Grass and Forage Science
The Journal of the British Grassland Society | The Official Journal of the European Grassland Federation

Effects of *in situ* freezing on soil net nitrogen mineralization and net nitrification in fertilized grassland of northern China

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Abstract

Effects of soil freezing on nitrogen (N) mineralization have been the subject of increased attention in the ecological literature, though fewer studies have examined N mineralization responses to successive mild freezing, severe freezing and cyclic freeze–thaw events. Even less is known about relationships of responses to soil N status. This study measured soil N mineralization and nitrification in the field along an experimental N gradient in a grassland of northern China during the dormant season (October 2005–April 2006), a period in which freezing naturally occurs. Net N mineralization exhibited great temporal variability, with nitrification being the predominant N transformation process. Soil microbial biomass C and N and extractable NH_4^+ pools declined by 40, 52, and 56%, respectively, in April 2006, compared with their initial concentrations in October 2005; soil NO_3^- pools increased by 84%. Temporal patterns of N mineralization were correlated with soil microbial biomass C and N. N mineralization and nitrification increased linearly with added N. Microbial biomass C in treated soils increased by 10% relative to controls, whereas microbial N declined by 9%. Results further suggest that freezing events greatly alter soil N dynamics in the dormant season at this site, with considerable available N accumulating during this period.

Keywords: nitrogen cycling, nitrification, soil freezing, nitrogen deposition, litter production, grassland

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Received 5 October 2010; revised 22 January 2011

Introduction

Mineralization of organic nitrogen (N) into inorganic forms plays a central role in supplying available N for plant growth, ultimately influencing primary productivity of terrestrial ecosystems (Reich *et al.*, 1997). Seasonal patterns of soil N mineralization have been well-studied in the field (Gilliam *et al.*, 2001; Schimel *et al.*, 2004), with most such studies focusing on soil N dynamics during the growing season (Van Der Krift and Berendes, 2001). Recently, soil N processing during dormant seasons has received increased attention because of the potential for N made available throughout this period either being available for spring ephemeral plant growth or leached from the system (Muller, 2003; Zhao *et al.*, 2010).

Freezing events of a range of intensity – from mild to severe and including repeated freeze/thaw cycles – are common during the non-growing season in most high latitude (35–65°N) ecosystems. Such events have the potential to affect release of plant-available soil nutrients (Schimel and Clein, 1996; Grogan *et al.*, 2004). Effects of freezing on soil N mineralization, with a focus on both steady freezing and freeze–thaw cycles, are well documented for a variety of northern latitude ecosystems (Deluca *et al.*,1992; Schimel and Clein, 1996; Neilsen *et al.*, 2001). Results of these *in situ* incubation (field) studies rarely exhibit consistent seasonal patterns in soil N dynamics when they experience sequential events of mild freezing, severe freezing, and freeze–thaw events, precluding broad generalizations of mechanisms of N mineralization and changes in available N supply as a result of freezing.

Soil N mineralization and nitrification are microbial processes that are regulated by a variety of abiotic and biotic factors (Hart *et al.*, 1994; Tietema, 1998). Some studies have reported rates of N mineralization/nitrification to be significantly correlated with either soil temperature, soil moisture, or both in growing season (Gilliam *et al.*, 2001; Wang *et al.*,

2006) and non-growing season (Zhao *et al.*, 2010), whereas the function of microbial biomass to regulate N dynamics is less clear.

Mongolian pasture constitutes a large part of the contiguous Eurasian steppe in northern China, covering about 1·13 million km^2 or 11·8% of the national land area. Severe land degradation in the Inner Mongolian plateau has occurred since the late 1970s, resulting in great reductions in grassland productivity and ecosystem stability (Bai *et al.*, 2004; Liu *et al.*, 2006). Land-use practices, such as grazing, which can be extensive in these Inner Mongolian grasslands, have been demonstrated to severely alter N dynamics of soil (Wolf *et al.*, 2010). Wolf *et al.* (2010) showed that spring thaw in these Inner Mongolian grasslands can result in a pulse of N_2O emissions. To stabilize these ecosystems and improve their productivity, some overgrazed steppes have been fenced and fertilized for restoration since 2000. In addition, based on long-term data (1952–2005), mean monthly temperatures from November to March at this site are <0°C, indicating the likelihood of freezing of surface soil several times during the non-growing season, suggesting the potential for freezing-mediated alteration in soil N dynamics at this site (Zhao *et al.*, 2010). During this time, virtually no data have been collected on soil N mineralization during non-growing season in the fertilized grassland, particularly in the context of interactions with soil freezing. Understanding temporal dynamics of soil N in winter is important not only as basic information regarding N cycling, but also to develop synchronized strategies for regeneration and management of degraded lands (Wardle, 1998; Barbhuiya *et al.*, 2004).

The purpose of this study was to examine effects of freezing on the dynamics of soil N cycling, including N transformations and soil microbial biomass, by utilizing a predictable series of freezing events (mild freezing, severe freezing and freeze–thaw cycles) in the field during the non-growing season. We used an existing N fertilization experiment to examine freezing effects along a gradient of N availability. We also compared the effects of N fertilization during the restoration of degraded pasture.

