Soil Carbon, Nitrogen and Phosphorus Dynamics in Response to
Warming and Grazing in Alpine Meadow Ecosystem of the Tibet
Plateau

Yichao Rui

B.Sc (2006)

School of Biomolecular and Physical Sciences
Science, Environment, Engineering and Technology
Griffith University

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Abstract

Ongoing global climate change is predicted to substantially impact on terrestrial ecosystems. The Qinghai-Tibet plateau is very sensitive to climate change. Alpine meadows, one of the major ecosystems on the Qinghai-Tibet Plateau, is very vulnerable and can be sensitive to both climate change and human disturbance. Grazing is the main land use mode of the alpine meadow ecosystem. Soil carbon (C), nitrogen (N), and phosphorus (P) availability are key to ecosystem productivity, while the alterations in soil C, N and P dynamics can affect ecosystem substantially. Investigation on the response of soil C, N and P cycling and the associated functional microorganisms in the alpine meadow can provide insights into soil function under warming and grazing conditions and sustainable management of the ecosystem.

In this thesis, the study was conducted based on a controlled warming-grazing experiment in the Haibei Alpine Meadow Ecosystem Station, Chinese Academy of Sciences (CAS). A free-air temperature enhancement (FATE) system and a moderate grazing intensity were applied to simulate the effects of warming and grazing. Four treatments including no warming with no grazing (NWNG), no warming with grazing (NWG), warming with no grazing (WNG) and warming with grazing (WG) were applied. Soil samples were taken after three years of warming. Soil total N (TN), inorganic N, microbial biomass C and N (MBC and MBN), soluble organic C and N (SOC and SON), as well as natural abundances of C and N stable isotopes were examined. A fractionation method was applied to investigate the sizes of different soil inorganic and organic P fractions and the activities of acid and alkaline phosphomonoesterase were studied. In order to assess the response of microorganisms
to simulated warming and grazing, the diversity of bacteria was investigated using denaturing gradient gel electrophoresis (DGGE). Abundances of N-cycling related functional microbial groups were measured with real-time quantitative PCR. In order to assess the warming effects on soil N-cycling related gene abundance in different ecosystems on a broader scale, we conducted another comparative study in the semiarid steppe of Inner Mongolia Plateau. The main results are listed as follows:

(1) After three years of treatment, warming and grazing affected soil C and N pools differently and these effects varied with soil depth. Warming significantly increased TN, MBC, MBN and SON and decreased δ¹³C at the 10–20 and 20–30 cm soil depths, while grazing generally decreased SON at 10-20 and 20-30 cm and MBC at 20-30 cm. At the 0–10 cm depth, neither warming nor grazing alone affected these soil parameters significantly. However, grazing alone increased NO₃⁻-N, total inorganic N, SOC and δ¹⁵N at the 0–10 cm depth.

(2) Both warming and grazing significantly decreased the quantity of organic P extracted by first NaOH (N(I)Po), as well as the total extractable organic P (TPo) at the 0-10 cm depth. Warming also decreased the total P of soil at 0-10 cm. The combined warming and grazing treatment (WG) led to the reduction of major soil organic P fractions (N(I)Po, TPo) by 40-48% and 28-32%, respectively compared with other treatments at 0-10 cm. The activities of acid and alkaline phosphomonoesterase (AcPME and AlPME) were both enhanced by warming and grazing, and their interaction. Decreased concentrations of soil N(I)Po and TPo were accompanied by increased AcPME activity (P < 0.01) and soil temperature (P < 0.05), indicating the enhanced mineralization of organic P under warming. Meanwhile, leaf biomass P of two
major species (*Potentilla anserine* and *Gentiana straminea*) within these plots were significantly enhanced by either grazing or warming.

(3) Warming significantly reduced bacterial diversity and abundance, based on 1.2-1.7 °C temperature increase, while the effects of warming and grazing on N-cycling microbial gene abundance varied with soil depths and between the drought year of 2009 and the normal precipitation year of 2010. Despite the lack of significant treatment effects in 2009, warming significantly reduced abundance of N-cycling related genes at the 0-10 cm soil depth in 2010, while grazing alone markedly increased the abundance of denitrifying genes *narG*, *nirS* and *nosZ* by 27-46% at 10-20 cm, which effect was surprisingly dampened by warming. Plant species diversity explained 54.5% of the variation of bacterial diversity over the two years, while soil moisture and nitrate concentration explained 39-53% of the variation of the abundance of total bacteria and denitrifiers.

(4) In order to compare the warming effects in other similar ecosystems and also test the conclusion that soil water availability plays a critical role in affecting the microbial abundance of C and N cycling, I took advantage of a 6 years’ multi-factorial experiment in the semiarid steppe of Inner Mongolia Plateau, and studied the independent and combined effects of experimental warming and increased precipitation on N-cycling gene abundance. Large inter-annual variation was observed between 2010 and 2011, as the N-cycling gene abundance surged in the second year along with the elevated seasonal precipitation and ammonium concentration. However, abundance of total bacteria and N-cycling genes generally showed little response to warming over the two years, while increased precipitation markedly elevated abundance of total bacteria,
*nifH, narG* and *nosZ* at 0-10 cm in the relatively dry year of 2010, but reduced the abundance of *nifH, nirK, nirS* and *nosZ* at 10-20 cm in 2011. The significant interaction between warming and increased precipitation in 2010 demonstrated that warming could counteract the positive effects of increased precipitation.

In conclusion, warming and grazing affected labile C, N and P fractions significantly but differently: warming tended to enlarge labile C and N pools through increased litter inputs, while grazing tended to directly increase inorganic N pools, decrease SON and accelerate N cycling; while the microbial mineralization of soil organic P could be strongly increased under combined warming and grazing conditions as driven by increasing plant demand for P and enhanced microbial activities. As for the microbial communities, warming mainly affected soil bacterial diversity and abundance indirectly through its impact on plant community and soil moisture, and might reduce the positive effects of grazing on denitrifiers’ abundance and N\textsubscript{2}O production in wet conditions in this alpine meadow. Meanwhile, in the semiarid steppe, increased precipitation could exert a positive impact on microbial abundance under xeric conditions in a dry year; while the functional microbial abundance was driven by and could respond rapidly to seasonal precipitation variation. In both ecosystems, soil moisture and precipitation are of great importance in controlling microbial abundance.
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Declaration of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Yichao Rui
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<th>Meaning</th>
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<tr>
<td>MBC</td>
<td>Microbial biomass carbon</td>
</tr>
<tr>
<td>MBN</td>
<td>Microbial biomass nitrogen</td>
</tr>
<tr>
<td>SOC</td>
<td>Soluble organic carbon</td>
</tr>
<tr>
<td>SON</td>
<td>Soluble organic nitrogen</td>
</tr>
<tr>
<td>NPP</td>
<td>Net primary production</td>
</tr>
<tr>
<td>AOA</td>
<td>Ammonia-oxidizing archaea</td>
</tr>
<tr>
<td>AOB</td>
<td>Ammonia-oxidizing bacteria</td>
</tr>
<tr>
<td>HBAMERS</td>
<td>Haibei Alpine Meadow Ecosystem Research Station</td>
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<tr>
<td>FATE</td>
<td>Free-air temperature enhancement</td>
</tr>
<tr>
<td>TN</td>
<td>Total nitrogen</td>
</tr>
<tr>
<td>δ^{13}C</td>
<td>Natural abundance of stable isotope^{13}C</td>
</tr>
<tr>
<td>δ^{15}N</td>
<td>Natural abundance of stable isotope^{15}N</td>
</tr>
<tr>
<td>SIN</td>
<td>Soil inorganic nitrogen</td>
</tr>
<tr>
<td>TSN</td>
<td>Total soluble nitrogen</td>
</tr>
<tr>
<td>WUE</td>
<td>Water use efficiency</td>
</tr>
<tr>
<td>DOC</td>
<td>Dissolved organic carbon</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>TP</td>
<td>Total phosphorus</td>
</tr>
<tr>
<td>TPo</td>
<td>Total extractable organic phosphorus</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>N(I)Po</td>
<td>Organic phosphorus extracted by first NaOH</td>
</tr>
<tr>
<td>API</td>
<td>Inorganic phosphorus extracted by NH$_4$Cl</td>
</tr>
<tr>
<td>B Pi</td>
<td>Inorganic phosphorus extracted by NaHCO$_3$</td>
</tr>
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<td>BPo</td>
<td>Organic phosphorus extracted by NaHCO$_3$</td>
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<td>N(I)Pi</td>
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<td>HPi</td>
<td>Inorganic phosphorus extracted by HCl</td>
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<tr>
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<td>Inorganic phosphorus extracted by second NaOH</td>
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<tr>
<td>N(II)Po</td>
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<tr>
<td>AcPME</td>
<td>Acid phosphomonoesterase</td>
</tr>
<tr>
<td>AlPME</td>
<td>Alkaline phosphomonoesterase</td>
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<tr>
<td>pNPP</td>
<td>Para-nitrophenyl phosphate</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>TEG</td>
<td>Total extractable glomalin</td>
</tr>
<tr>
<td>EEG</td>
<td>Easily extractable glomalin</td>
</tr>
<tr>
<td>PLFA</td>
<td>Phospholipid fatty acid</td>
</tr>
<tr>
<td>ANPP</td>
<td>Aboveground net primary production</td>
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<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic units</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<td>SIP</td>
<td>Stable isotope probing</td>
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Chapter 1 Introduction

1.1 Background

The global surface temperature has been increasing since the industrial revolution. The Intergovernmental Panel on Climate Change (IPCC) pointed out that, the global surface mean temperature had risen by 0.74°C(±0.18°C) in the past century (1906-2005), while this trend had become more obvious since the 1950s (IPCC 2007). A temperature increase of 1.4-5.8°C was predicted from 2000 to 2100, based on modeling. This significant temperature increase, which can be attributed to the drastic increase of greenhouse gas emissions caused by intensified human activity since the 1840s, has already brought significant influence on the global environment. Besides the direct impact on melting icecaps and the rising sea levels, climate change could also give rise to the increase of frequency of extreme weathers, hence affect people’s lives. Therefore, climate change has become the main challenge that mankind is facing in the 21st century, and the principal global environment issue recognized internationally (Oreskes 2004).

Global climate change also has direct and indirect influence on human society by affecting ecosystem productivity (Schimel et al. 2000), cycling of carbon (C), nitrogen (N), phosphorus (P) and sulfur (S), etc. (Melillo et al. 2002) and biodiversity. The changes in regional climate, especially rising temperature, has already had substantial influence on the structure and function of various natural ecosystems in many regions in the world. These include retreating icecaps, melting permafrost, extending growing
season in high-latitude areas, the migration of animals and plants to high-latitude and high-altitude areas, declining number of some plant and animal species. How to secure the sustainable development of human society and environment, and to mitigate the negative influence on environment, has received strong public attention.

A great number of studies have demonstrated that the high-altitude and high-latitude ecosystems are responding to rising temperature more sensitively and swiftly (Chapin et al. 1992). In the past century, the air temperature of China has also increased 0.5-0.8°C, whereas the temperature of the Qinghai-Tibet Plateau is increasing faster than elsewhere in China. Since the 1950s, the glaciers have retreated by 21%, and the permafrost soil has become 4-5 m thinner. Because of its typical fragility and sensitivity to climate change, the Qinghai-Tibet plateau has become a key area that receives wide attention.

The Qinghai-Tibet alpine meadow, the main ecosystem on this plateau, is sensitive to global climate change (Thompson et al. 1993). Moreover, much greater than average increase in surface temperature was predicted to occur in this region in the future (Giorgi et al. 2001). Grazing is the main land-use mode, and there are about 13.3 million domestic yaks and 50 million sheep on the Qinghai-Tibet plateau, and grazing pressure will increase due to increasing population (Yao et al. 2006). A number of simulated experiments have been carried out to study the impacts of global warming and grazing on terrestrial ecosystems all over the world. However, few studies have used infrared heaters in the Qinghai-Tibet Plateau to simulate relevant ecosystem
This research is based on a simulated warming-grazing experiment established in the Haibei Alpine Meadow Ecosystem Research Station, Chinese Academy of Sciences (CAS). The aims of this study is to examine the influence of climate change and land-use management, the two main disturbance factors, on the dynamics of soil C, N and P. The associated microbial groups and pools were measured to explain the macro-scale phenomenon. The research is conducted to provide scientific basis for predicting the impact of global climate change and human activity, and appropriately managing the grazing regime.

1.2 Terrestrial C, N and P cycling in a changing world

Terrestrial ecosystems hold a lot of carbon—about 500 Pg C in plant biomass, and 2000 Pg C in soil organic matter. Land use management and predicted global climate change, through their effects on net primary productivity, the plant community and soil conditions, may have important effects on the size of the soil organic C (SOC) pool in the soil and therefore directly affect the atmospheric concentration of these trace gases (Melillo et al. 2002). However, humans have increasingly distorted the balance, by modifying land use and by injecting fossil C back into the cycle. The changing carbon cycle poses new questions for scientists. Depletion of SOC pool has contributed 78±12 Pg of C to the atmosphere (Lal 2004). Soil C sequestration could restore degraded soils, improve biomass production, and reduces the rate of enrichment of atmospheric CO₂ by
offsetting emissions due to fossil fuel. Thus, adoption of a restorative land use and recommended management practices can mitigate the rate of enrichment of atmospheric CO$_2$ and have positive impacts on food security, soil quality and the environment.

Nitrogen cycling is the pivotal process to ecosystem since N availability is essential to plant growth, subsequently the net primary production of ecosystem. The inorganic N (mainly NH$_4^+$ and NO$_3^-$) which plants can utilize are usually proportionately much less than organic N and are always in the quick transition, being mineralized from organic N by the microorganisms through ammonification and nitrification, then immobilized through microbial assimilation and plant uptake back to organic form, or transformed into N$_2$ and N$_2$O back to the atmosphere through denitrification.

