mean significant difference between ambient and +N treatment at $P < 0.05$ determined by t-tests.

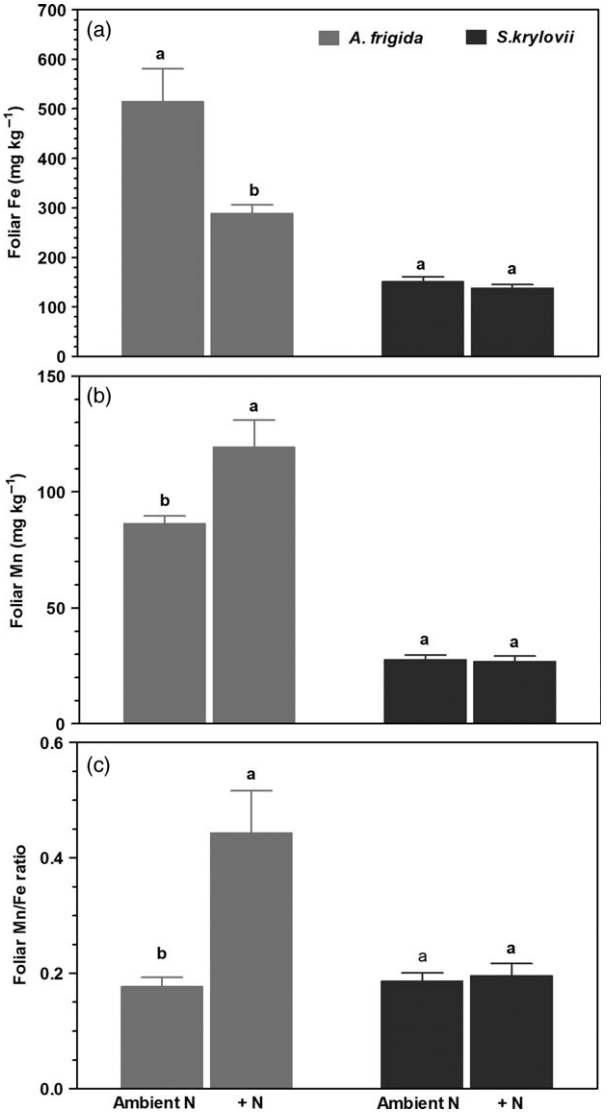

Fig. 5. Effect of 8-year N amendment on foliar Fe (a) and Mn (b) concentrations and Mn/Fe ratio (c) of *Artemisia frigida* and *Stipa krylovii*. Data are means ± SE (n = 8). Different letters above the bars mean significant difference between ambient N level and N amendment (+N).

EFFECTS OF MN ON GROWTH AND PHOTOSYNTHETIC RATES OF *A. FRIGIDA* AND *S. KRYLOVII* SEEDLINGS

The observations that foliar Mn and Pn of *A. frigida* differed from those of *S. krylovii* in response to the N amendment prompted us to hypothesize that the two species may have differential sensitivity to elevated soil Mn concentrations, which in turn result in differential root responses due to different changes in C allocation patterns. To test this hypothesis, we conducted pot experiments in the glasshouse to evaluate effects of exogenous application of $MnCl_2$ on growth and photosynthetic rates of *A. frigida* and *S. krylovii* seedlings. As shown in Fig. 6, treatment with $MnCl_2$ led to a significant reduction in photosynthetic rate and growth of both shoot and root in *A. frigida*, whereas the same treatment had no effect on *S. krylovii* in terms of photosynthetic rates, shoot and root growth. Therefore, these results support that the reduction in root productivity of the steppe community by N amendment may result from suppression of root growth in forbs due to inhibition of photosynthetic rates.