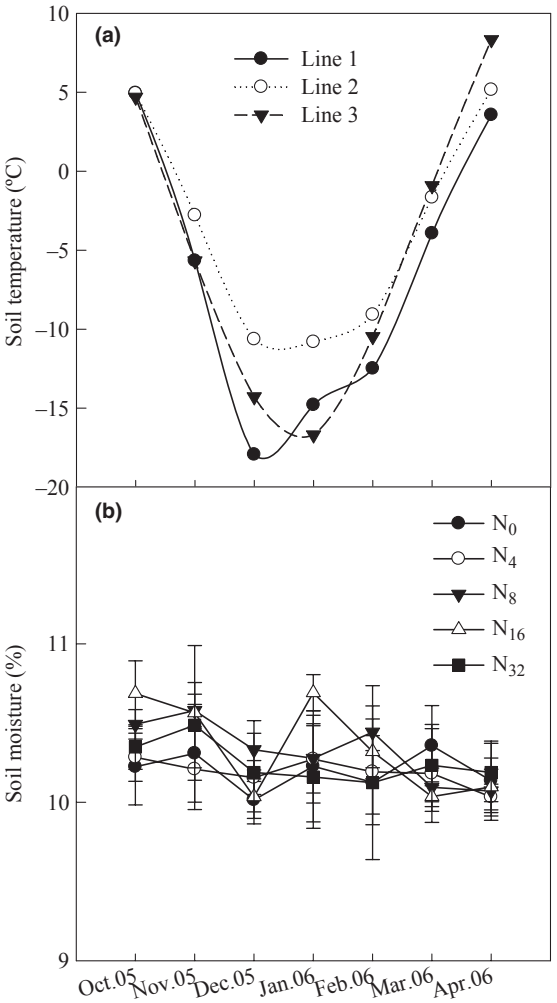

Figure 1 Changes in mean monthly soil temperatures at the surface and 5-cm depth (a) and soil moisture (b) of the study site. Line 1: mean monthly soil surface temperature during a non-growing season (October 2005–April 2006). Line 2: mean monthly soil temperature at 5-cm depth during the non-growing season (October 2005–April 2006). Line 3: long-term (1994–2003) mean monthly soil surface temperature (Zhao *et al.*, 2010).

Material and methods

Description of study site and N fertilizer experiment

The field experiment was conducted in Duolun Restoration Ecology Research Station, the Chinese Academy of Sciences (CAS), located in Duolun County (116°41′E, 41°02′N, altitude 1380 m above mean sea level), in the centre of the Inner Mongolia Autonomous Region, China. This typical grassland is dominated by *Stipa krylovii* Roshev., and *Artemisia frigida* Willd. Mean annual temperature is 5·3°C, with mean minimum temperatures of –16·7°C in January and mean maximum temperature of 24·3°C in July. Mean annual precipitation is 401 mm. Over the period of field incubation (October 2005–April 2006), soil temperature (5 cm in depth) ranged from –16·4°C in February 2006 to 11·3°C in October 2005 (Figure 1). A complete micrometeorological system was installed on eddy towers near the fertilizer treatment,

with soil temperatures being measured by 107 temperature probes (Zhang *et al.*, 2007). Basic soil characteristics are summarized in Table 1.

The N fertilization treatments were established in 2002, with the entire area fenced to exclude any grazing by vertebrate herbivores. Treatments were administered by adding urea in July of each year at the following rates: 0 (N_0: control), 4 g N m^{-2} $year^{-1}$ (N_4), 8 g N m^{-2} $year^{-1}$ (N_8), 16 g N m^{-2} $year^{-1}$ (N_{16}), and 32 g N m^{-2} $year^{-1}$ (N_{32}). All treatments were replicated five times, resulting in a total of 25 treatment plots. Nitrogen was added as urea and broadcast prior to rains in July of each year to minimize leaching/runoff loss of fertilizer. Plots were randomly located, each being 10 m × 15 m in size with a minimum of 4 m between each plot as buffer (Huang *et al.*, 2008).

Net N mineralization and microbial biomass in mineral soil

Net N mineralization in mineral soil during the non-growing season (October 2005–April 2006) was measured monthly by an intact soil-core incubation technique (Raison *et al.*, 1987). Because of the difficulty in collecting soil samples from frozen soil, all cores for the incubation were installed on 1 October 2005. Seven PVC tubes (5·0 cm in diameter and 15 cm in length) in each plot were driven into the soil for 10 cm, and kept 5 cm above the soil surface. One tube in each plot was removed immediately, and taken back to the laboratory for the measurement of soil inorganic N (NH_4^+–N + NO_3^-–N) concentration and microbial biomass. The remaining tubes in each plot were incubated in the field and sampled monthly on the first day of each month from (and including) November 2005 to April 2006.

Thawed soils were mixed thoroughly by hand, whereas frozen soils (November, December, January and February) were reduced to small pieces, with the pieces being homogenized to the extent possible (Schimel *et al.*, 2004). Immediately following, inorganic N concentrations were measured by extracting 10 g fresh soil with 50 mL 2 M KCl for 1 h on a Variable Speed Reciprocal Shaker (Apparatus Co. Ltd., Changzhou, China). Soil slurries were filtered through 45 μm filter paper, and the extract was analysed by a Segment Flow Analyser (the Skalar autoanalyser system, Breda, The Netherlands) for NH_4^+–N and NO_3^-–N in the laboratory of the Institute of Botany, CAS. Net N mineralization, nitrification, and ammonification rates were calculated from the month-to-month accumulation of NH_4^+–N and NO_3^-–N concentrations in the incubated soil cores (Groffman *et al.*, 2001; Zhao *et al.*, 2010). Because N mineralization is the sum of both nitrification and ammonification, these latter two processes were calculated and reported separately.