Nitrogen cycling, especially its key process of N mineralization, can be influenced by many environmental factors (soil temperature and moisture), soil properties (pH, soil structure, etc.), quality of organic matters (litter, tanning, etc.) and soil animals and microorganisms (structure, biomass and activity) (Rustad et al. 2001). Thus, most environmental changes which can affect these factors may give rise to the shift in net N mineralization, consequently the soil N availability, as well as N$_2$O flux and leaching of nitrate, which are also important issues in environment protection. A number of studies have reported that N mineralization could be accelerated by warming in various ecosystems from forests to grasslands and tundra. A meta-analysis of 22 studies has shown that across different experimental sites, 2–9 years of experimental warming in
the range 0.3–6.0 °C significantly increased net N mineralization rates by 46% (Rustard et al. 2001). However, in many ecosystems N may remain to be a limiting factor. According to some models, land ecosystems can sequester C fast enough to help to counteract CO₂ emissions (Hungate et al. 2003). But ecosystem C accumulation may be constrained by nutrients, particularly N, through mechanisms that are not well developed in or absent from the models (Nadelhoffer et al. 1994). Some experimental studies show that when microbial decomposition is enhanced by elevated temperature or CO₂ more N is required by microorganisms, which can reduce net N mineralization, the main source for plant growth (Gill et al. 2002). Therefore, a thorough understanding of the mechanisms that N cycling respond to environmental changes such as increased temperature and CO₂ can provide better insights into the long-term impact of global change.

Phosphorus as one of the major soil nutrients in terrestrial ecosystems is essential to plant growth. Under natural conditions, the loss of P can be replenished only by the release of P from primary minerals such as apatite, resulting in a gradual decrease of total P in the soil with time (Filippelli 2008). In many ecosystems P is in short supply and tends to limit the net primary production, N fixation and C storage in the long term (Vitousek and Howarth 1991; Tiessen 2008). Soil P cycling is controlled mainly by geochemical and biological processes, and can be vulnerable to global climate change and land-use management (Papanikolaou et al. 2010; Jouany et al. 2011). Microbial mineralization of organic P, producing available inorganic P for plants, is thought to be
the key process of soil P cycling in unfertilized/natural ecosystems. Soil phosphatases, produced by both microbes and plants, are involved in the P mineralization. Increased temperature can stimulate soil phosphatase activity (Papanikolaou et al. 2010), and accelerate P cycling by enhancing plant growth which increases the demand for nutrients, quantity and quality of litter inputs, root turnover and exudation (Sardans et al. 2006), and by increasing microbial biomass and activity (Raghothama 1999). Meanwhile, the effects of warming can be controlled by soil water availability (Sardans et al. 2006). In grassland ecosystems, land-use management such as grazing can substantially affect P cycling through its direct control on plant and animal residues (Williams and Haynes 1990). Grazing also promotes the nutrient cycling rates, and increases soil nutrient heterogeneity through the uneven deposition of faeces and urine (Carline et al. 2005), which can modify the proportions of organic and inorganic soil P fractions (Galvao and Salcedo 2009). The combined direct (grass consumption) and indirect (recycling through urine and/or faeces deposition) effects of grazing have been studied extensively. However, the net effect of grazing on P cycling and its consequence for primary productivity seem to vary with locations and to be dependent on other factors such as the climate (Jouany et al. 2011). Exploring the mechanisms driving P availability can provide insights into the coupling of C, water and nutrient cycles, and ultimately the responses of ecosystems to climate change (Vandecar et al. 2009).

1.3 Effects of warming on terrestrial ecosystems
Many simulated experiments have been carried out to study the impact of global warming on terrestrial ecosystems all over the world (Rustad et al. 2001). Although ecosystem responses to global warming will be complex and varied, these ecosystem warming experiments still hold great potential for providing insights on ways terrestrial ecosystems will respond to upcoming decades of climate change.

Generally, warming tends to affect most soil processes. Temperature is a key factor that regulates many terrestrial biogeochemical processes, such as soil respiration, litter decomposition, N mineralization and nitrification, denitrification, \( \text{CH}_4 \) emission, plant productivity, and plant nutrient uptake. Data have shown that rates of soil respiration generally increase under warmer temperatures (Raich and Schlesinger 1992), but the responses of other ecosystem processes to warming have been more variable, and general response patterns have been difficult to identify. However, results from a meta-analysis demonstrate that across a wide range of ecosystem types and experimental methodologies, experimental warming in the range of 0.3–6.0 °C significantly increases rates of soil respiration, net N mineralization, and aboveground plant productivity (Rustad et al. 2001), which is consistent with the conceptual model that warming directly increases rates of microbial processes including litter decomposition and N mineralization, thereby increasing the availability of nutrients, and, particularly in nutrient limited ecosystems, increasing plant productivity. The increase in plant productivity may also be a result of a direct effect of warming on rates of net photosynthesis, or an extension of the growing season. In addition, warming can
affect net primary production (NPP) and heterotrophic respiration (Rh) indirectly by
altering the ecosystem's moisture regime or species composition (Melillo et al. 2002).

Plant and microbial community composition can shift towards organisms better
suited to new environmental conditions in response to environmental stress (Balser et al.
2001). It has been documented that ammonia-oxidizing archaea (AOA) and
ammonia-oxidizing bacteria (AOB) groups respond to warming quickly (Avrahami et al.
2003; Horz et al. 2004), which may affect AOB community structure directly through
the effects on environmental factors such as moisture or temperature, and indirectly
through increased plant production and hence substrate availability for microbial growth
(Anderson 1992). In the longer term, another effect of warming on soil microbial
community can occur indirectly via a shift in the functional composition of vegetation
(Wardle et al. 1998) as climate change is likely to alter species distribution and
biodiversity (Davis et al. 1998). As plant functional groups vary in resource acquisition
mode and as species differ in quality and quantity of their root exudates, litter, dead
roots and shoots, they can also affect AOB through their impact on resource
availability. Plants can also alter environmental factors like soil pH and moisture, which
have recently been shown to alter AOB community structure (Nicol et al. 2008).
Adaptation of microbial communities to altered environmental factors and resources
regimes may condition their response to perturbations (Balser et al. 2001). However, as
the effects of warming on abundance and activity of AOA and AOB vary with soil types
and the presence of other environmental changes (Horz et al. 2004), it is still requisite to
understand the more universal mechanisms of warming’s impact on ammonia oxidizers
and their responses in specific ecosystems.

A variety of warming methods have been employed, including electric resistance
heating, infrared irradiation, reciprocal transplants, and open-top and closed-top field
greenhouses (Luo et al. 2001; Melillo et al. 2002; Schmidt et al. 2002; Beier et al. 2008).
Some experiments have warmed only portions of the ecosystem. Others have warmed
the ecosystem for only part of each day or year. No one method perfectly simulates the
expected climatic change, but all have yielded useful results that, with cautious
interpretation, have begun to reveal similarities, differences, and patterns of response
among the ecosystems studied. Future research should include manipulations designed
to provide focused insights into individual ecosystems and key processes of ecosystem
responses.

1.4 Grazing and its influence on soil processes

Grazing, the main land-use form of many grassland ecosystems, is also known to play
an important role in regulating nutrient cycling and other soil processes. Grazing can
quickly lead to changes in nutrient pools and fluxes, in vegetation cover, and in plant
community composition in grasslands. It can also contribute to higher \( N_2O \) fluxes by
creating excreta patches which increase \( NO_3^- \)-N in soils. Grazing could affect the
nutrient cycling mainly in two ways: through transforming plants into urine and fecal
material and by influencing litter mass losses (Olofsson et al. 2001). Saliva of
herbivores may contain certain enzymes directly, while it is also reported that grazing
could increase the activities of soil enzymes that are fundamental to the cycling of N. As
well, sheep trampling can compact the soil consequently reduce soil aeration. Grazing
also affects the quantity and chemical composition of soil organic matter and the
distribution of C and N in the soil profile, thus influencing the largest reservoir of C and
N in the soil-plant system. Grazing has been shown to influence litter dynamics, its rate
of decomposition, and its subsequent effects on herbage production (Naeth et al. 1991).

The direct consumption of plants by herbivores normally intensifies nutrient
cycling, as the nutrient release from feces tends to be faster than the nutrient release
from litter (Ruess et al. 1999). However, the direct effect of herbivores on nutrient
cycling is not always positive as the release of nutrients from feces produced when
grazers are facing nutrient shortage is slower than the release from plants and plant litter
(Pastor et al. 1993), because much of the nutrient content in the plant material is taken
up by the herbivore during digestion (Pastor et al. 1993). Because N is the limiting
factor for primary production in many ecosystems, grazing-induced enhancement of soil
N dynamics is a key mechanism through which herbivores can influence plant
productivity in such a way to alleviate nutrient deficiencies (McNaughton et al. 1997).
In particular, the regulation of nitrification has often been regarded as the key for an
efficient N cycling in this context, as in contrast to most other steps in N turnover only a
limited number of microbes are able to convert ammonium into nitrate. Enhanced
nitrification in response to grazing pressure has been reported in different unfertilized grassland ecosystems (Le Roux et al. 2003; Patra et al. 2005). These changes can be explained by factors such as urine and dung input by herbivores and changes in competition for N between microorganisms and plants. It has been further shown that grazing-induced differences in nitrification levels in grasslands are associated with changes in the abundance and community structure of AOB that were thought to be the key players of the first step of nitrification: oxidation of ammonium into nitrite. However, the role of the latter group in soil N dynamics is still unclear, as factors influencing AOA and AOB abundance and activity still need to be assessed.

1.5 Qinghai-Tibet plateau under warming and grazing conditions

The Qinghai-Tibet Plateau, covering the majority of Qinghai Province and Xizang (Tibet) Autonomous Region of China, is located in central Asia with a mean elevation of more than 4000 m and an area of about 2,300,000 km². Surrounded by the Earth’s highest mountains such as the Himalayas, Pamir, Kunlun Shan and others, it is the highest and most extensive plateau in the world and has long been known as the roof of the world. Many studies show strong evidence that this plateau exerts profound thermal and dynamical influences on the local weather and climate as well as on atmospheric circulation in the Northern Hemisphere (Kutzbach et al. 1993). This region presents a unique natural environment, serving specific ecological functions critical for water
conservation in the large headwaters, abundant in natural resources, and diversified in
species and germplasm resources (Liu, 1996; Li and Zhou, 1998). Therefore, this
plateau has become the focus of public concern and received considerable attention.
However, owing to the lack of data at the spatial and temporal resolution levels needed
for climate research, it is only in the last a few years that investigations into
contemporary climate change over this plateau have been carried out (Tang et al. 1998).

Recent works based on reconstructed climatic data have demonstrated that there has
been a warming trend over the plateau during the last 90 years and suggested that it
could be one of the most sensitive areas to global change. Ice cores also reflect a
significant increase in temperature over the last few decades (Thompson et al. 1993;
Yao et al. 2006), which is associated with a retreat of most mountain glaciers in this
region (Tang et al. 1998). Several studies (Liu and Chen 2000; Liu et al. 2006) found
that the Qinghai-Tibet Plateau has experienced distinct warming in recent decades,
especially for cold seasons, and the trend of warming over the plateau is usually larger
than that of the surrounding areas. The headwaters region of the Yangtze and Yellow
River, located in the interior of the Qinghai–Tibet Plateau, represents a distinct
cryospheric environment, housing a number of typical alpine ecosystems including
alpine meadows and alpine steppes. Such ecosystems are quite sensitive to global
climate change, which has impacted the region's environment and altered its water cycle
(Li and Zhou 1998).

In the Qinghai-Tibet Plateau, which is being grazed by 13.3 million domestic yaks
and 50 million sheep that could cause large amounts of animal excrete directly depositing on it, there is a scarcity of available information about N cycling under warming and grazing conditions. Alpine meadow ecosystem occupies a large part of the plateau. It is similar to boreal forests and arctic tundra ecosystems, with a large stock of N in the soils, 95% of which is organic N, including free amino acids. Previous research on N cycling in these ecosystems reported a lack of inorganic N and plant growth limited by N availability. However, organic N might cover the N requirements of plants in these alpine meadow ecosystems. The grassland, however, is characterized by the long cold and dry, short wet seasons, with the growing season of 90-150 days, resulting in lower primary productivity in alpine *Kobresia* meadow. It has long been known that the productivity of cold ecosystems is limited by short growing season and reduced soil nutrient availability. Plant growth in cold ecosystems might also be influenced by soil moisture availability. Research showed that the seasonal changes of aboveground biomass and biomass composition of different vegetation types are different in plant growing season, and the belowground biomass is distributed mainly in 0-10 cm soil and has vertical distributive characteristics.
Based on the information above, our research questions are listed as follows:

1) In the Qinghai-Tibet alpine meadow, will warming and grazing lead to symmetrical changes in major labile C and N pools and higher N availability?

2) Under warming and grazing conditions, will soil organic P mineralization be increased to meet microbial and plants’ requirements? Will there be a gradual P depletion?

3) How will warming and grazing affect the population size and composition of N-associated functional microbial groups such as AOA and AOB? Is soil moisture a
driving factor controlling the microbial response?

1.7 Reference


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Chapter 2  Experimental site description

2.1 Experimental site

The experimental site is located at the Haibei Alpine Meadow Ecosystem Research Station (HBAMERS), a facility run by the Northwest Institute of Plateau Biology, Chinese Academy of Sciences (CAS). The station is situated at latitude 37° 37’N, longitude 101° 12’E, and the mean elevation of the valley bottom is 3200 m. The site is located at Menyuan County, Qinghai Province. This station is within a flat valley, surrounded by mountains. The mountain of LenglongLing is lying on the north, average 4600 m, covered with snow across the whole year. The mountain of Daban is lying on the south, average 4000 m;

![Location of Haibei Alpine Meadow Ecosystem Research Station, CAS](image)

Fig. 2.1 The location of Haibei Alpine Meadow Ecosystem Research Station, CAS

Lying in a large valley surrounded by the Qilian Mountains on the northeast of the Qinghai-Tibetan Plateau, the station experiences a typical plateau continental climate which is dominated by the southeast monsoon in summer, from May to September and high pressure from Siberia in winter. Summers are short and cool, and
winters are long and severely cold. Mean annual temperature is \(-2^\circ\text{C}\), while mean annual precipitation is 500 mm, over 80% of which falls during the summer monsoon season. The relative air humidity is 67%, and the mean pressure of 691 hPa. Across the year there is no absolute frost-free period, and the relatively frost-free period is about 20 days. In the summer season, there still can be frost, ice, snow, and winter weather phenomenon. The cold season is dry and long, and the warm season is moist and short.