Discussion

Many studies have investigated the impacts of N deposition on ecosystem processes such as ANPP, biodiversity and C and N cycling in the N-limited Inner Mongolia steppes by adding mineral N with varying doses (Xia & Wan 2008; Niu *et al.* 2010; Lan & Bai 2012). Yet below-ground processes such as root productivity, though important in magnitude to overall ecosystem productivity and long-term carbon sequestration, remain poorly understood. Moreover, to more realistically simulate atmospheric N deposition, long-term N amendment with low doses may be more relevant for our understanding and predicting how N deposition impacts ecosystem processes. In the present study, we evaluated the responses of below-ground productivity to chronic (8 years), low N amendment rate (20 kg ha^{-1} $year^{-1}$) by measuring root traits (productivity, root biomass and root/shoot ratio) in the temperate steppe of Inner Mongolia grasslands at the levels of ecosystem, functional group (forbs vs. grasses) and individual species (*Artemisia frigida* vs. *Stipa krylovii*) using several methods including rhizotron, ingrowth core and traditional soil monoliths. Our findings reveal, for the first time, that although N amendment up to 8 by years had no impact

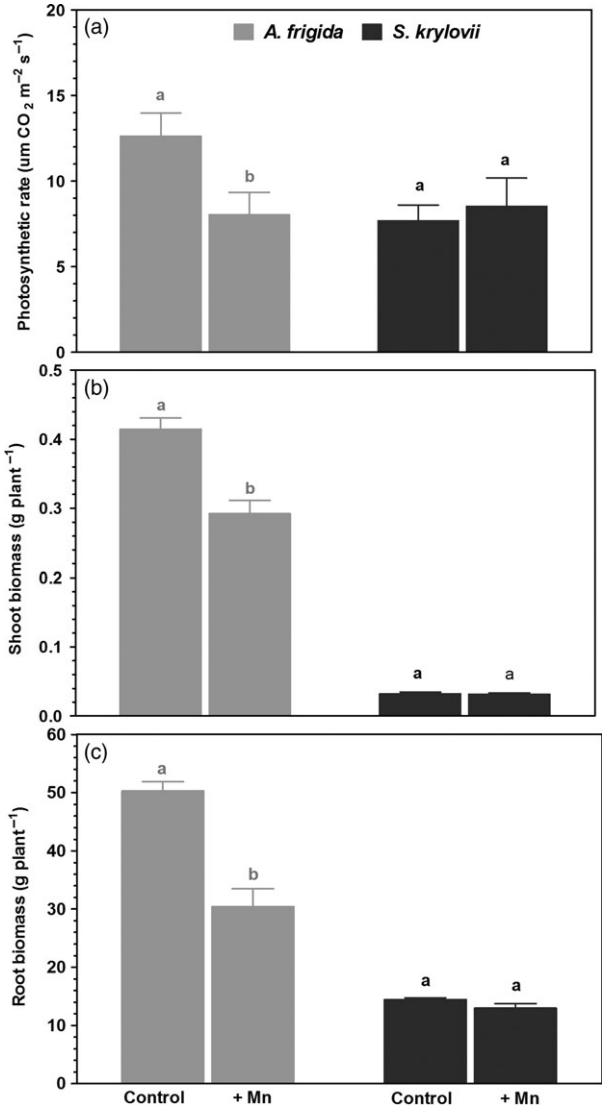

Fig. 6. Effect of exogenous supply of 50 μM $MnCl_2$ on photosynthetic rate (a), shoot biomass (b) and root biomass (c) of *Artemisia frigida* and *Stipa krylovii* seedlings grown in vermiculite glasshouse. Data are means ± SE (n = 6). Different letters above the bars column mean significant difference between control and irrigation with solution supplemented with 50 μM $MnCl_2$.

on ANPP and root biomass at the ecosystem level, it induced a marked reduction in root productivity (24–33%) (Fig. 1). These results highlight that long-term N deposition at a relatively low rate approximating current N deposition in this area can cause substantial changes in ecosystem below-ground productivity. These changes in below-ground productivity in turn may impact long-term soil C sequestration. We acknowledge that the steppe communities in our studies have experienced ambient N deposition rates of 16 kg ha^{-1} $year^{-1}$ (Zhang *et al.* 2008). Therefore, our observed responses of below- and above-ground traits to the N amendment are the consequences of total N input of 36 kg ha^{-1} $year^{-1}$. Further studies on steppe communities exposed to lower ambient N deposition rates are warranted to test whether a lower N input has a similar effect on the steppe communities.