Soil microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) were measured on these samples using a fumigation-extraction method (Brookes *et al.*, 1985). Fresh subsamples (approximately 20 g dry weight equivalent) were fumigated with ethanol-free $CHCl_3$, and were incubated at 25°C for 24 h, whereas a similar amount of subsample was not fumigated. Extracts from fumigated and unfumigated samples were obtained by shaking soil with 0·5 M K_2SO_4 for 30 min. Extracts were filtered through 0·45 μm filters and frozen at –20°C prior to analysis; extractable N and C were analysed using an automatic analyser (liquiTOC, Hanau, Germany). MBN and MBC were calculated from the difference between extractable N and C contents in the fumigated and the unfumigated samples using conversion factors (*k*EN and *k*EC) of 0·45 and 0·38, respectively. Soil was also analysed for moisture content after drying at 105°C for 24 h.

Basic soil properties, litter production and root biomass

Subsamples of soil from each treatment were collected in October 2005, air dried, and used to measure total nitrogen (TN), soil organic carbon (SOC) and pH. Soil moisture, an important driver of N dynamics in these

Table 1 Basic soil characteristics (0–10 cm) of five nitrogen fertilizer treatments measured on 1 October 2005 (the beginning date of the study) in Inner Mongolia grassland.

Treatment	N_0	N_4	N_8	N_{16}	N_{32}
SOC (g kg^{-1})	22·4 ± 1·7a	22·9 ± 1·3a	24·5 ± 0·7a	24·4 ± 1·0a	25·6 ± 1·0a
TN (g kg^{-1})	2·1 ± 0·2a	2·2 ± 0·2ab	2·3 ± 0·1ab	2·5 ± 0·1ab	2·6 ± 0·1b
C/N	10·5 ± 0·6a	10·6 ± 0·2a	11·0 ± 0·5a	10·0 ± 0·5a	10·0 ± 0·4a
pH (1:2W/V H_2O)	7·30 ± 0·05c	6·97 ± 0·06c	7·03 ± 0·22c	6·38 ± 0·19b	5·85 ± 0·15a
Litter biomass (g m^{-2})	154·1 ± 41·2a	163·8 ± 29·4a	196·6 ± 23·2a	195·3 ± 50·3a	260·7 ± 32·0a
Root biomass (g m^{-2})	1676·1 ± 301·7a	1110·0 ± 262·2a	1124·5 ± 357·4a	1162·1 ± 148·2a	927·0 ± 114·7a

Different letters indicate statistical significance among the five fertilizer treatments at $P < 0·05$ ($n = 5$).
TN, total N; SOC, soil organic carbon.

Inner Mongolian grasslands (Wolf *et al.*, 2010), was determined gravimetrically by comparing moist and air-dry soil weight. Soil TN was analysed using Kjeldahl acid-digestion method (Gallaher *et al.*, 1976) with an Alpkem autoanalyser (Kjeltec System 1026 Distilling Unit, Hillerød, Denmark), whereas SOC was analysed using the H_2SO_4–$K_2Cr_2O_7$ oxidation method (Nelson and Sommers, 1982). Soil pH was determined on mixtures of fresh soil with deionized water (1:2 w/v) using glass electrode.

In 2005, field sampling was conducted mid-August, typically the period of maximum aboveground biomass (Bai *et al.*, 2004). In each plot, a 1-m × 1-m sampling quadrat was established, and the above-ground biomass in the quadrat was clipped to ground level. Following oven drying, this quantity was considered equal to the above-ground net primary productivity of the current year. Plant litter was also collected from one 1-m^2 quadrat within each of the 25 plots. Litter biomass was determined by oven drying litter material to a constant mass at 70°C. Root material was taken by sampling 8-cm diameter soil cores to 10 cm depth and separating roots from mineral soil; two cores were sampled in each plot. Root biomass was determined by oven drying at 70°C to constant mass.

Statistical analysis

Two-way analysis of variance was used to examine the effects of N fertilization, freezing (as sampling date), and their interactions on soil moisture, mineral N pools, mineralization rate, nitrification rate, ammonification rate, microbial biomass C and microbial biomass N. For a specific sampling date, Duncan's multiple range test was used to separate treatments means. Pearson product–moment correlation was used to test the relationship of soil N mineralization, nitrification and ammonification rates with microclimatic factors, soil characteristics and microbial biomass, respectively. Linear stepwise regression procedures were used to determine the correlations between basic soil properties and microbial biomass or soil net N transformation. All statistical analyses were performed using the software program SPSS, ver. 10·0 (SPSS Inc., Chicago, IL, USA).