The Qinghai-Tibet Plateau covers a vast area of 2.9 million square kilometers. Surrounded by massive mountain ranges, A great number of rivers including the Yangtze river, Yellow river, and Mekong river etc. flow from the west to the east. As a result, the plateau is of vital importance to the lives and cultures in East Asia.

The special natural environment of the Tibet Plateau has led to the development of perennial herbaceous plant communities to adapt to cold and wet weather. The constructive plant communities include the *Kobresia Humilis*, *Potentilla fruticosa*, *Kobresia pygmaea*, and *Kobresia tibetica meadows*. The community structure is relatively simple, with a low primary production. In this research, we choose the *Kobresia Humilis* meadow as the major objective, with the major species including *Elymus nutans*, *Stipa aliena*, *Poa pratensis*, *Festuca ovina*, *Carex scabrirostris*, *Scripus distigmaticus*, *Gentiana straminea*, *Gentiana grumii*, *Saccssurea superb*, *Potentilla anserine*, *Potentilla nivea* etc.

The main soil type of this site is Mat-Cryic Cambisols (FAO classification), which is relatively newly developed, shallow and rich in soil organic matter content. Table 2.1 shows the principal physical and chemical properties of the soil at this site.
Table 2.1 Chemical and physical properties of the Mat-Cryle Cambisols (Wu et al. 2005)

<table>
<thead>
<tr>
<th>Soil depth (cm)</th>
<th>pH</th>
<th>Bulk density (g .cm⁻³)</th>
<th>Organic C (%)</th>
<th>Total N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-8</td>
<td>8.0</td>
<td>0.88</td>
<td>7.27</td>
<td>0.53</td>
</tr>
<tr>
<td>8-32</td>
<td>8.5</td>
<td>1.19</td>
<td>3.32</td>
<td>0.33</td>
</tr>
<tr>
<td>32-71</td>
<td>8.7</td>
<td>1.16</td>
<td>2.65</td>
<td>0.27</td>
</tr>
<tr>
<td>71-95</td>
<td>8.7</td>
<td>1.25</td>
<td>2.20</td>
<td>0.21</td>
</tr>
</tbody>
</table>

2.2 Experimental design

The warming-grazing site (Fig. 2.2) was used for winter grazing for many years before the experiment. At the beginning of the experiment, the site is fenced to prevent the damage from the animals.

Fig. 2.2 The study site of the warming and grazing experiment

The infrared heating system (Fig. 2.3), herein called a free-air temperature enhancement (FATE), has been set up since May 2006. The setpoint differences between heated and corresponding reference plots were 1.2 °C during daytime and 1.7 °C at night in summer, which fell within limits of predicted temperature increases for this century (1.5–5°C). The soil temperature at depths of 5, 10 and 20 cm were measured automatically using type-K thermocouples (Campbell Scientific, Logan,
Utah, U.S.A), which were connected to a CR1000 datalogger (Fig. 2.4). Meanwhile the soil temperatures at 0 and 40 cm depth were manually monitored using mercury-in-glass thermometers. Warming significantly increased soil temperatures for 0–40 soil depths in 2006, 2007 and 2008. Mean seasonal soil temperature increases in the warmed plots above the reference plots were 0.8–1.3, 1.4–1.5, 1.2–1.4, 1.1–1.2 and 0.5–0.7 °C at 0, 5,10, 20 cm depths, and at 40 cm soil depth in 2006 and 2007, respectively.

Fig. 2.3 The equipments of Free Air Temperature Enhancement. A: ceramic heater; B: ceramic heaters and infrared thermometer

Fig. 2.4 Thermal image of the warming pattern produced by the heaters. A: hanging one Kalglo infrared heater; B: combining six of ceramic heaters in a hexagonal array

A two factorial design (warming and grazing) was used with four replicates for
each of four treatments, i.e. no warming with no grazing (NWNG); no warming with grazing (NWG); warming with no grazing (WNG); and warming with grazing (WG). In total, 16 circular plots of 3-m diameter were used in a randomized block design in the field.

Initially, one adult Tibetan domestic sheep (*Ovis aries*) was fenced in the grazing plots on the morning of 15 August 2006 for approximately 2 hours. The canopy height was 8–9 cm and 4–5 cm before and after grazing, respectively. Two sheep were fenced for approximately 1 hour in the grazing plots on the mornings of 12 July, 3 August and 12 September 2007, and 8 July and 20 August in 2008. The canopy height of the vegetation was measured at 50 points within the plots before and after grazing, and the sheep were removed from the grazing plots when the canopy height was reduced to approximately half of the initial height, which generally corresponded to a moderate stocking rate in the region. All experimental sheep were fenced into 3 additional 5 × 5 m fenced plots for a day before beginning the grazing experiment to help them adapt to small plots.

![Fig 2.5 Distributing figure of the plots in the warming and grazing experiment. NWNG: no warming and no grazing; NWG: no warming and grazing; WNG: warming and no grazing; WG: warming and grazing.](image_url)
2.3 Reference

Chapter 3  The effects of warming and grazing on soil labile carbon and nitrogen pools in the alpine meadow ecosystem of the Tibet Plateau

3.1 Abstract

Small but highly bio-active labile carbon (C) and nitrogen (N) pools are of great importance in controlling terrestrial C and N fluxes while long-term C and N storage is determined by less labile but relatively large size of C and N pools. Little information is available about the effects of global warming and grazing on different forms of C and N pools in the Qinghai-Tibet Plateau of China. In this controlled warming-grazing experiment, the effects of warming and grazing on various C and N pools were studied. After three years of warming, soil samples were taken from the four-treatment plots: no warming with no grazing (NWNG); no warming with grazing (NWG); warming with no grazing (WNG); and warming with grazing (WG). Warming and grazing treatments affected soil C and N pools differently and these effects varied with soil depth. Warming significantly increased total nitrogen (TN), microbial biomass carbon (MBC), microbial biomass nitrogen (MBN) and soluble organic nitrogen (SON) and decreased natural abundance of stable isotope $^{13}$C ($\delta^{13}$C) at the 10–20 and 20–30 cm soil depths, while grazing generally decreased SON at 10-20 and 20-30 cm and MBC at 20-30 cm. At the 0–10 cm depth, neither warming nor grazing alone affect these soil parameters significantly - the large error bars indicated that there could be considerable perturbation on the soil surface and the effects of warming on these carbon and nitrogen pools might be interfered by other factors. However, grazing alone increased NO$_3^-$-N, total inorganic N, soluble organic carbon (SOC) and natural abundance of stable isotope $^{15}$N ($\delta^{15}$N) at the 0–10 cm
depth. Correlations between MBC and SOC; and TN and MBN or SON were positive. However, SON was less well correlated with TN and MBN, compared with the highly positive correlations between SOC and MBC. It is clearly demonstrated that warming and grazing affected labile C and N pools significantly but differently after three years of treatments: warming tended to enlarge labile C and N pools through increased litter inputs, while grazing tended to increase inorganic N pools, decrease SON and accelerate N cycling. Grazing might have modified the mode that warming affected soil C and N pools through its strong impacts on microbial processes and N cycling.

3.2 Introduction

Labile carbon (C) and nitrogen (N) pools, including inorganic N, microbial biomass C and N (MBC and MBN) and soluble organic C and N (SOC and SON) all play vital roles in soil C and N cycling, structure and functioning of ecosystems through their impacts on turnover and supply of nutrients to vegetation, and can be vulnerable to climate change and disturbance (Pastor and Post 1986; Zak et al. 1993; Hu et al. 1997; Pu et al. 2001; Chen et al. 2002; Blumfield and Xu 2003; Burton et al. 2007). Terrestrial C and N budgets are largely controlled by the small but highly bio-active, labile pools of these elements in soils (Mathers et al. 2003; Chen and Xu 2005; Belay-Tedla et al. 2009; Xu et al. 2009). Being a direct reservoir of readily available substrates and nutrient, labile C and N pools are especially important and may exert considerable control on productivity, community structure and functioning of ecosystems through their impacts on turnover and supply of nutrients to vegetation (Pastor and Post 1986; Chen et al. 2003; Xu and Chen 2006; Burton et al. 2010). Global warming could stimulate C sequestrations in soil through enhanced primary production but there is still uncertainty due to potential increase of soil respiration
(Rustad et al. 2001). Relative small changes in the sizes and distribution of soil C and N pools may therefore induce substantial effects on atmospheric greenhouse gas concentrations and on global C and N cycling at large (Belay-Tedla et al. 2009; Xu and Chen 2006). Such alterations in labile and less labile C and N pools may not only lead to new dynamic processes in the short term, but can also influence the long-term terrestrial C and N storage and consequently feedback to the atmosphere (Xu et al. 2008, 2009; Burton et al. 2010; Jiang et al. 2010; Xing et al. 2010). Studies have also demonstrated that labile C and N pools in some situations could be sensitive to alterations in soil moisture, temperature and plant community structure resulting from climate change (Zak et al. 1993; Xu et al. 2009). Hence, measurements of the labile C and N pools may provide insights into early indications of impacts of climate change on soil C and N dynamics and the consequent ecosystem functioning (Xu et al. 2008, 2009; Ge et al. 2010).

Inconsistent results on the responses of soil C and N dynamics to climate change have been observed so far (Rustad et al. 2001; Xu et al. 2009; Liu et al. 2010). Warming enhanced plant productivity (Chapin et al. 1995; Hartley et al. 1999; Jonasson et al. 1999) and litter mass losses (Luo et al. 2010), and thus a higher flux of carbon dioxide into the atmosphere (Meentemeyer 1978; Berg et al. 1993; Shaw and Harte 2001; Liski et al. 2003). These effects are reported most likely to take place in cold biomes (high-latitude and high-altitude sites) because the greatest warming is predicted to occur there, and decomposition in these regions is strongly temperature-limited (Hobbie and Chapin 1998; Robinson 2002; Aerts 2006). Grazing could affect the nutrient cycling mainly through providing plants with urine and fecal material, and also by influencing litter mass losses (Hobbs et al. 1991; Bardgett et al. 2001; Olofsson et al. 2001), and its role depended on plant and litter quality. However,
there is a scarcity of information about labile C and N pools and their coupling relations under future warming and grazing conditions in the Qinghai-Tibet Plateau of China and even in the world. Effects of warming and grazing on soil labile C and N pools may occur in four ways: 1) by altering rates of litter mass loss directly at short-time scales through changes in soil temperature and moisture (Kalbitz et al. 2000; Aerts 2006; Luo et al. 2010); 2) by decreasing litter mass with the increase in grazing intensity at short time scales (Shariff et al. 1994; Olofsson et al. 2001); 3) by changing plant litter quantity and quality indirectly at longer time-scales (Aerts 2006); and 4) by changing indirectly the species composition and structure of the decomposer and detritivore communities in the long term (Kalbitz et al. 2000; Aerts 2006).

Global surface temperature is predicted to increase by 1.1 to 6.4 °C by the end of this century (IPCC 2007). The Qinghai-Tibet alpine meadow, unique among all ecosystems, is a sensitive region to global climate change (Thompson et al. 1993; Wang and French 1994; Thompson et al. 2000). Moreover, much greater than average increase in surface temperature was predicted to occur in this region in the future (Giorgi et al. 2001). Grazing is the main land-use mode, and there are about 13.3 million domestic yaks and 50 million sheep on the Qinghai-Tibet plateau, and grazing pressure will increase due to increasing population (Gerald et al. 2003; Yao et al. 2006). A number of simulated experiments have been carried out to study the impacts of global warming and grazing on terrestrial ecosystems all over the world. However, this is the first time that infrared heaters were used in the Qinghai-Tibet Plateau to study relevant ecosystem responses.

We experimentally manipulated temperature by actively warming plots using infrared heaters in a free-air temperature enhancement (FATE) system since May 2006,
as described by Kimball et al. (2008), and applied a moderate grazing treatment to an alpine meadow on the Qinghai-Tibet Plateau. Interaction between warming and grazing on different soil C and N pools was studied at four soil depths: 0–10, 10–20, 20–30 and 30–40 cm. It is hypothesized that warming would increase the sizes of labile C pools by increasing plant biomass (Luo et al. 2009; Hu et al. 2010); while grazing would increase the sizes of N pools and the rate of N transformation, and modify the responses of soil C and N pools to warming. Grazing would have different effects with warming, and these effects could be different among the four soil depths.

The objectives of this study were to investigate through a controlled warming-grazing experiment: 1) the effects of warming and grazing on soil temperature and moisture, total N, C isotope composition (δ^{13}C) and N isotope composition (δ^{15}N) in the top 40 cm of the soil profile; 2) the effects of warming and grazing on various soil C and N pools including inorganic N, soluble organic C (SOC) and soluble organic N (SON), microbial biomass C and N (MBC and MBN); and 3) the interactions between warming and grazing on these different forms of C and N pools.

### 3.3 Materials and methods

#### 3.3.1 Experimental site

The experimental site and design were described in Chapter 2 and also by Kimball et al. (2008) and Luo et al. (2009, 2010). Warming significantly increased soil temperatures for 0–40 soil depths in 2006, 2007 and 2008. Mean seasonal soil temperature increases in the warmed plots above the reference plots were 0.8–1.3, 1.4–1.5, 1.2–1.4, 1.1–1.2 and 0.5–0.7 °C at 0, 5, 10, 20 cm depths, and at 40 cm soil
depth in 2006 and 2007, respectively. More data can be found in our published results (Luo et al. 2009, 2010; Hu et al. 2010).