Our results indicating that N amendment had no impact on ANPP of these grasslands are in contrast to the paradigm that N input can enhance productivity in N-limited ecosystems (Vitousek & Howarth 1991). To elucidate the underlying mechanisms, we examined the responses of different functional groups as well as dominant species to the N amendment. One important finding in the present study is that N amendment had opposite effects on ANPP of forbs and grasses, such that N amendment reduced forb ANPP by 27.9% yet enhanced grass ANPP by 31.7% (Fig. 2). The N-induced increase in grass ANPP thus may offset the N-induced reduction in forb ANPP, leading to no change in the overall community ANPP after N amendment for 8 years. Moreover, the dominant grass species *S. krylovii* and dominant forb species *A. frigida* can account for up to 80% of the community ANPP in the control, ambient N plots (Fang *et al.* 2012). ANPP of the two species also exhibited opposite trends in response to the N amendment, that is enhanced ANPP in *S. krylovii* by 28.7% and reduced ANPP in *A. frigida* by 22.8% (Fig. 2). These results indicate that the observed effects of N amendment on community ANPP are combined results of opposite responses of the dominant species.

Although N amendment caused no significant changes in ANPP and root biomass of the community, different causes may be responsible for the observations. The lack of N-induced significant changes in community ANPP is likely used by offsetting opposite effects of N amendment on ANPP of grasses and ANPP of forbs. Root biomass is a standing crop accumulated over times and is determined by root turnover (McCormack & Guo 2014). Despite a greater reduction in root biomass of forbs than increase in root biomass of grasses by the N amendment (Fig. 3b), the reduced C allocation into roots of forbs (Fig. S1 in Supporting Information) can lead to an increase in root life span and a slow root turnover for those remaining roots of forbs (Eissenstat & Yanai 1997; Farrar & Jones 2000; Bai *et al.* 2010a). In contrast to forbs, the increased ANPP of grasses by N amendment can lead to more C allocation into their roots (Fig. S1), stimulating root turnover (Eissenstat & Yanai 1997; Farrar & Jones 2000; Bai *et al.* 2010a). Therefore, the N-induced reduction in root turnover of forbs may counteract the N-induced increase in root turnover of grasses, leading to no significant change in the overall standing crop of root biomass. Moreover, the N-induced increase in grass root biomass was less than the decrease in root biomass of forbs (i.e. increase 47.3% vs. decrease 58.9%), but the death of overall grass roots may also be reduced because grass roots generally have slower turnover than forb roots (Peek 2007; Chen & Brassard 2013). This may also explain no significant changes in root biomass of the community following chronic N amendment.

This observation may also imply that root processes in the temperate steppe are more complex in response to chronic N deposition than ANPP. Therefore, results obtained from the use of root biomass to estimate below-ground productivity in response to N deposition should be treated with caution. Given that root productivity is closely related to C allocation

(Eissenstat & Yanai 1997; Farrar & Jones 2000; Bai *et al.* 2010a), we speculate that reduced C allocation of forbs to roots may be an important mechanism underlying the observed N-induced reduction in root production. To test this hypothesis, we examined the effect of N amendment on photosynthetic rates in the two dominant species. An important finding in the present study is that chronic N amendment led to a reduction in Pn of the forb *A. frigida* by 53.7%, while the same treatment had no impact on Pn of the grass *S. krylovii* (Fig. 4). It has been suggested that 50–60% of photoassimilates can be used for root production (Jackson, Mooney & Schulze 1997; Peek 2007). Therefore, the 53.7% reduction in Pn by N amendment is expected to reduce root productivity of forbs by 26–32%. This estimation matches well with our observed N-induced reduction in root productivity of the ecosystem by 24–33%, implying that the reduction in the community root productivity by N amendment is likely to result from suppression of root growth of *A. frigida*. Therefore, our findings highlight that reduction in C allocation into roots due to marked inhibition of Pn is an important mechanism by which chronic N amendment reduced below-ground productivity of temperate steppes.