Results

Net rates of N mineralization, nitrification and ammonification

Net N mineralization, nitrification and ammonification rates in soils of the five fertilizer treatments varied significantly during the period October 2005 to April 2006 (Figure 2). During this time, soil net N mineralization in the five treatments declined gradually from October to November, increasing from December (mean rate was 0·06 $\mu g\ g^{-1}\ d^{-1}$) – January (mean rate was 0·05 $\mu g\ g^{-1}\ d^{-1}$) – February (mean rate was 0·09 $\mu g\ g^{-1}\ d^{-1}$), and again in April. The overall daily rate ranged from –0·29 to 0·51 $\mu g\ g^{-1}\ d^{-1}$ during the study period (Figure 2a). Relative to the control (N_0), net N mineralization and accumulations of mineral N increased significantly with increasing N fertilization (Tables 2 and 3).

Net nitrification varied significantly with incubation period, N fertilization, and the interaction of period and fertilization (Table 2). Net nitrification ranged from –0·18 to 0·32 $\mu g\ g^{-1}\ d^{-1}$ (Figure 2b) during the study, with mean monthly rates being positive, except in March. Relative to N_0, net nitrification and accumulation of nitrate increased by 9 and 880%, respectively, with increased N fertilization (Table 3).

Net ammonification in the fertilizer treatments showed similar temporal patterns to that of N mineralization, with mean ammonification rate ranging from – 0·23 to 0·26 $\mu g\ g^{-1}\ d^{-1}$(Figure 2c). Ammonification and accumulation of ammonium did not vary significantly among the five fertilizer treatments (Tables 2, 3 and Figure 5).

Variation of soil microbial biomass during the non-growing season

Soil microbial biomass C and N showed temporal variations during the sample period, varying significantly with sampling time and N treatment (Table 2 and Figure 3). Microbial biomass C ranged from 185 to 2464 $\mu g\ g^{-1}$, initially declining gradually in the five fertilizer treatments, then increasing drastically with highest values for N_8, N_{16} and N_{32} treatments in February, and in March for N_0 and N_4 treatments (Figure 3a). Microbial biomass N varied from 9 to 132 $\mu g\ g^{-1}$, increasing from October to November and declining in December. Following an increase in January, microbial biomass N declined until April (Figure 3b).

Soil microbial biomass C increased linearly with N fertilization up to the 16 g N m^{-2} treatment, declining to a minimum at the 32 g N m^{-2} treatment (Figure 4a). In contrast, microbial biomass N declined linearly throughout the range of added N (Figure 4b).

Seasonal variations of soil mineral N pool

Available pools of NH_4^+–N and NO_3^-–N varied significantly with sampling date and N fertilization (Table 2). Concentrations of NH_4^+–N in the five treatments declined gradually, and by as much as 46 to 69% by the end of the incubation, compared to initial values (Figure 5a). In contrast, NO_3^-–N concentrations

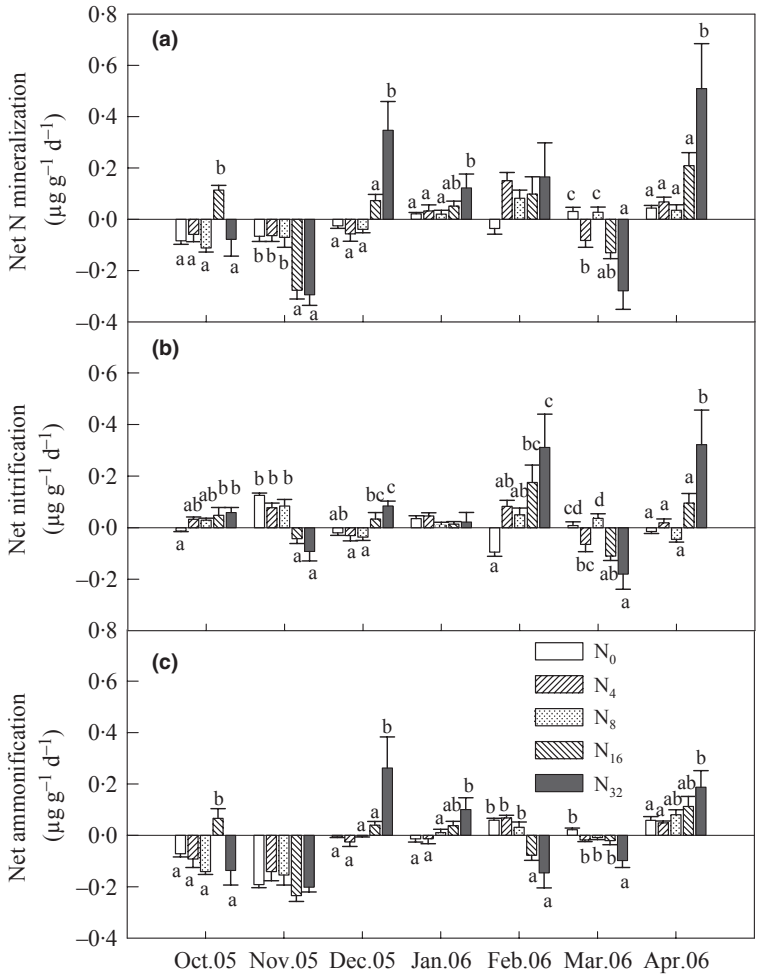

Figure 2 Changes in soil net N mineralization rate (a), net nitrification rate (b) and net N ammonification rate (c) in five N fertilizer treatments during the non-growing season (October 2005–April 2006). Values are means (n = 5), standard errors shown. Significant differences among fertilizer treatments are indicated by different letters at $P < 0{\cdot}05$. The periods of October–November and November–December correspond to mild freezing, January–February, and March–April corresponds to deep freezing and freeze–thaw phases.