3.3.2 Soil sampling
Soil samples from each plot were collected on 2 August 2009 using a drill. Five soil cores were randomly collected within each plot and bulked as a single sample. As the impact of infrared radiation on soil temperature reached the depth of 40 cm (Luo et al. 2010), soil samples from four soil depths 0–10, 10–20, 20–30 and 30–40 cm were taken. All soil samples were sent to the laboratory and sieved through a 2 mm screen and stored in a refrigerator at 4 °C prior to analyses. Soil sub-samples were extracted within 24 h for NH$_4^+$-N and NO$_3^-$-N analysis.

3.3.3 Soil analysis
Soil pH was determined in 1:5 (v/v) soil/water extracts using a combination glass electrode, and soil gravimetric moisture was determined by drying at 105 °C for 24 h. Soil NH$_4^+$-N and NO$_3^-$-N were determined in 2 M KCl extracts by LACHAT Quickchem Automated Ion Analyzer (QuickChem Method 10-107-06-04-D for NH$_4^+$-N and QuickChem Method 12-107-04-1-B for NO$_3^-$-N). In addition, a low-temperature incubation experiment was carried out in the laboratory, as the 0–10 cm soil samples were incubated for 3 months at 4 °C, which was close to in situ mean annual temperature of this region. N transformation during the 3 months was analysed by calculating the difference of inorganic N concentrations before and after this period. So in this incubation, we aimed to look at the potential mineralization of different field treatments (NWNG, NWG, WNG and WG) under a low temperature condition. Net ammonification, net nitrification, and net N mineralization were calculated as the differences of soil NH$_4^+$-N, NO$_3^-$-N and total inorganic N before and
after this period.

Soil total N (TN), $\delta^{13}$C and $\delta^{15}$N were determined using an isotope ratio mass spectrometer with a Eurovector Elemental Analyzer (Isoprime-EuroEA 3000, Milan, Italy).

Soil microbial biomass C and N (MBC and MBN) were measured using the fumigation-extraction method described by Vance et al. (1987) and Brookes et al. (1985). In brief, fumigated and non-fumigated soils (4 g dry weight equivalent) were extracted with 20 ml of 0.5 M K$_2$SO$_4$ (soil/extractant ratio 1:5). The fumigation lasted for 16 h. Samples were shaken for 1 h and filtered through a Whatman 42 filter paper. Soluble organic C and total soluble N (TSN) in the fumigated and non-fumigated samples were determined using a SHIMADZU TOC-VCPH/CPN Analyzer. NH$_4^+$-N and NO$_3^-$-N concentrations in 0.5 M K$_2$SO$_4$ extracts were determined by LACHAT Quickchem Automated Ion Analyzer while SON was calculated as the difference between TSN and soil inorganic N (SIN). MBC and MBN were calculated using a conversion factor for C (Ec) of 2.64 (Vance et al. 1987) and for N (En) of 2.22 (Brooks et al. 1985).

3.3.4 Statistical analysis

Statistical significances of the effects of warming and grazing on soil moisture at different soil depths, inorganic N concentrations, TN, $\delta^{13}$C, $\delta^{15}$N, MBC, MBN, SOC and SON were determined separately by analysis of variance (ANOVA) using Statistix for Windows version 8.0 (Analytical Software, Tallahassee, FL), with warming and grazing as the main factors. Least significance difference (LSD) was used to separate the means when differences were significant. Significance was assumed at the $p=0.10$ level as there might be unavoidable disturbance in the field. Simple correlations between all these variables were performed.
3.4 Results

3.4.1 Soil temperature, moisture, TN and C and N isotope compositions

Warming significantly increased soil temperature at the 0-40 cm depth during the growing season of 2009 (Fig. 3.1). No interaction between warming and grazing was found on soil temperature although NWG also increased it by decreasing vegetation canopy height, probably through increased solar radiation. WG caused the largest increase in soil temperature compared with other treatments.

![Fig. 3.1 Soil temperature (°C) of growing season (A) and gravimetric moisture content (%) (B) at different depths under different warming and grazing regimes in 2009. NWNG: no warming with no grazing; NWG: no warming with grazing; WNG: warming with no grazing; and WG: warming with grazing. Different letters mean significant differences between treatments at p<0.05 for each soil depth. Mean ± SE is shown in the figure.](image)

Warming significantly decreased soil moisture at all depths (F=59.59, p<0.01) (Table 3.1, Fig. 3.1B). No direct influence of grazing and interaction between warming and grazing on soil moisture was found. However, the interactions between warming and depth, grazing and depth, and warming, grazing and depth on soil
moisture were all significant ($p<0.001$, $p<0.10$ and $p<0.05$ respectively) (see Table 3.1).

Generally, the effect of warming on TN varied with soil depths (Table 3.2). Warming significantly increased TN at the 10–20 cm ($F=21.9$, $p=0.018$) and 20–30 cm ($F=7.77$, $p=0.069$) depths (Table 3.2, Tables 3.3 and 3.5), but the interaction between warming and grazing was not significant. At 0–10 cm, however, the effects of warming and grazing were not significant.

Warming decreased $\delta^{13}$C at the 10–20 cm ($F=9.99$, $p=0.051$) and 20–30 cm ($F=9.35$, $p=0.055$) depths (see Table 3.2 and Table 3.5). Warming did not affect $\delta^{15}$N significantly. However, at 0–10 cm there was a significant increase of $\delta^{15}$N in the grazing plot ($F=6.5$, $p=0.084$). Compared with NWNG, NWG increased the $\delta^{15}$N by 18.6% (see Table 3.2 and Table 3.5). The interactions between warming and grazing on $\delta^{15}$N were not significant at the 0–10 cm ($F=5.54$, $p=0.100$) and 10–20 cm ($F=4.15$, $p=0.134$) depths (see Table 3.5).
Table 3.1 Analysis of variance (ANOVA) for moisture content and inorganic nitrogen (N) in the 40 cm soil profile under different warming and grazing regimes.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Moisture (%)</th>
<th>NH$_4^+$-N (mg kg$^{-1}$)</th>
<th>NO$_3^-$-N (mg kg$^{-1}$)</th>
<th>Inorganic N (mg kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>F</td>
<td>P</td>
<td>DF</td>
</tr>
<tr>
<td>Warming</td>
<td>1</td>
<td>59.59</td>
<td>&lt;0.001***</td>
<td>1</td>
</tr>
<tr>
<td>Grazing</td>
<td>1</td>
<td>0.67</td>
<td>0.427</td>
<td>1</td>
</tr>
<tr>
<td>Warming*Grazing</td>
<td>1</td>
<td>0.24</td>
<td>0.630</td>
<td>1</td>
</tr>
<tr>
<td>Depth</td>
<td>3</td>
<td>77.30</td>
<td>&lt;0.001***</td>
<td>3</td>
</tr>
<tr>
<td>Warming*Depth</td>
<td>3</td>
<td>9.21</td>
<td>&lt;0.001***</td>
<td>3</td>
</tr>
<tr>
<td>Grazing*Depth</td>
<td>3</td>
<td>2.44</td>
<td>0.081*</td>
<td>3</td>
</tr>
<tr>
<td>Warming<em>Grazing</em>Depth</td>
<td>3</td>
<td>3.02</td>
<td>0.042**</td>
<td>3</td>
</tr>
</tbody>
</table>

* *, ** and *** indicate significance at $p<0.10$, $p<0.05$ and $p<0.01$, respectively.
Table 3.2 Mean values for total nitrogen (TN), C isotope composition ($\delta^{13}\text{C}$), N isotope composition ($\delta^{15}\text{N}$), microbial biomass C (MBC), microbial biomass N (MBN), soluble organic C (SOC) and soluble organic N (SON) in the 40 cm soil profile under different warming and grazing regimes (Standard errors are shown in brackets)

<table>
<thead>
<tr>
<th>Treatments(^a)</th>
<th>TN (%)</th>
<th>$\delta^{13}\text{C}$ (%)</th>
<th>$\delta^{15}\text{N}$ (%)</th>
<th>MBC (mg kg(^{-1}))</th>
<th>MBN (mg kg(^{-1}))</th>
<th>SOC (mg kg(^{-1}))</th>
<th>SON (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NWNG</td>
<td>0.693 (0.04)</td>
<td>-26.1 (0.28)</td>
<td>3.35 (0.15) B (^b)</td>
<td>2245 (322)</td>
<td>274 (60)</td>
<td>573 (74) b</td>
<td>26.2 (6.4)</td>
</tr>
<tr>
<td>NWG</td>
<td>0.690 (0.03)</td>
<td>-26.0 (0.19)</td>
<td>3.98 (0.13) A</td>
<td>2101 (133)</td>
<td>283 (57)</td>
<td>607 (42) ab</td>
<td>24.4 (3.1)</td>
</tr>
<tr>
<td>WNG</td>
<td>0.661 (0.03)</td>
<td>-25.9 (0.23)</td>
<td>3.50 (0.21) B</td>
<td>2211 (369)</td>
<td>249 (41)</td>
<td>560 (53) b</td>
<td>27.3 (3.6)</td>
</tr>
<tr>
<td>WG</td>
<td>0.755 (0.04)</td>
<td>-26.2 (0.14)</td>
<td>3.53 (0.21) AB</td>
<td>2407 (244)</td>
<td>316 (33)</td>
<td>718 (113) a</td>
<td>37.6 (11.3)</td>
</tr>
<tr>
<td>10-20 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NWNG</td>
<td>0.449 (0.01) B</td>
<td>-23.9 (0.25) A</td>
<td>4.23 (0.16)</td>
<td>1098 (155) b</td>
<td>131 (18)</td>
<td>465 (61)</td>
<td>27.9 (2.8)</td>
</tr>
<tr>
<td>NWG</td>
<td>0.449 (0.01) B</td>
<td>-24.0 (0.14) AB</td>
<td>4.45 (0.04)</td>
<td>1049 (132) b</td>
<td>111 (12)</td>
<td>427 (22)</td>
<td>25.2 (2.3)</td>
</tr>
<tr>
<td>WNG</td>
<td>0.470 (0.02) AB</td>
<td>-24.2 (0.20) AB</td>
<td>4.29 (0.09)</td>
<td>1251 (116) ab</td>
<td>154 (16)</td>
<td>433 (20)</td>
<td>28.2 (3.4)</td>
</tr>
<tr>
<td>WG</td>
<td>0.499 (0.02) A</td>
<td>-24.7 (0.26) B</td>
<td>4.23 (0.15)</td>
<td>1373 (139) a</td>
<td>173 (35)</td>
<td>472 (37)</td>
<td>28.0 (4.3)</td>
</tr>
<tr>
<td>20-30 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NWNG</td>
<td>0.375 (0.02) ab</td>
<td>-22.7 (0.30) AB</td>
<td>4.75 (0.09) ab</td>
<td>904 (77) AB</td>
<td>119 (16)</td>
<td>381 (31)</td>
<td>24.0 (1.7) A</td>
</tr>
<tr>
<td>NWG</td>
<td>0.347 (0.01) b</td>
<td>-22.4 (0.24) A</td>
<td>4.95 (0.23) a</td>
<td>783 (71) B</td>
<td>88.9 (15)</td>
<td>360 (29)</td>
<td>22.5 (1.6) AB</td>
</tr>
<tr>
<td>WNG</td>
<td>0.385 (0.02) AB</td>
<td>-22.9 (0.32) AB</td>
<td>4.30 (0.30) b</td>
<td>1012 (137) A</td>
<td>117 (18)</td>
<td>369 (19)</td>
<td>23.7 (2.7) AB</td>
</tr>
<tr>
<td>WG</td>
<td>0.390 (0.02) a</td>
<td>-23.1 (0.12) B</td>
<td>4.63 (0.10) ab</td>
<td>950 (134) AB</td>
<td>115 (10)</td>
<td>384 (38)</td>
<td>21.6 (2.3) B</td>
</tr>
<tr>
<td>30-40 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NWNG</td>
<td>0.301 (0.01)</td>
<td>-20.8 (0.36)</td>
<td>4.83 (0.17)</td>
<td>572 (45) B</td>
<td>73.6 (10)</td>
<td>320 (26)</td>
<td>16.8 (1.1)</td>
</tr>
<tr>
<td>NWG</td>
<td>0.299 (0.02)</td>
<td>-20.7 (0.39)</td>
<td>4.69 (0.17)</td>
<td>547 (49) B</td>
<td>67.4 (16)</td>
<td>312 (26)</td>
<td>19.4 (2.4)</td>
</tr>
<tr>
<td>WNG</td>
<td>0.323 (0.03)</td>
<td>-21.4 (0.56)</td>
<td>4.59 (0.26)</td>
<td>720 (94) A</td>
<td>81.1 (19)</td>
<td>319 (37)</td>
<td>19.3 (2.3)</td>
</tr>
<tr>
<td>WG</td>
<td>0.303 (0.02)</td>
<td>-20.8 (1.00)</td>
<td>4.72 (0.11)</td>
<td>613 (82) AB</td>
<td>68.8 (6)</td>
<td>310 (26)</td>
<td>20.0 (2.1)</td>
</tr>
</tbody>
</table>

\(^a\) NWNG: no warming with no grazing; NWG: no warming with grazing; WNG: warming with no grazing; WG: warming with grazing.