Mineral nutrients such as Fe and Mn are essential microelements for plant growth and development and closely associated with chloroplast development and photosynthetic processes (Marschner 1995). Among the mineral nutrients examined, we detected that foliar Mn and Fe concentrations in the two species exhibited marked differences in response to chronic N amendment, such that N amendment specifically enhanced foliar accumulation of Mn and suppressed Fe accumulation in *A. frigida*, while no significant changes in foliar Mn and Fe concentrations were detected in *S. krylovii* under the same regime of N amendment (Fig. 5). These differential patterns of foliar Fe and Mn accumulation in the two species may account for the significant reductions in chlorophyll concentrations in *A. frigid*a, and the reduced chlorophyll concentration may lead to the observed inhibition of Pn in *A. frigida*. Although Mn is an essential mineral nutrient for plant growth and development, excessive accumulation by plants can be toxic to plants by targeting photosynthetic apparatus and processes (Foy, Chaney & White 1978).

To further explore the mechanism by which *A. frigida* accumulates Mn in the N-amended plots, we first tested whether N amendment alters soil Mn concentrations and whether forbs and grasses differ in their acquisition of Mn. Indeed, we found that the N amendment significantly enhanced exchangeable Mn^{2+} concentrations in soil (Table S1). Because soil Mn^{2+} concentration is negatively correlated with soil pH (Marschner 1995), we determined the effect of N amendment on soil pH and observed a significant reduction in soil pH by the N amendment (Fig. S2). These results suggest that mobilization of soil Mn^{2+} by N amendment is likely to be caused by soil acidification. In addition to mobilization of Mn^{2+}, soil acidification-mediated release of Fe^{3+} and Al^{3+} has been used to explain N deposition-induced species loss (Aber *et al.* 1989; Stevens *et al.* 2004; Bowman *et al.* 2008). However, in our system, we found a decrease in soil Al^{3+} and no change in soil Fe^{3+} (Table S1). A significant mobilization of Al^{3+} often occurs in acidic soil with pH less than 5.0 (Tyler 1996). Despite soil acidification, soil pH in the N-amended plots was higher than 6 (Fig. S2). This may explain our results that N amendment did not induce increases in soil Al^{3+} concentrations. Soil acidification can also increase soil Fe^{3+} (Marschner 1995). However, only a significant increase in soil Mn^{2+}, but not Fe^{3+} concentration, was detected in our system (Table S1), suggesting that mobilization of soil Mn^{2+} is more sensitive to the reduction in soil pH than that of Fe^{3+}. Because dicots and monocots differ in their acquisition systems for Fe and Mn, such that the dicot *A. frigida* takes up Fe^{2+} from rhizosphere after reduction of Fe^{3+} to Fe^{2+} by ferric reductase in roots (Marschner 1995; Curie & Briat 2003), while the monocot *S. krylovii* can directly take up Fe^{3+} from soils (Marschner 1995; Curie & Briat 2003), the increased soil Mn^{2+} concentration directly competes with Fe^{2+}, leading to accumulation of Mn and concurrent suppression of Fe acquisition in *A. frigida*. In contrast, the N-induced increases in soil Mn^{2+} concentration may have little effect on Fe^{3+} acquisition by *S. krylovii*, thus resulting in higher foliar Mn/Fe ratio in *A. frigida* than in *S. krylovii* (Fig. 5). Fe and Mn are involved in regulation of chloroplast development and photosystem I (PSI)-dependent cyclic flow (Marschner 1995; Millaleo *et al.* 2013). The enhanced accumulation of Mn and subsequent suppression of Fe acquisition can impair chloroplast development and photosynthetic rates, leading to the observed reductions in photosynthetic rates and chlorophyll contents in *A. frigida* under conditions of N amendment.