Table 2 *F*-ratios of the two-way ANOVA of sampling dates and N fertilizer treatments on soil moisture (SM), NH_4^+-N, NO_3^--N, soil N mineralization rates, nitrification rates, ammonification rates, microbial biomass C and microbial biomass N during a non-growing season in Inner Mongolian grassland, China.

		Soil moisture	Mineral N pool (mg N m^{-2})		Net rate (ug g^{-1} d^{-1})			Microbial biomass (ug g^{-1})	
		(%)	NH_4^+-N	NO_3^--N	Mineralization	Nitrification	Ammonification	C	N
Sampling date	*F*	1·46	16·98	15·03	23·31	8·89	37·62	225·37	73·48
	P	0·19	0·001	0·001	0·001	0·001	0·001	0·001	0·001
Fertilizer	*F*	0·65	48·03	196·88	3·11	3·23	0·53	19·87	2·59
	P	0·63	0·001	0·001	0·02	0·01	0·72	0·001	0·04
Fertilizer * date	*F*	0·37	1·02	4·49	6·52	6·28	5·62	51·18	2·85
	P	0·99	0·45	0·001	0·001	0·001	0·001	0·001	0·001

Significance at $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ and $***P < 0{\cdot}001$, (n = 5).

Table 3 Comparisons of the mean soil moisture, mineral N pool (NH_4^+-N, NO_3^--N), accumulative mineralized N (Accum min N), nitrified N (Accum nit N) and ammonified N (Accum amm N) among five treatments in Inner Mongolia, China during a non-growing season.

Fertilizer	N_0	N_4	N_8	N_{16}	N_{32}
Soil moisture (%)	10·2 ± 0·1a	10·2 ± 0·1a	10·3 ± 0·1a	10·4 ± 0·1a	10·3 ± 0·1a
NH_4^+-N pool (mg N m^{-2})	497·6 ± 58·4a	532·6 ± 62·1a	579·9 ± 55·8a	914·2 ± 71·0b	1621·1 ± 145·1c
NO_3^--N pool (mg N m^{-2})	331·2 ± 35·4a	396·5 ± 38·9a	513·8 ± 41·0b	827·3 ± 61·5c	1608·0 ± 75·0d
Accum mineral N (μg g^{-1})	–3·6 ± 1·4a	–0·4 ± 2·5a	–2·1 ± 2·3a	5·8 ± 3·4ab	17·3 ± 11·4b
Accum nitrif N (μg g^{-1})	0·8 ± 0·3a	5·1 ± 1·7a	3·7 ± 1·6a	6·9 ± 1·5ab	16·9 ± 7·3b
Accum ammon N (μg g^{-1})	–4·4 ± 1·2a	–5·5 ± 1·4a	–5·8 ± 1·5a	–1·2 ± 2·2a	0·4 ± 5·3a

Significant differences among the treatments are indicated by different letters at $P < 0·05$ ($n = 5$).

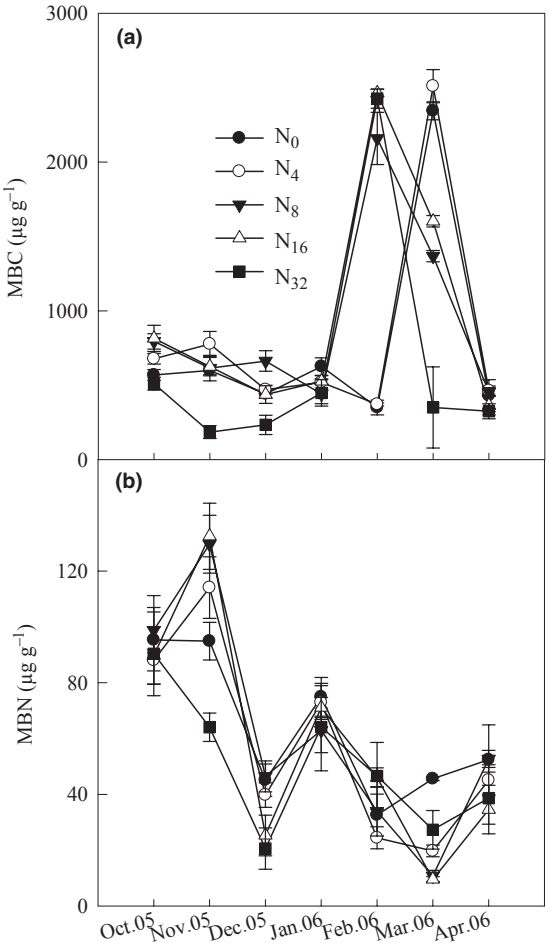

Figure 3 Temporal variations of soil microbial biomass C (a) and microbial biomass N (b) during the non-growing season. Values are means ($n = 5$), standard errors shown.