\(^b\) Where values are followed by different upper case or lower case letters for each soil depth this indicates that treatment means are significantly different from each other at \(p<0.05\) or \(p<0.10\) respectively.
Table 3.3 Analysis of variance (ANOVA) for total nitrogen (TN), C isotope composition ($\delta^{13}$C) and N isotope composition ($\delta^{15}$N) in the 40 cm soil profile under different warming and grazing regimes $^a$

<table>
<thead>
<tr>
<th>Factors</th>
<th>Total N (%)</th>
<th>$\delta^{13}$C (%)</th>
<th>$\delta^{15}$N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>F</td>
<td>P</td>
</tr>
<tr>
<td>Warming</td>
<td>1</td>
<td>2.55</td>
<td>0.137</td>
</tr>
<tr>
<td>Grazing</td>
<td>1</td>
<td>0.42</td>
<td>0.529</td>
</tr>
<tr>
<td>Warming*Grazing</td>
<td>1</td>
<td>1.5</td>
<td>0.243</td>
</tr>
<tr>
<td>Depth</td>
<td>3</td>
<td>312.18</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Warming*Depth</td>
<td>3</td>
<td>0.28</td>
<td>0.841</td>
</tr>
<tr>
<td>Grazing*Depth</td>
<td>3</td>
<td>1.92</td>
<td>0.143</td>
</tr>
<tr>
<td>Warming<em>Grazing</em>Depth</td>
<td>3</td>
<td>1.48</td>
<td>0.238</td>
</tr>
</tbody>
</table>

$^a$, ** and *** indicate significance at $p<0.10$, $p<0.05$ and $p<0.01$, respectively.

Table 3.4 Analysis of variance (ANOVA) for microbial biomass carbon (MBC), microbial biomass nitrogen (MBN), soluble organic carbon (SOC) and soluble organic nitrogen (SON) in the 40 cm soil profile under different warming and grazing regimes $^a$

<table>
<thead>
<tr>
<th>Factors</th>
<th>Microbial biomass C (mg kg$^{-1}$)</th>
<th>Microbial biomass N (mg kg$^{-1}$)</th>
<th>Soluble organic C (mg kg$^{-1}$)</th>
<th>Soluble organic N (mg kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>F</td>
<td>P</td>
<td>DF</td>
</tr>
<tr>
<td>Warming</td>
<td>1</td>
<td>1.86</td>
<td>0.198</td>
<td>1</td>
</tr>
<tr>
<td>Grazing</td>
<td>1</td>
<td>0.4</td>
<td>0.841</td>
<td>1</td>
</tr>
<tr>
<td>Warming*Grazing</td>
<td>1</td>
<td>0.29</td>
<td>0.601</td>
<td>1</td>
</tr>
<tr>
<td>Depth</td>
<td>3</td>
<td>95.59</td>
<td>&lt;0.001***</td>
<td>3</td>
</tr>
<tr>
<td>Warming*Depth</td>
<td>3</td>
<td>0.16</td>
<td>0.924</td>
<td>3</td>
</tr>
<tr>
<td>Grazing*Depth</td>
<td>3</td>
<td>0.2</td>
<td>0.897</td>
<td>3</td>
</tr>
<tr>
<td>Warming<em>Grazing</em>Depth</td>
<td>3</td>
<td>0.38</td>
<td>0.769</td>
<td>3</td>
</tr>
</tbody>
</table>

$^a$, ** and *** indicate significance at $p<0.10$, $p<0.05$ and $p<0.01$, respectively.
Table 3.5 Analysis of variance (ANOVA) for total nitrogen (TN), C isotope composition ($\delta^{13}$C) and N isotope composition ($\delta^{15}$N) in 0-10, 10-20, 20-30 and 30-40 cm soil layers respectively under different warming and grazing regimes $^a$.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Total N (%)</th>
<th>$\delta^{13}$C (%)</th>
<th>$\delta^{15}$N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>F</td>
<td>P</td>
</tr>
<tr>
<td>0-10 cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warming</td>
<td>1</td>
<td>0.50</td>
<td>0.529</td>
</tr>
<tr>
<td>Grazing</td>
<td>1</td>
<td>3.98</td>
<td>0.140</td>
</tr>
<tr>
<td>Warming*Grazing</td>
<td>1</td>
<td>4.53</td>
<td>0.123</td>
</tr>
<tr>
<td>10-20 cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warming</td>
<td>1</td>
<td>21.9</td>
<td>0.018 **</td>
</tr>
<tr>
<td>Grazing</td>
<td>1</td>
<td>3.6</td>
<td>0.154</td>
</tr>
<tr>
<td>Warming*Grazing</td>
<td>1</td>
<td>3.73</td>
<td>0.149</td>
</tr>
<tr>
<td>20-30 cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warming</td>
<td>1</td>
<td>7.77</td>
<td>0.069 *</td>
</tr>
<tr>
<td>Grazing</td>
<td>1</td>
<td>1.46</td>
<td>0.313</td>
</tr>
<tr>
<td>Warming*Grazing</td>
<td>1</td>
<td>3.01</td>
<td>0.181</td>
</tr>
<tr>
<td>30-40 cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warming</td>
<td>1</td>
<td>1.13</td>
<td>0.366</td>
</tr>
<tr>
<td>Grazing</td>
<td>1</td>
<td>0.78</td>
<td>0.442</td>
</tr>
<tr>
<td>Warming*Grazing</td>
<td>1</td>
<td>0.52</td>
<td>0.521</td>
</tr>
</tbody>
</table>

$^a$ * and ** indicate significance at $p<0.10$ and $p<0.05$, respectively.
3.4.2 NH$_4^+$-N, NO$_3^-$-N and total inorganic N

Generally, soil inorganic N concentrations were more affected by grazing than by warming (Fig. 3.2A and B). No significant effect of warming on NH$_4^+$-N, NO$_3^-$-N and SIN concentrations was found, but soil depth had a significant effect. Concentration of NH$_4^+$-N among all soil depths ranged from 16.61 to 25.52 mg kg$^{-1}$ while that of NO$_3^-$-N ranged from 1.43 to 43.17 mg kg$^{-1}$.

![Graph showing NH$_4^+$-N, NO$_3^-$-N, and total inorganic N concentrations at different soil depths under different treatments.](image)

Fig. 3.2 NH$_4^+$-N (A), NO$_3^-$-N (B) and total inorganic N (C) concentrations at 0-10, 10-20, 20-30 and 30-40 cm soil depths under different warming and grazing regimes; and net N transformations in the 0-10 cm soil under laboratory low-temperature conditions (4°C) for three months (D). NWNG: no warming with no grazing; NWG: no warming with grazing; WNG: warming with no grazing; and WG: warming with grazing. Different letters mean significant differences between treatments at $p<0.05$ for each soil depth (A-C) and each N transformation process (D). Mean ± SE is shown in the figure.
Grazing increased NO$_3^-$-N overall (F=4.88, $p=0.047$) and at the 0–10 cm (F=5.69, $p=0.097$), 10–20 cm (F=6.99, $p=0.078$) and 20–30 cm (F=7.40, $p=0.073$) depths, as well as increasing SIN overall (F=5.07, $p=0.044$) (see Fig. 3.2B and C). Specifically, at 0–10 cm, NWG increased NO$_3^-$-N by over 2 folds compared with NWNG, while WG drastically increased NO$_3^-$-N by over 6 folds compared with WNG. As well, NWG increased SIN by 21.3% compared with NWNG, while WG increased it by 145% compared with WNG at 0–10 cm. Warming tended to decrease NH$_4^+$-N, NO$_3^-$-N and SIN while grazing tended to increase them; however WG caused the largest increases in NO$_3^-$-N and SIN (see Fig. 3.2B and C).

3.4.3 MBC, MBN, SOC and SON

The effects of warming on MBC and MBN varied with soil depth. Warming significantly increased MBC at the 10–20 cm (F=11.1, $p=0.045$) and 20–30 cm (F=8.22, $p=0.024$) depths (Table 3.6). Grazing decreased MBC significantly at 20–30 cm (F=7.94, $p=0.067$). No significant interactions between warming and grazing on MBC were found among any of the depths. Although the effect was not significant, warming increased MBN at the 10–20 cm (F=5.88, $p=0.094$) depth (see Table 3.6). No significant effects of warming and grazing or interaction between them on MBN were found. However, at the 0–10 cm WG tended to increase MBN by 15.3%, 11.7% and 27.0%, compared with NWNG, NWG and WNG, respectively.
Table 3.6 Analysis of variance (ANOVA) for microbial biomass carbon (MBC), microbial biomass nitrogen (MBN), soluble organic carbon (SOC) and soluble organic nitrogen (SON) in 0-10, 10-20, 20-30 and 30-40 cm soil layers respectively under different warming and grazing regimes \(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Microbial biomass C (mg kg(^{-1}))</th>
<th>Microbial biomass N (mg kg(^{-1}))</th>
<th>Soluble organic C (mg kg(^{-1}))</th>
<th>Soluble organic N (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>F</td>
<td>P</td>
<td>DF</td>
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<tr>
<td>0-10 cm</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Warming</td>
<td>1</td>
<td>0.35</td>
<td>0.596</td>
<td>1</td>
</tr>
<tr>
<td>Grazing</td>
<td>1</td>
<td>0.01</td>
<td>0.917</td>
<td>1</td>
</tr>
<tr>
<td>Warming*Grazing</td>
<td>1</td>
<td>0.55</td>
<td>0.513</td>
<td>1</td>
</tr>
<tr>
<td>10-20 cm</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Warming</td>
<td>1</td>
<td>11.13</td>
<td>0.045**</td>
<td>1</td>
</tr>
<tr>
<td>Grazing</td>
<td>1</td>
<td>0.27</td>
<td>0.638</td>
<td>1</td>
</tr>
<tr>
<td>Warming*Grazing</td>
<td>1</td>
<td>1.43</td>
<td>0.318</td>
<td>1</td>
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<tr>
<td>20-30 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warming</td>
<td>1</td>
<td>8.22</td>
<td>0.024**</td>
<td>1</td>
</tr>
<tr>
<td>Grazing</td>
<td>1</td>
<td>7.94</td>
<td>0.067*</td>
<td>1</td>
</tr>
<tr>
<td>Warming*Grazing</td>
<td>1</td>
<td>0.85</td>
<td>0.424</td>
<td>1</td>
</tr>
<tr>
<td>30-40 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warming</td>
<td>1</td>
<td>4.85</td>
<td>0.115</td>
<td>1</td>
</tr>
<tr>
<td>Grazing</td>
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<td>1.84</td>
<td>0.268</td>
<td>1</td>
</tr>
<tr>
<td>Warming*Grazing</td>
<td>1</td>
<td>0.71</td>
<td>0.461</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\) * and ** indicate significance at \(p<0.10\) and \(p<0.05\), respectively.
Warming and grazing affected SOC and SON differently and inconsistently among soil depths. At 0–10 cm grazing increased SOC (F=6.56, p=0.083), but there was no interaction between warming and grazing on it (F=2.75, p=0.196) (see Table 3.6). At 10–20 cm the effects of warming (F=12.11, p=0.040) in increasing SON and the effects of grazing (F=10.7, p=0.047) in decreasing SON were both significant (see Table 3.6). At the 20–30 cm depth, grazing also decreased SON (F=292, p=0.013) (see Table 3.6).

3.4.4 N transformation under low temperature

After 3 months’ incubation, the composition of inorganic N had changed greatly (see Fig. 3.2D), as low concentration of NH$_4^+$-N and abundance in NO$_3^-$-N were observed, which was opposite to the state before incubation. The 4 °C incubation experiment had shown that warming (particularly with grazing) led to net immobilization of N, but no-warming treatments led to net N mineralization (see Fig. 3.2D). However, WG caused the largest net N immobilization, and nitrification was strong across all these different treatments (see Fig. 3.2D).

3.4.5 Relationships among the soil C and N pools.

There were positive correlations between TN and MBN ($R^2=0.76, p<0.001$), and between TN and SON ($R^2=0.24, p<0.001$) (Fig. 3.3A). Positive correlation was also found between MBC and SOC ($R^2=0.53, p<0.001$) and between MBN and SON ($R^2=0.21, p<0.001$) (see Fig. 3.3C and D). However, SON was less well correlated with TN and MBN, compared with the highly positive correlations between SOC and
MBC.

Fig. 3.3 Relationships between (A) total nitrogen (TN) (%) and microbial biomass N (MBN) (mg kg\(^{-1}\)) and soluble organic N (SON) (mg kg\(^{-1}\)); (B) MBC (mg kg\(^{-1}\)) and SOC (mg kg\(^{-1}\)); and (C) MBN (mg kg\(^{-1}\)) and SON (mg kg\(^{-1}\)).

3.5 Discussion

As the interaction between warming and grazing on C and N pools is not significant, the discussion is separated into two parts: the effects of warming on C and N pools, and the effects of grazing on C and N pools.
3.5.1 Effects of warming on C and N pools

Some studies reported that labile C and N pools obtained through different hydrolysis methods as well as microbial biomass C and N pools were significantly increased by experimental warming and elevated CO$_2$ (Belay-Tedla et al. 2009; Zak et al. 1993) through accelerated decomposition rates or enhanced substrate inputs. According to our published results (Luo et al. 2009), WNG increased dissolved organic C (DOC) in 2006 and 2007. Here we also found warming increased various C and N pools including MBC, MBN and SON, as well as total N in the soil.

Microbial biomass C and N pools are vital components of ecosystem cycling and serve as a source (mineralization) or a sink (immobilization) of labile nutrients (Hu et al. 1997). Microbial biomass could respond rapidly to changes in soil moisture (Skopp et al. 1990) and soil temperature (Fang et al. 2005). A lag time for microbial biomass C and N in response to experimental warming had been reported in field experiments in subarctic soils (Ruess et al. 1999). However, in our study warming increased both MBC and MBN at the 10-20 and 20-30 cm depths, suggesting that microbial immobilization of C and N was significantly enhanced by warming despite the effect of decreased soil moisture content on microorganisms. This was consistent with the results from the low-temperature incubation where warming led to net immobilization of N (Fig. 3.2D). Microorganisms, especially nitrifiers, were largely stimulated by warming as NH$_4^+$-N was continuously being consumed (Fig. 3.2D), indicating a great demand by microbes in the warming plots to take up inorganic N. Besides the
increase in the microbial biomass C and N pools, how warming affects the nitrifying groups and other microorganisms will be crucial for understanding the mechanisms of microbial-mediated C and N cycling. Further studies are required to investigate the effects of warming and grazing on nitrifying and denitrifying communities.