Thus, our tests of various possibilities of metal toxicity to plants strongly support the hypothesis that reduction in ANPP and root productivity of forbs may result from inhibition of C assimilation by reduced photosynthetic rates due to accumulation of Mn by forbs. Our results from pot experiments (Fig. 6) provide strong evidence to support the idea that mobilization of soil Mn by chronic N amendment may be an important event to trigger the observed N-induced reduction in root productivity of forbs. Reductions in ANPP and species richness invoked by N deposition have been ascribed to toxicities of aluminium (Duprè *et al.* 2010; Lieb, Darrouzet-Nardi & Bowman 2011) as well as depletion of base cations (Ca^{2+}, Mg^{2+}, K^{+}) (Stevens *et al.* 2004; Stevens, Dise & Gowing 2009) in grasslands. In the present study, we did not detect changes in soil exchangeable Ca^{2+} and Mg^{2+} concentrations (Table S1), while N amendment led to significant reductions in soil exchangeable and foliar Al concentration (Tables S1 and S2). Despite N amendment induced a reduction in soil exchangeable K^{+} concentration (Table S1), foliar K concentrations in both *A. frigida* and *S. krylovii* remained relatively constant (Table S2). Therefore, the reductions in ANPP and root productivity by N amendment are unlikely to be caused by the depletion of soil base cations and phytotoxicity of Al^{3+}.

In conclusion, our results demonstrated that a chronic, low N amendment rate had contrasting effects on root traits (root productivity, root/shoot ratio, root-mediated metal acquisition)

of grasses and forbs, leading to a net reduction in root productivity by 24–33% of Inner Mongolia grasslands. Moreover, we discovered that N amendment suppressed photosynthetic rates of forbs, but it had no impact on photosynthetic rates of grasses. This differential response provides a mechanistic explanation of the differential responses of ANPP and root productivity of the two functional groups to N amendment. We further revealed that N amendment significantly acidified soils and mobilized soil Mn concentrations because Mn^{2+} concentration in soil is negatively correlated with soil pH. The enhanced soil Mn^{2+} concentration results in greater accumulation of foliar Mn^{2+} than in grasses due to fundamental differences in their metal transport system. The higher foliar Mn and subsequent inhibition of foliar Fe accumulation in forbs inhibit their photosynthetic rates, thus reducing root productivity due to less allocation of photoassimilates into roots. Our study is among the first to elucidate the mechanisms by which chronic, low-dose N deposition reduces root production and below-ground productivity of temperate steppes at levels of individual species, functional groups and ecosystem by linking those below-ground events (soil acidification, metal mobilization and root acquisition) to above-ground events (foliar metal accumulation, chlorophyll biosynthesis and photosynthesis). Our findings highlight the integration of species, functional groups, above-ground and below-ground traits in studying grassland C cycles under N deposition scenario.

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Data accessibility

Data are available at http://dx.doi.org/10.5061/dryad.42ph5.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Effect of long-term low dose of N amendment on concentrations of mineral nutrients in the surface soil (0–10 cm).

Table S2. Effect of long-term low dose of N amendment on concentrations of mineral nutrients in leaves of *A. frigida* and *S. krylovii*.

Figure S1. The ratio of root biomass to shoot biomass under ambient and long-term addition of N at the rate of 20 kg ha^{-1} $year^{-1}$ (+N) in community and forbs and grasses.

Figure S2. Soil (0–10 cm) pH value under ambient and long-term addition of N at the rate of 20 kg ha^{-1} $year^{-1}$ (+N) in 2011.