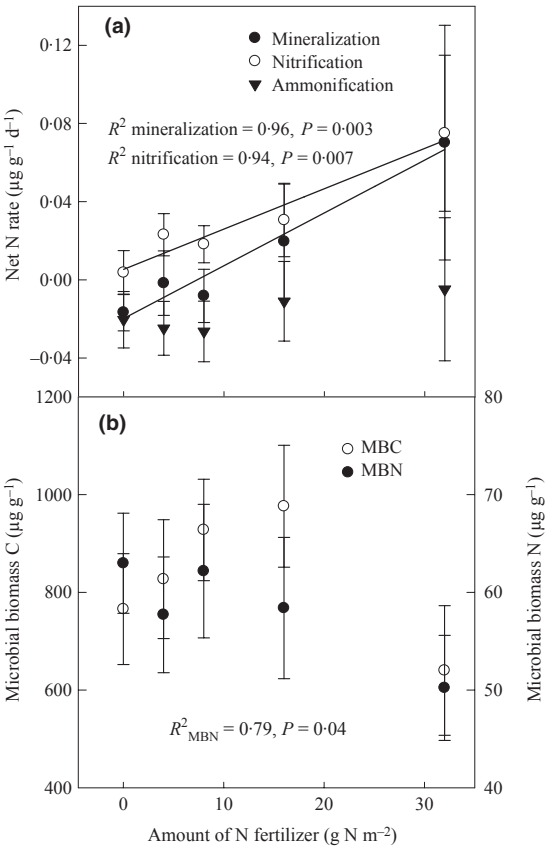

Figure 4 Relationships between soil N mineralization rate (a) and microbial biomass (b) with N fertilization ($n = 5$).

increased gradually over time (Figure 5b) by as much as 127% by the end of the period. Relative to the control, mean NH_4^+–N and NO_3^-–N concentrations in the four fertilized plots increased by 153 and 83%, respectively.

Correlations between N mineralization and climatic factors, soil characteristics and microbial biomass

Net rates of N mineralization, nitrification and ammonification exhibited contrasting relationships with microclimatic factors, microbial biomass, and soil characteristics (Table 4). Net N mineralization was

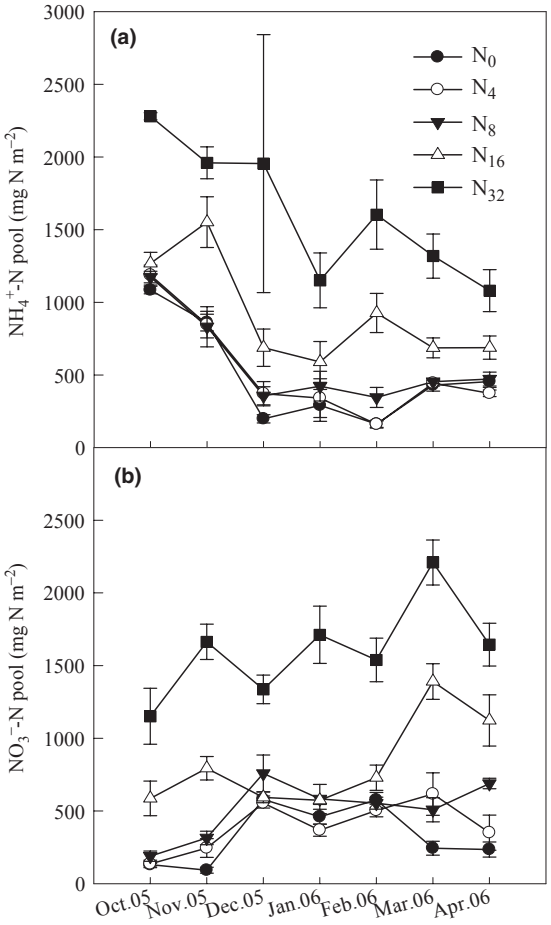

Figure 5 Temporal variation in soil NH_4^+-N (a) and NO_3^--N (b) pools in five N treatments during non-growing season (October 2005–April 2006). Periods of October/November–November/December correspond to mild freezing, January/February–March/April correspond to deep freezing/freeze–thaw phases.

significantly and negatively correlated with MBN, C/N, pH and positively correlated with litter biomass. Net nitrification was significantly correlated with pH and litter biomass, and net ammonification was negatively correlated with MBN and C/N. Linear regression analysis showed that soil microbial biomass N explained 36% ($F = 4{\cdot}78$, $P = 0{\cdot}04$) of temporal variation in net N mineralization and soil microbial biomass C and microbial biomass N explained 60% ($F = 18{\cdot}16$, $P < 0{\cdot}0001$) and 67% ($F = 13{\cdot}31$, $P < 0{\cdot}0001$) of seasonal patterns in ammonification. Net N mineralization, nitrification and ammonification rates were negatively correlated with C/N ratio and pH, and positively correlated with litter biomass. Linear regression analysis also indicated that litter biomass explained 52% ($F = 8{\cdot}30$, $P = 0{\cdot}008$) of

Table 4 Linear correlations of soil N mineralization, nitrification and ammonification with temperature ($n = 35$), microbial biomass ($n = 35$), or basic soil properties ($n = 25$) during the non-growing season.