SOC and SON, often considered as direct substrates of N mineralization, were small but highly bio-active pools and were important in controlling short-term terrestrial C and N cycling (Chen and Xu 2008; Belay-Tedla et al. 2009). Several results had confirmed that some plants were able to directly utilize and generally prefer amino acid over inorganic N (Schimel and Chapin 1996; Chen and Xu 2008). It was also reported that in the alpine meadow of the Qinghai-Tibet plateau organic N uptake by plants was quantitatively significant under field conditions (Xu et al. 2004, 2006). Most of SOC and SON in soils were derived from root exudation, litter decomposition, transformation of organic matters and immobilization of inorganic C and N (Chen and Xu 2006, 2008). Therefore, quantity and quality of organic and inorganic inputs and the associated microbially-mediated processes, which were always controlled by temperature and moisture, were important in determining the sizes of SOC and SON. In our study, warming did not affect SOC but significantly increased SON at 10-20 cm depth, implying that both pools might not simply be controlled by a single factor, but by a combination of temperature, moisture, substrate inputs and microbial processes. According to our previous findings (Luo et al. 2009), the direct contribution of temperature and moisture on DOC was small, while biotic factors (i.e. quality of standing dead and below-ground biomass) were the main
controls on DOC in the soil. Belay-Tedla et al. (2009) also suggested that the increases in labile and microbial biomass C and N pools under warming conditions largely resulted from increased aboveground and belowground biomass. On the other hand, higher soil temperature in warmed plots might stimulate decomposition of the recalcitrant pools (Knorr et al. 2005), which could increase labile C and N levels, while decreased soil moisture under warming might restrict decomposition of the labile and recalcitrant pools (Skopp et al. 1990). So the overall direct effects of temperature and moisture on labile C and N pools might be relatively small compared with the impact of changes in substrate inputs. However, the increased SON indicated a larger source for direct plant uptake or N mineralization under warming conditions.

The effect of warming in increasing total N, especially at 10–20 and 20–30 cm depths, might result from the shift in plant composition because warming significantly increased the growth of N-fixing plants in our study (data not shown). Despite the scarcity of published information about this effect, it was very likely that warming would cause change in plant community composition, which would lead to shifts in the composition of microorganisms through alterations in above-ground inputs and rhizospheric exudates that might favour different microorganisms. This “plant-microbe” interaction could feedback in regulating C and N cycling in the long term (Xu and Chen 2006; Xu et al. 2009). In addition, at 0-10 cm warming did not affect total N significantly, implying that near the soil surface there could be large disturbance like grazing, causing considerable perturbations in top soil N contents. Warming tended to affect δ^{13}C by decreasing it significantly at 10–20 and 20–30 cm
depths. Previous studies suggested that soil $\delta^{13}C$ was highly related with plant $\delta^{13}C$ through plant litter inputs, which had been demonstrated to be positively related to plant water use efficiency (WUE) and photosynthesis (Xu et al. 2000; Huang et al. 2008a, b; Tutua et al. 2008; Sun et al. 2010). Farquhar et al. (1989) indicated that the decrease in soil moisture under warming conditions could result in a greater WUE of plants, as well as enhanced C$_4$ photosynthesis, which could cause $\delta^{13}C$ to increase (Farquhar et al. 1989). However, within this region, C$_3$ species were believed to dominate. The decrease in $\delta^{13}C$ here under warming conditions was more likely to be caused by the increase in annual net primary production (data not shown), which was a direct result of enhanced photosynthesis. Therefore, discrimination against $^{13}C$ by the carboxylating enzyme in C$_3$ plants (Farquhar et al. 1982; Dawson et al. 2002) could be enhanced as a result of stimulated photosynthesis, leading to the decrease of $\delta^{13}C$ in plants and subsequently in soil. The decrease of $\delta^{13}C$ suggested that water content might not act as a limiting factor to plants here. The C isotope composition of plants could provide an opportunity to assess long-term stability of plant communities and climate of a region (Nordt et al. 1994). Therefore, the signature of soil $\delta^{13}C$ under warming conditions in our study reflected a significant implication of warming on plants of this ecosystem.

3.5.2 Effects of grazing on C and N pools

It was well documented that grazing could enhance decomposition and mineralization of SOM through its impact on quality and quantity of litter (Hobbs et al. 1991; Shariff
et al. 1994; Bardgett et al. 2001; Olofsson et al. 2001), but inconsistent results had been reported on the effects of grazing on C and N pools. Some suggested grazing could increase C and N pools (Reeder et al. 2004); while others showed opposite results (Stark et al. 2003; Golluscio et al. 2009), indicating that the effects of grazing on C and N pools could vary with ecosystems. Here we found in alpine meadow ecosystem of the Qinghai-Tibet Plateau, grazing greatly increased inorganic N which effect was intensified by warming. Grazing also increased SOC and $\delta^{15}$N at 0-10 cm but decreased SON at 10-20 and 20-30 cm depths and MBC at 20-30 cm.

The significant increase in NO$_3^-$-N concentrations under NWG and WG compared with non-grazing treatments could be explained by the direct effect of sheep dung deposition which probably contain high concentration of inorganic N and could rapidly increase nitrate concentration on the soil surface (Hobbs et al. 1991; Bardgett et al. 1997; Olofsson et al. 2001). Microbial activity could also be stimulated by saliva excreted by grazing animals. In addition, there could be a general positive feedback as herbivory could promote plant regrowth as well as energy and nutrient flow in grazed plots (Hamilton and Frank 2001). Although NWG did not affect the total inorganic N significantly, WG would increase it significantly, implying that the effects of grazing on inorganic N could be intensified by warming. The interaction between warming and grazing could possibly stimulate microbial activities and N mineralization strongly (Rustad et al. 2001; Melillo et al. 2002), resulting in a substantial feedback in inorganic N pools. The effects of grazing on inorganic N were more significant at 0–10 cm depth, implying the direct impact of grazing on the
highly dynamic and transient turnover of labile N near the soil surface.

The effects of warming are quite consistent, as warming generally increased labile C and N pools. While the effects of grazing on these pools are inconsistent: grazing increased SOC at 0-10 cm but decreased SON at 10-20 and 20-30 cm depths and MBC at 20-30 cm. Grazing affected the quantity and quality of litter matter input. The removal of aboveground biomass by grazing would directly decrease the litter mass input therefore SON and MBC; however, the quality change resulted from grazing might enhance the short-term turnover hence increase SOC in the topsoil. The decreases in SON and MBC could be explained by less litter inputs as well as the effects of grazing in enhancing mineralization (Shariff et al. 1994; Hamilton and Frank 2001). The regrowth of plants and the flow of nutrients promoted by grazing, as well as the enhanced microbial activity stimulated by enzymes contained in saliva and dung would greatly promote plant uptake of SON and microbial mineralization of it. These results suggest that, unlike the effects of warming which tended to favour increases in labile C and N pools through increasing substrate input, grazing would affect them by influencing the quality of litter input and stimulating the nutrient and energy flows in the soil (Hamilton and Frank 2001). However, SON was less well correlated with TN and MBN, compared with the highly positive correlations between SOC and MBC, indicating the strong influences of grazing on N cycling. Further investigations should be conducted into C and N cycling and storage under future warming and grazing conditions in the alpine meadow of the Qinghai-Tibet plateau.

Unlike warming, grazing did not affect δ^{13}C but increased δ^{15}N at 0-10 cm depth
which might also imply that N cycling could be directly accelerated by grazing rather than warming. Soil $\delta^{15}$N was presumed to be an index of N cycling as a higher cycling rate might yield greater N loss (Nadelhoffer and Fry 1994; Dawson et al. 2002). The change in soil $\delta^{15}$N under grazing conditions could be attributed to both the N inputs in the forms of sheep excreta, as well as the shift in the compositions of the plants and microbes because of grazing (Frank and Evans, 1997). Soil could become enriched in $^{15}$N when $^{15}$N-depleted products (i.e., NH$_3$, NO$_3^-$, N$_2$O, N$_2$), resulting from fractionation occurring during soil N transformations, were lost from the soil system (Evans and Ehleringer 1993; Nadelhoffer and Fry 1994). Our previous study indicated that NWG and WG significantly increased the average annual N$_2$O flux (57.8% and 31.0%) compared with NWNG and WNG, respectively (Hu et al. 2010). Thus, considering there is now increasing grazing pressure in this region, N cycling could be accelerated in the long term, resulting in higher N availability as well as larger N$_2$O emissions.

The one time sampling in this study may weaken the conclusion to some extent. However, although not always significant, the results obtained in this study were very indicative: the effects of warming in increasing C and N pools were consistent while the effects of grazing were inconsistent. In some studies, more than a decade is required to detect the significant effects of warming in arctic ecosystems (Lamb et al. 2011). As a result, we may need to wait for a longer term to observe the significant results.
3.6 Conclusions

After 3 years’ treatment warming and grazing affected labile C and N pools significantly and differently. Warming increased TN, MBC, MBN and SON and decreased δ^{13}C at the 10–20 and 20–30 cm soil depths, while grazing generally decreased SON at 10-20 and 20-30 cm and MBC at 20-30 cm depth. Grazing alone increased NO_{3−}-N, total inorganic N, SOC and δ^{15}N at the 0–10 cm depth. Strong interactive effects of warming and grazing on soil C and N pools found in this study might have significant implications for the long-term C and N storage and productivity of the alpine meadow ecosystem in the Qinghai-Tibet Plateau of China. In future works, how seasonal patterns of labile C and N, as well as microbial communities that mediate the key processes in soil C and N cycling respond to warming and grazing, will be crucial to increase our knowledge on mechanisms and long-term effects of warming and grazing on this plateau.

3.7 References


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Chapter 4 The effects of warming and grazing on soil phosphorus cycling in the alpine meadow ecosystem of Tibet Plateau

4.1 Abstract

Little is known about the soil phosphorus (P) biogeochemical cycling in response to combined warming and grazing, especially in the alpine meadow ecosystem of the Qinghai-Tibet Plateau. Here, we used a free-air temperature enhancement system in a controlled warming-grazing experiment to test the hypothesis that combined warming and grazing would significantly accelerate mineralization of soil organic P. A two factorial design of warming (1.2-1.7 °C temperature increase) and moderate grazing was utilized. A fractionation method was applied to investigate the sizes of different soil inorganic and organic P fractions. Results showed that both warming and grazing significantly decreased the quantity of organic P extracted by first NaOH (N(I)Po), as well as the total extractable organic P (TPo) at the 0-10 cm depth. Warming also decreased the total P of soil at 0-10 cm. The combined warming and grazing treatment (WG) led to the reduction of major soil organic P fractions (N(I)Po, TPo) by 40-48% and 28-32%, respectively compared with other treatments at 0-10 cm. The activities of acid and alkaline phosphomonoesterase (AcPME and AlPME) were both enhanced by warming and grazing, and their interaction. Decreased concentrations of soil N(I)Po and TPo were accompanied by increased AcPME activity (P < 0.01) and soil temperature (P < 0.05), indicating the enhanced mineralization of organic P under
rising temperature. Meanwhile, leaf biomass P of two major species (*Potentilla anserine* and *Gentiana straminea*) within these plots were significantly enhanced by either grazing or warming. The microbial mineralization of soil organic P could be strongly increased under combined warming and grazing conditions as driven by increasing plant demand for P and enhanced microbial activities.

### 4.2 Introduction

Phosphorus (P), one of the major soil nutrients in terrestrial ecosystems, is essential to plant growth. Under natural conditions, the loss of P can be replenished only by the release of P from primary minerals such as apatite, resulting in a gradual decrease of total P in the soil with time (Filippelli 2008). In many ecosystems P is in short supply and tends to limit the net primary production (NPP), nitrogen (N) fixation and carbon (C) storage in the long term (Vitousek and Howarth 1991; Tiessen 2008; Wang et al. 2010).

Global surface temperature is predicted to increase by 1.1 to 6.4 °C by the end of this century (IPCC 2007). Soil P cycling is controlled mainly by geochemical and biological processes (Frossard et al. 1995), and can be vulnerable to global climate change and land-use management (Williams and Haynes 1990; Papanikolaou et al. 2010; Jouany et al. 2011). Microbial mineralization of organic P, producing available inorganic P for plants, is thought to be the key process of soil P cycling in unfertilized/natural ecosystems (Chen et al. 2004). Soil phosphatases, produced by both microbes and plants, are involved in the P mineralization. Increased temperature
can stimulate soil phosphatase activity (Papanikolaou et al. 2010), and accelerate P cycling by enhancing plant growth which increases the demand for nutrients, quantity and quality of litter inputs, root turnover and exudation (Sardans et al. 2006), and by increasing microbial biomass and activity (Raghothama 1999). Meanwhile, the effects of warming can be affected by soil water availability (Sardans et al. 2006). In grassland ecosystems, land-use management such as grazing can substantially affect P cycling through its direct control on plant and animal residues (Williams and Haynes 1990). Grazing also promotes the nutrient cycling, and increases soil nutrient heterogeneity through the uneven deposition of faeces and urine (O’Connor 1981; Carline et al. 2005), which can modify the proportions of organic and inorganic soil P fractions (Galvao and Salcedo 2009; Hao et al. 2008). The combined direct (grass consumption) and indirect (recycling through urine and/or faeces deposition) effects of grazing have been studied extensively (O’Connor 1981; Williams and Haynes 1990; Carline et al. 2005). However, the net effect of grazing on P cycling and its consequence for primary productivity seem to vary with locations and to be dependent on other factors such as the climate (Jouany et al. 2011). Exploring the mechanisms driving P availability can provide insights into the coupling of C, water and nutrient cycles, and ultimately the responses of ecosystems to climate change (Vandecar et al. 2009).