	Mineralization	Nitrification	Ammonification
Soil temperature	–0·06	0·005	–0·09
Air temperature	–0·08	0·02	–0·14
MBC	0·02	0·22	–0·16
MBN	–0·36*	0·09	–0·59**
C/N ratio	–0·39*	–0·28	–0·48*
pH	–0·46*	–0·49*	–0·27
Litter biomass	0·52**	0·52**	0·38

Values shown are Pearson product–moment correlation coefficients.
TN, total soil N; SOC, soil organic C.
Significance at $*P < 0{\cdot}05$ and $**P < 0{\cdot}01$.

variation in soil N mineralization rate among different treatments. Both litter biomass and pH explained 52% ($F = 8{\cdot}31$, $P = 0{\cdot}008$) and 62% ($F = 7{\cdot}03$, $P = 0{\cdot}004$), respectively, of variation in N nitrification rate, while soil C/N explained 48% of N ammonification rate ($F = 6{\cdot}93$, $P = 0{\cdot}02$).

Discussion

Temporal patterns of soil N mineralization and mineral N pools

In this study, net N mineralization rates in the fertilizer treatments showed significant temporal variation during the non-growing season, with much of this variation related to the fact that these soils experienced three distinct freezing events over time. These include mild freezing (end of October–November 2005), deep freezing (December 2006–February 2006), and rapidly alternating freeze–thaw events (March–April 2006) (Figure 1). During mild freezing, soil temperatures declined to –2°C, and net N mineralization was negative (i.e. net N immobilization) (Figure 2a). This is consistent with results of other studies showing available N to either be immobilized or exhibit minimal change in response to mild freezing (Groffman *et al.*, 2001; Neilsen *et al.*, 2001; Gilliam *et al.*, 2010; Zhao *et al.*, 2010). Gilliam *et al.* (2010) suggested that mild freezing may inhibit N mineralizing microbes and stimulate N immobilizing groups. This is consistent with the increase in microbial biomass N in our study (Figure 3b). Decreasing microbial biomass C (Figure 3a) also suggests that more available C was released after mild freezing to

stimulate microbial activities, and resulting in more mineral N being immobilized by soil microbes (Corre *et al.*, 2002). Root mortality may also enhance N immobilization, since available C is released when plant roots freeze, stimulating microbial activity (Groffman *et al.*, 2001). Lower microbial biomass in the treatments appeared related to faster net N mineralization, whereas higher microbial biomass was correlated with N immobilization, consistent with the findings of Gilliam *et al.* (2011).

Severe freezing occurred when soil temperatures declined below –10°C (Figure 1), a time during which net N mineralization in the five treatments was positive (Figure 2a). These results are consistent with published findings that freezing soil at temperatures between –10 and –15°C substantially increases net N mineralization (Deluca *et al.*, 1992; Neilsen *et al.*, 2001; Zhang *et al.*, 2008; Gilliam *et al.*, 2010; Zhao *et al.*, 2010). Observed declines in microbial biomass C and N (Figure 3) are likely related to microbial mortality at these severe freezing temperatures (Edwards and Cresser, 1992). Cells and associated protoplasm from fine roots killed by severe freezing also might be a source of N substrate available for surviving microbial biomass (Campbell and Biederbeck, 1972). In addition, microbial activity may be enhanced from more C available from detritus and microbes killed by freezing (Christensen and Tiedje, 1990). Such interactions among soils, roots, and microbes may explain increases of soil N mineralization (Figure 2), consistent with the results of Neilsen *et al.* (2001), who found that mild freezing had few effects on N mineralization, whereas severe freezing at –13°C stimulated N mineralization.

The drastic change in microbial C and N and, more important, the lack of consistency of temporal patterns between two microbial characteristics that are commonly closely correlated, is notable (Figure 3). As already suggested, this may be related in part to freezing-mitigated microbial dynamics, wherein cell lysis may cause changes in soil N without changes in soil C. Indeed, working in similar Inner Mongolian steppes, Wolf *et al.* (2010) found microbial populations decreased by 80% with the onset of winter, consistent with patterns displayed in Figure 3b.

Perhaps more likely, however, is that these patterns are related to profound shifts in soil microbial community structure, particularly a shift from bacterial- to fungal-dominated communities. Gilliam *et al.* (2011) reported that soils with low N availability had higher microbial biomass (530 μmol PLFA kg^{-1} soil) and fungal:bacterial ratios of (24·2), whereas high N soils had lower microbial biomass (399 μmol PLFA kg^{-1} soil) and fungal:bacterial ratios (12·1), supporting the pattern shown in Figure 4b. Working in North American grasslands, McCulley and Burke (2004) found similar shifts towards fungal dominance in response to changes in soil N status. This potential change in microbial community structure (i) underlines the dynamic nature of soil microbes, particularly those associated with N processing and (ii) has important implications for restoration and management of these grasslands. Accordingly, we suggest that further work be done to specifically examine and characterize soil microbial communities and their potential change with freezing and N additions.

From March to April 2006, these soils experienced alternating freeze–thaw events, with soil temperatures ranging from –2 to 5°C. Repeated freezing and thawing can represent both a physical and biological disturbance of soil, leading to physical disruption of soil microbial cells and release of substrates protected by soil aggregates (Edwards and Cresser, 1992).

Net nitrification was positive in each monthly incubation period, except for March (Figure 2), a pattern that was correlated with neither soil temperature nor microbial biomass (Table 4), suggesting that soil nitrification was only indirectly regulated by biotic or abiotic factors. During this time, nitrifier activity continued at lower temperatures, with nitrifying bacteria likely acclimating to changes in temperature, which changed gradually in the field (Cookson *et al.*, 2002). As a result, nitrification rates varied slowly (Figure 2b) (Zhao *et al.*, 2010) and, without plant uptake during this dormant period, accumulation of soil NO_3^- increased.

Soil NH_4^+ decreased and NO_3^- increased during the non-growing season (Figure 5), consistent with Schimel *et al.* (2004) and Zhao *et al.* (2010). Seasonal patterns of NH_4^+ and NO_3^- may be attributed to freezing, which can disrupt coupling between soil N mineralization and microbial immobilization of N (Maithani *et al.*, 1998; Gilliam *et al.*, 2010). During the study period, nitrification was higher than mineralization and ammonification in the five treatments, suggesting that nitrification is the predominant N transformation process, simultaneously increasing NO_3^- and decreasing NH_4^+ in the soil.

Comparison of N mineralization and nitrification among N fertilizer treatments

Fertilizer N significantly affected soil N mineralization, with both net N mineralization and nitrification increasing linearly with the N fertilizer added. Soil biochemical characteristics, such as C/N ratio and pH, and soil microbial communities likely contributed to this pattern. Soil C/N ratios have been shown to play an important role in regulating N mineralization (Lovett *et al.*, 2002; Christenson *et al.*, 2009). Significant negative correlation between C/N ratio and soil N mineralization indicated that the treatments with

higher C/N had lower rates of N mineralization. Under conditions of lower C/N ratios (indicating higher N availability), microbial growth is more limited by C availability in soil organic matter. Because N availability is sufficient, N mineralization is generally stimulated. In contrast when C/N ratios are higher, microbes become N-limited, resulting in greater N immobilization (Nadelhoffer *et al.*, 1991; Steltzer and Bowman, 1998).

Soil pH has been shown to be another factor potentially controlling net N mineralization and nitrification (Gilliam *et al.*, 2004). Significant negative correlations between soil pH and N mineralization/nitrification suggest that soil pH influences soil N mineralization directly or indirectly during the non-growing season (Table. 4). Soil pH declined with increased N loading (Guo *et al.*, 2010), and enhanced soil acidity may suppress directly the functioning of the microbial community (Compton *et al.*, 2004).

Litter production increased with N application rates (Table 1), which also stimulated soil microbial activity (Bowman *et al.*, 2004), affected soil microbial biomass (Fisk and Fahey, 2001; Aerts *et al.*, 2006; Liu *et al.*, 2006) and regulated soil N mineralization. Positive correlation between N mineralization and litter biomass suggests that increased litter biomass may stimulate soil N mineralization during the non-growing season. Increasing litter biomass generally increases litter decomposition, which influences soil microbial activity, mediating conversion of soil organic N to inorganic forms (NO_3^- and NH_4^+) (Weintraub and Schimel, 2003; Sun *et al.*, 2004), and regulating soil N availability directly (Huang *et al.*, 1998; Liu *et al.*, 2000). Changes in litter chemistry, which was not determined in this study, can also greatly influence temporal patterns of net N mineralization and nitrification and further mitigate responses to N fertilization (Bowman *et al.*, 2004; Aerts *et al.*, 2006). Changes in root biomass could also affect microbial biomass through altering the availability of C (Treseder, 2008) and physical soil environment (Wallenstein *et al.*, 2006). The relationship between microbial biomass C and root biomass was not significant (Table 3), suggesting that C limitation to soil microbial biomass growth in the fertilizer treatments might not occur in this grassland (Zhang *et al.*, 2008).

Conclusion: Linkage of N fertilization and effects of soil freezing

The need to fertilize degraded Inner Mongolian grasslands for the purpose of restoration is inextricably linked to the phenomenon of soil freezing and, ultimately, its effects on soil N dynamics. This region experiences ambient air temperatures <0°C for a considerable part of the non-growing season, a time during which plant uptake of mineral N is minimal. As shown in this and other studies (Groffman *et al.*, 2001; Gilliam *et al.*, 2010), freezing can increase the size of available soil N pools, leading to accumulation of mineral N beyond demand by plants. In this way, freezing can both mimic and exacerbate effects of N fertilization. Although it was not within the scope this study to measure other soil responses, the potential for base cation leaching associated with mobile NO_3^- in the soil must be considered when addressing restoration strategies.

Soil N mineralization showed distinct temporal variation during the non-growing season, a pattern that appeared regulated by microbial biomass. Net nitrification was the dominant form of N transformation. Considerable available N accumulated during this dormant period, which would be immediately available for plant uptake in the following growing season. Effects of N fertilization on soil N mineralization were related to variation in soil pH, C/N ratio, and plant biomass. Management of these and similar grasslands, especially those which utilize additions of fertilizer N, should take account of the non-growing season effects of changes in ambient temperatures on soil N dynamics, which can mitigate plant response to N additions. Future work will investigate ecosystem-level implications for these treatments in the context of soil freezing, including net primary productivity and potential changes in plant community structure and composition.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China under the Innovative Research Group Project (30521002) and from the Chinese Academy of Sciences under the Key Research Project (KSCX2-SW-127).

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