The Qinghai-Tibet alpine meadow has been reported to be an extremely sensitive region to global climate change (Thompson et al. 1993, 2000; Wang and French 1994). Moreover, 40% greater than average increase in surface temperature (1.3 to 6.9 °C)
was predicted to occur in this region in the future (Giorgi et al. 2001). Concurrent with climate change, increased grazing pressure is also causing profound changes to pastoral land-use dynamics in this alpine meadow ecosystem (Zhou et al. 2005). Such changes, together with increased temperature, will strongly influence litter decomposition and nutrient mineralization, and all other important soil processes (Ineson et al. 1998 a, b; Schmidt et al. 2004). Although climate change experiments in alpine ecosystems in the temperate zone are sparse (Luo et al. 2009), many studies from tundra ecosystems suggest that the altered nutrient cycling in alpine ecosystems may be a key response to climate and grazing perturbations (Jonasson et al. 1993; Shaver et al. 1998). There have been a number of studies on the responses of P cycling to warming or grazing, but in this region, the combined influence of warming and grazing on P cycling has never been investigated.

We experimentally manipulated temperature by actively warming plots using infrared heaters in a free-air temperature enhancement (FATE) system in an alpine meadow on the Qinghai-Tibet Plateau since May 2006, as described by Kimball et al. (2008), and applied a moderate grazing treatment to it. We hypothesized that warming and grazing would decrease the sizes of some organic P pools by stimulating P cycling and increasing plant and microbial demands for P, and increase phosphatase activities; and grazing could modify the response of P cycling to warming through decreased litter inputs and uneven faeces and urine deposition. The objectives of this study were to investigate through a controlled warming-grazing experiment: 1) the effects of warming and grazing on various inorganic and organic soil P fractions
obtained through a sequential extraction scheme; 2) the effects of warming and grazing on the activities of soil acid and alkaline phosphatase; and 3) the relationships between soil P fractions, phosphatase activities and other environmental factors and soil biochemical properties.

4.3 Materials and methods

4.3.1 Experimental site

The experimental site and design were described in Chapter 2.

4.3.2 Soil sampling

Soil samples from each plot were collected on 2 August 2009 using a 5 cm-diameter corer. Five soil cores were randomly collected within each plot and bulked as a composite sample. Soil samples from the soil depths of 0-10 and 10-20 cm were taken. Outside the fenced warming-grazing plots, there was an area under free grazing (FG) condition. This area was grazed by animals freely during the winter season which resulted in approximately complete removal of litter. Soil samples were also taken from the free grazing area in order to study the effects of FG on soil P. All soil samples were sent to the laboratory and sieved through a 2 mm screen and stored in a refrigerator at 4 °C prior to analyses of soil P fractions and enzymatic activities. Soil temperature, soil moisture, NH$_4^+$-N, NO$_3^-$-N, microbial biomass C and N (MBC and MBN), soluble organic C and N (SOC and SON), pH and total N of soil were analyzed and had already been reported by Rui et al. (2011).
4.3.3 Soil P fractionation

A modified version of Hedley et al. (1982) fractionation scheme was used to sequentially extract various forms of inorganic (Pi) and organic (Po) soil P. In brief, 1.0 g of finely ground (<150 um) air-dried soil was extracted with 30 ml of each extractant solution in an end-to-end shaker for 16 h. Tubes were then centrifuged at 10,000 RPM for 10 min, and the supernatant was decanted and saved for measurement of P.

The extractant solutions were 1 M ammonium chloride (NH₄Cl) (APi), 0.5 M sodium bicarbonate (NaHCO₃) (pH 8.5) (BPi, BPo), first 0.1 M sodium hydroxide (NaOH) (N(I)Pi, N(I)Po), 1M hydrochloric acid (HCl) (HPi) and second 0.1 M sodium hydroxide (NaOH) (N(II)Pi, N(II)Po). The concentration of inorganic P in the extracts was determined after the precipitation of organic matter by acidification, and the concentration of total P in the extracts was determined after persulfate oxidation, while the concentration of organic P in the extracts was calculated as the difference between total P and inorganic P (Tiessen and Moir 1993). The P in the extracts was determined by the method of Murphy and Riley (1962). Soil total P (TP) was determined using nitric acid (HNO₃) – perchloric acid (HClO₄) digestion (Olsen and Sommers 1982).

4.3.4 Determination of soil acid and alkaline phosphatase activities

Acid phosphomonoesterase (AcPME) (EC 3.1.3.2) and alkaline phosphomonoesterase (AlPME) (EC 3.1.3.1) activities were determined by the methods described by
Tabatabai and Bremner (1969) using para-nitrophenyl phosphate (pNPP) as an orthophosphate monoester substrate. In brief, soil sample was incubated at 37 °C for 1 h (AcPME-pH 6.5, AlPME-pH 11). The enzymatic reaction was stopped by addition of NaOH and CaCl$_2$, and the absorbance of the pNPP produced was measured spectrophotometrically at a wavelength of 400 nm.

4.3.5 Plant leaf biomass P measurement

Two major species within the plots, Potentilla anserine and Gentiana straminea, were selected for plant leaf biomass P measurements. The aboveground biomass was estimated by a non-destructive sampling method described by Klein et al. (2007) within 1x1 m quadrats of these plots. Leaf biomass of each species was determined as total aboveground biomass multiplied by proportion of this species within all species and the proportion of leaf biomass in total plant biomass of individual species. The leaf P concentration was determined after the HNO$_3$–HClO$_4$ digestion of 1.0 g dry leaf (Olsen and Sommers 1982). The total leaf biomass P was calculated as leaf biomass multiplied by leaf P concentration.

4.3.6 Statistical analyses

Statistical significances of the effects of warming and grazing on soil P fractions and phosphatase activities at different soil depths and leaf biomass P were determined separately by two-way analysis of variance (ANOVA) using Statistix for Windows version 8.0 (Analytical Software, Tallahassee, FL), with warming and grazing as the
main factors. Multi-comparison of each treatment was conducted using one-way ANOVA. Least Significant Difference (LSD) was used to separate the means when differences were significant at the \( P < 0.05 \) level. Linear regression and redundancy analysis (RDA) was used to examine the relationship between environmental and biochemical parameters (soil moisture, pH, total N, C/N ratio, \( \text{NH}_4^+ \)-N, \( \text{NO}_3^- \)-N, MBC, MBN, SOC and SON), soil P fractions and phosphatase activities, using Canoco Software 4.5 (Microcomputer Power, USA). Principal component analysis (PCA) was applied with a correlation similarity matrix to determine the major factors influencing all these P-associated parameters, using IBM SPSS Statistics 19 (SPSS Inc., USA).

4.4 Results

4.4.1 Soil P fractions

The sizes of soil P fractions at the 0-10 cm depth were generally larger than at 10-20 cm (Fig. 4.1). The soil TP ranged from 786 to 833 \( \mu g \ g^{-1} \) and 721 to 746 \( \mu g \ g^{-1} \) for the 0-10 and 10-20 cm depths, respectively (Fig. 4.2). \( \text{N(I)Po} \), ranging from 101 to 260 \( \mu g \ g^{-1} \), comprised the largest proportion (12.6 % - 32.7 % of TP) among all extracted fractions for both soil depths, followed by the \( \text{N(II)Po} \) (77 to 161 \( \mu g \ g^{-1} \)) and \( \text{HPI} \) (65 to 184 \( \mu g \ g^{-1} \)) while the \( \text{API} \) (1.6 to 8.5 \( \mu g \ g^{-1} \)) constituted the smallest portion (0.22 % - 1.03 %) of TP (Fig. 4.1).
Fig. 4.1 Inorganic and organic phosphorus (P) fractions (µg g⁻¹) of the 0-10 cm (A) and 10-20 cm (B) soil depths extracted using a P fractionation scheme. NWNG: no warming with no grazing; NWG: no warming with grazing; WNG: warming with no grazing; WG: warming with grazing; and FG: free grazing. Different letters indicate significant differences between treatments at $p<0.05$ for each soil depth. Mean ± SE values are shown.
Fig. 4.2 Total inorganic P and total organic P (µg g⁻¹) extracted using a P fractionation scheme, and total P (µg g⁻¹) measured with a digestion method for the 0-10 cm (A) and 10-20 cm (B) soil depths. Different letters indicate significant differences between treatments at p<0.05 for each soil depth. Mean ± SE are shown. See Fig. 2 for explanations of the abbreviations.

Warming and grazing affected the soil P fractions mainly at 0-10 cm depth, while at 10-20 cm these fractions were less affected (Table 4.1 and Figs. 4.1 and 4.2).

Warming and grazing significantly affected soil organic P fractions. The ANOVA results have shown that both warming and grazing reduced concentrations of N(I)Po (P = 0.01, Table 4.1). There was no significant interaction between warming and grazing on N(I)Po (p=0.16), but the WG treatment caused the largest decrease of it, by 48.3% compared with NWNG (Fig. 4.1), suggesting that the effects of warming and grazing on N(I)Po were additive. WG also significantly decreased N(II)Po compared with WNG at 0-10 cm (Fig. 4.1 A). In addition, total extractable Po (TPo) was significantly reduced by both warming and grazing treatments at 0-10 cm.
Table 4.1 P values for soil at two depths (0-10 cm and 10-20 cm) from two-way ANOVAs of soil phosphorus (P) fractions using warming (W) and grazing (G) as main factors. APi: inorganic P extracted by 1 M ammonium chloride (NH₄Cl); B Pi: inorganic P extracted by 0.5 M sodium bicarbonate (NaHCO₃); N(I)Pi: inorganic P extracted first by 0.1 M sodium hydroxide (NaOH); H Pi: inorganic P extracted by 1M hydrochloric acid (HCl); N(II)Pi: inorganic P extracted second by 0.1 M NaOH; B Po: organic P extracted by 0.5 M NaHCO₃; N(I)Po: organic P extracted first by 0.1 M NaOH; N(II)Po: organic P extracted second by 0.1 M NaOH; Total Pi: total inorganic P; Total Po: total organic P; AcPME: acid phosphomonoesterase; and ALPME: alkaline phosphomonoesterase.

<table>
<thead>
<tr>
<th></th>
<th>APi</th>
<th>BPi</th>
<th>N(I)Pi</th>
<th>H Pi</th>
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<th>N(I)Po</th>
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<th>Total Pi</th>
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<th>AcPME</th>
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<td>&lt;0.01*</td>
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<td>0.20</td>
<td>0.69</td>
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<tr>
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<td>0.87</td>
<td>0.97</td>
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"*" indicates significance at P < 0.05.
(p=0.01 and p<0.01, respectively) (Table 4.1 and Fig. 4.2 A). Interaction between warming and grazing was found on TPo (p=0.01) at 0-10 cm, and WG decreased it by 31.6% compared with NWNG. Among all treatments, WG led to the reduction of major soil organic P (N(I)Po, TPo) fractions by 40-48% and 28-32%, respectively. Moreover, soil TP was reduced by warming at 0-10 cm (p=0.045) (Table 4.1), and TP under WNG was significantly lower than NWG and FG (Fig. 4.2 A).

Inorganic P fractions and total extractable Pi (TPI) were generally less affected by warming and grazing treatments (Table 4.1 and Figs. 4.1 and 4.2) compared with the organic P fractions. APi, the solution of Pi extracted by NH₄Cl, had the minimum P concentration, ranging from 1.56 to 8.48 µg g⁻¹. BPi, N(I)Pi, HPi and N(II)Pi were larger fractions compared with APi, but were also little affected by warming and grazing (Fig. 4.1). However, FG had significant influence on these labile P fractions, as FG significantly increased APi at 0-10 cm compared with WNG and WG, and increased APi and BPi at 10-20 cm compared with NWNG, NWG and WNG (Fig. 4.1). FG also decreased BPo at 0-10 cm compared with WNG and N(II)Pi at 10-20 cm compared with NWNG (Fig. 4.1).

4.4.2 Soil phosphatase activities

The activities of soil alkaline phosphatase ranged from 8.7 to 26.9 µg p-NP g⁻¹ h⁻¹ for the 0-10 and 10-20 cm depths, generally higher than soil acid phosphatase (5.5-14.5 µg p-NP g⁻¹ h⁻¹). Both warming and grazing strongly affected the activities of soil acid and alkaline
phosphatase. At the 0-10 cm soil depth, warming, grazing and their interaction increased AcPME activity ($p<0.01$), while warming treatments and the interaction between warming and grazing also increased AlPME activity ($p=0.02$ and $p<0.01$ respectively) (Table 4.1 and Fig. 4.3). Compared with other treatments, WG caused the largest increases in both AcPME and AlPME activities at 0-10 cm, by 117.8% and 36.8% respectively (Fig. 4.3). At 10-20 cm, the effect of grazing on the activity of AcPME was significant according to factorial two-way ANOVA ($p=0.01$), while little effect of warming was found (Table 4.1). FG also increased the activity of AcPME compared with NWNG, NWG and WNG at 0-10 cm (Fig. 4.3).

![Fig. 4.3](image)

**Fig. 4.3** Acid phosphomonoesterase (AcPME) (A) and alkaline phosphomonoesterase (AlPME) (B) activities in soil from 0-10 and 10-20 cm depths. Different letters indicate significant differences between treatments at $p<0.05$ for each soil depth. Mean ± SE values are shown. See Fig. 2 for explanations of the abbreviations.

4.4.3 Plant leaf P concentration and leaf biomass P

Both *Potentilla anserine* and *Gentiana straminea* were important species within these
plots. However, grazing favored the abundance of *Potentilla anserine*, while warming tended to favor *Gentiana straminea*. The leaf P concentration of *Potentilla anserine* was generally higher than *Gentiana straminea* (Fig. 4.4 A). WNG led to the lowest leaf P concentration of *Potentilla anserine* (1.08 mg g⁻¹), which was significantly lower compared with NWNG (1.33 mg g⁻¹) (Fig. 4.4 A). Grazing (NWG and WG) significantly increased leaf biomass P of *Potentilla anserine*, which rose from 13.4 and 15.2 mg m⁻² under NWNG and WNG treatments to 40.3 and 36.9 mg m⁻² under NWG and WG treatments (Fig. 4.4 B). WNG significantly affected leaf biomass P of *Gentiana straminea* compared with NWG, by increasing it from 12.6 to 24.0 mg m⁻² (Fig. 4.4 B).

![Graph A](image1.png)

**Fig. 4.4** Plant leaf P concentration (mg g⁻¹) (A) and leaf biomass P (mg m⁻²) (B) of two major species, *Potentilla anserine* and *Gentiana straminea* for different treatments. Different letters indicate significant differences between treatments at *p*<0.05. Mean ± SE are shown. See Fig. 2 for explanations of the abbreviations.
4.4.4 Relationships between environmental factors and soil P fractions and phosphatase.

N(I)Po and TPo were negatively correlated with the AcPME activity and soil temperature at 0-10 cm. The activity of AcPME and soil temperature explained 44% and 38% of the variation of N(I)Po (Fig. 4.5 A and B), and 54% and 29% of the variation of TPo (Fig. 4.5 C and D), respectively.

![Fig. 4.5 Relationships between AcPME activity and N(I)Po (A), soil temperature and N(I)Po (B), AcPME activity and TPo (C), and soil temperature and TPo (D) at 0-10 cm soil depth.](image)

Figure 4.6 demonstrates the relationships between environmental and soil biochemical factors, and soil P fractions and phosphatase activities. The approximately same direction between the arrows that soil P parameters are pointing and that of environmental and soil biochemical parameters indicates a high positive correlation (the longer the arrow, the stronger the relationship; Kennedy et al. 2004). TP was highly and
positively correlated with soil moisture, as their arrows have approximately same
direction and length. AlPME was highly and positively correlated with TN, NH$_4^+$-N and
MBN, while AcPME was highly and positively correlated with NO$_3^-$-N and SOC. The
N(I)Po was negatively correlated with NO$_3^-$-N and SON. The PCA has showed that the
clear separation of WG samples from other treatments along the PC2 axis. The PC1 and
PC2 axes explained 37.6% and 21.8% of the variance of these parameters, respectively
(Fig. 4.7).

![Biplot of redundancy analysis (RDA) of the relationships between environmental factors
and soil P fractions and phosphatases activities. TN: soil total N; MBC: microbial biomass C;
MBN: microbial biomass N; SOC: soluble organic C; SON: soluble organic N; TP: total P; TPo:
total organic P; BPI: inorganic P extracted by 0.5 M sodium bicarbonate (NaHCO$_3$); BPO:
organic P extracted by 0.5 M NaHCO$_3$; HPI: inorganic P extracted by 1M hydrochloric acid
(HCl); N(I)Po: organic P extracted first by 0.1 M sodium hydroxide (NaOH); N(II)Po: organic P
extracted second by 0.1 M NaOH; AcPME: Acid phosphomonoesterase; and AlPME: alkaline
phosphomonoesterase.](image)
Fig. 4.7 Ordination plot of principal component analysis (PCA) of all P-associated parameters under different warming and grazing regimes (A) and loading values of the individual parameter for PC1 and PC2 (B). Numbers in parentheses are percentage variance for each principal component. LBP1: Leaf biomass P of *Potentilla anserine*; LBP2: Leaf biomass P of *Gentiana straminea*; APi: inorganic P extracted by 1 M ammonium chloride (NH₄Cl); BPi: inorganic P extracted by 0.5 M sodium bicarbonate (NaHCO₃); N(I)Pi: inorganic P extracted first by 0.1 M sodium hydroxide (NaOH); HPi: inorganic P extracted by 1M hydrochloric acid (HCl); N(II)Pi: inorganic P extracted second by 0.1 M NaOH; BPo: organic P extracted by 0.5 M NaHCO₃; N(I)Po: organic P extracted first by 0.1 M NaOH; N(II)Po: organic P extracted second by 0.1 M NaOH; Total Pi: total inorganic P; Total Po: total organic P; AcPME: acid phosphomonoesterase; and ALPME: alkaline phosphomonoesterase. See Table 1 for explanations of other abbreviations.
4.5 Discussion

4.5.1 Effects of warming on P cycling in the alpine meadow ecosystem on the Qinghai-Tibet Plateau

The effects of warming on terrestrial P cycling have been widely studied, and results from a range of sites have reported that increased temperature could stimulate microbial and soil phosphatase activity, enhance microbial mineralization and plant uptake of P, hence result in an accelerated P cycling (Sardans et al. 2006; van Meeteren et al. 2006; Bell et al. 2010). Here we also found that warming significantly stimulated soil phosphatase activities and decreased soil organic P fractions, suggesting that on the Qinghai-Tibet Plateau, increased temperature could lead to fundamental changes in soil P cycling.

Soil P cycling and availability are controlled by a combination of biological processes (mineralization - immobilization) and chemical processes (adsorption - desorption and dissolution - precipitation) (Chen et al. 2004). Soil pH is one of the main parameters determining the adsorption/desorption equilibrium of phosphate in soil (Hinsinger 2001). However, as pH was little affected by warming and grazing in our study, due to the low rates of weathering of P-containing primary minerals in this region, the availability of P was controlled mainly by mineralization of mineralizable organic P (Schlesinger 1991). WG decreased N(I)Po, N(II)Po and TPo, and increased the activities
of AcPME and AlPME at the 0-10 cm depth (Figs. 4.1A, 4.2A and 4.3). The N(I)Po, often associated with Fe and Al hydrous oxides, was considered to be moderately mineralizable. The enhanced mineralization of soil organic P in the warming treatments, as indicated by a significant decrease in N(I)Po, can be attributed to increased plant and microbial demands under the warming conditions. Several studies have reported that the mineralization of organic P is controlled mainly by demand for P (McGill and Cole 1981; Chen et al. 2004). It was well documented that warming could enhance plant and microbial growth, leading to an increased excretion of phosphatase to catalyze the mineralization of organic P and to meet their demands for available P (Sardans et al. 2006). In our study, soil temperature explained 38% and 29% of the variation of N(I)Po and TPo, respectively. WNG significantly increased leaf biomass P compared with NWG in Gentiana straminea (Fig. 4.4 B), while NWG and WG significantly increased leaf biomass P compared with NWNG in Potentilla anserina. As the leaf P concentration of the two species was not affected by WNG (Potentilla anserina), the increases in leaf biomass P were mainly due to the increase in the leaf biomass under warming, considering that warming tended to favor the Gentiana straminea population while Potentilla anserina became dominant in grazing plots. However, warming also significantly increased total P content in plant litter in 2009 (Data not shown), directly revealing the increased plant demand for P under the warming conditions. Microbial activity was undoubtedly a key regulator of P availability and both microbial mineralization and immobilization occurred rapidly, and their net effect on P availability
varied with soil moisture, temperature, and soil organic matter quantity and quality (Vandecar et al. 2009). Microbes were thought to outcompete plants for available P over short timescales due to their high surface area to volume ratio, rapid growth, and high turnover rates (Cole et al. 1977; Schimel et al. 1989). Olander and Vitousek (2004) reported that microbial demand determined the partitioning of newly added P into biological vs geochemical sinks. Microbial biomass could act as a source (mineralization) or a sink (immobilization) for plant-available nutrients. In another study (Rui et al. 2011) we have reported that WG significantly increased MBC at 10-20 cm and SOC at 0-10 cm, suggesting a stronger demand for P by the microbes under the WG treatment. Nevertheless, the effects of warming in increasing P mineralization and plant and microbial uptake of P could be controlled simultaneously by the decreased soil moisture under the warming conditions. Lower water supply could limit P diffusion at the root interface and uptake by plants, and also hinder microbial activity (Bradford and Hsiao 1982). However, although warming significantly decreased soil moisture in our study, the lowest value of soil moisture of the two depths was still approximately 30%, indicating water was not a limiting factor in this region. Sardans et al. (2007) suggested that the greatest values of phosphatase activity were observed when intermediate values of soil water content coincided with intermediate values of temperatures in soil and air, which were the most suitable for active plant metabolism and soil microbial activity. However, the decreased soil moisture might restrict the solubility of P, and limit its availability for plants.
Warming decreased TP ($p=0.045$) at the 0-10 cm soil depth. Studies of Mediterranean shrubland (Sardans et al. 2006) and subarctic heath ecosystems (Rinnan et al. 2008) also suggested that warming could decrease soil total P, mainly through the increased accumulation of P in plant biomass. As we have observed a higher P content in the litter under warming, presumably the P is getting returned in litter in the long term. However, the various inorganic P fractions, including APi (solution Pi), BPi (labile Pi, which adsorbed on the surface of crystalline P compounds, sesquioxides or carbonate), N(I)Pi (moderately labile Pi, which was associated with amorphous and some crystalline Al, Fe hydrous oxides), HPi (less labile Pi, which was associated with primary calcium minerals) and N(II)Pi (stable Pi, which was adsorbed into the mineral structure of soil components, or occluded by Fe and Al coatings), remained unchanged under the warming conditions. This implied that these inorganic P fractions were in transient turnover, and might not be controlled by only a single factor but by multiple factors, while the depletion of available inorganic P with relatively smaller size, could be quickly replenished by mineralization of organic P under the warming conditions.

Soil enzymes integrated information on soil microbial status and soil physical-chemical conditions and thus were a useful sensor to study the effects of environmental changes (Wick et al. 1998; Chen et al. 2003). It had been suggested that warming could increase soil phosphatase activities, not only through its direct influence in stimulating microbial activity, but also through its impacts on plant root exudation and mycorrhizal fungi. The activity of AcPME explained 44% and 54% of the variation of
N(I)Po and TPo, respectively. Acid phosphatase was produced by bacteria, mycorrhizal fungi, yeasts, protozoa and plant roots. The symbiotic mycorrhizal fungi could coat plant rootlets, excreting phosphatase and organic acids to release P and provide an active uptake site of for rapid diffusion of P from soil pore spaces to the root surface (Filippelli 2008). Alkaline phosphatase was produced by bacteria, fungi and earthworms. Temperature increases by 5°C had been found to double the colonization of roots by mycorrhiza (Gavito et al. 2003). According to unpublished results of us, warming also significantly increased the concentration of total extractable glomalin (TEG) and easily extractable glomalin (EEG), glycoproteins produced by arbuscular mycorrhizal fungi which were important in enhancing soil aggregation, soil aeration, drainage and microbial activity (Jastrow and Miller 1997; Wright et al. 1999; Rillig et al. 2001), suggesting that the activities of soil phosphatase could be favored due to both direct and indirect effects of warming, therefore result in an accelerated P cycling.

4.5.2 Effects of grazing on P cycling in the alpine meadow ecosystem on the Qinghai-Tibet Plateau

The modern terrestrial P cycle is dominated by agriculture and human activity (Filippelli 2008). Grazing, the major land use mode of this meadow, can accelerate P cycling by promoting plant growth which will increase nutrients uptake and enhancing soil phosphatase activities (Williams and Haynes 1990; Hobbs 1996). Although NWG, the simulated moderate grazing treatment, did not affect various P fractions, WG decreased
sizes of organic P fractions (N(I)Po, N(II)Po and TPo) significantly and increased the activities of soil acid and alkaline phosphatase, indicating that the combined warming and grazing treatment would augment the effects of warming or grazing alone. NWG also increases soil temperature due to enhanced solar radiation, but WG caused the largest temperature increase. These results have profound implications on the alpine meadow in future warming conditions with continuous grazing, that P cycling may not be affected by warming or grazing alone, but can be significantly accelerated by combined effects of warming and grazing. Different from NWG, FG had significant influence on labile P fractions, as FG significantly increased APi and BPi at 0-10 and 10-20 cm, and decreased BPo at 0-10 cm and N(II)Pi at 10-20 cm, indicating that FG might be able to affect labile P fractions as a greater grazing intensity compared with NWG. These results in total suggested that the pattern or intensity of grazing was an important factor affecting P cycling.

In natural ecosystems, often both N and/or P availabilities are near limiting levels, but the dependence of biological N fixation on adequate P supply makes P the principal limiting element (Tiessen 2008). When P is limiting, nodule growth and N-fixation activity are limited (Haynes and Ludecke 1981), the diversity and productivity of plant community may be reduced (Falkengren-Grerup 1998; Wrage et al. 2010). Large amounts of N could stimulate phosphatase exudation and therefore plant uptake of P (Wrage et al. 2010). The activity of AcPME in our study was positively correlated with NO\textsubscript{3}\,-N (Fig. 7), which was significantly stimulated by grazing as reported previously at this experimental
site (Rui et al. 2011) and some other studies (e.g. Olofsson et al. 2001), suggesting that
the grazing induced increase in NO$_3^-$-N could have a positive effect on phosphatase
activity, consequently the P mineralization. Speirs and McGill (1979) also reported that
acid phosphatase activity increased 6-fold in soils supplemented with glucose and
NH$_4$NO$_3$. Turner et al. (2003) found that soil with a long history of N deposition in
northern England had low P concentrations and most P was in the form of relatively
stable organic P. However, P fertilization will favor the development of legumes on
grazed grasslands and rangelands (Aydin and Uzun 2005; Martiniello and Berardo 2007).
In another experiment within this region, we found that aboveground net primary
production (ANPP) did not respond to the addition of N fertilizer but increased
significantly in response to P fertilizer, implying that on this alpine meadow P rather than
N could be a principal limiting factor (Data not published). The increased availability and
total storage of N under warming and grazing conditions, and the possible depletion of
organic P, could bring about fundamental changes to the nutrients cycling and plant
diversity in the long term on the plateau.

Unlike warming, grazing did not affect soil TP, which could be attributed to the
quick return of P through deposition of animal faeces and urine, which contained both
inorganic and organic P. The release of N and P was much faster via the animal
decomposition pathway (Floate 1981), so that the accelerated P cycling might result in a
transient turnover of all labile forms of N and P. However, as the effects of warming and
grazing on soil P fractions and phosphatase were additive, the combined effects might
lead to a gradual decrease of mineralizable and total P in the soil. As the availability and total content of N have been increased under the warming and grazing conditions, whether the depletion of organic P will result in a P deficiency and act as a limiting factor to the C and N cycling requires further investigation in this ecosystem in the long term.

4.6 Conclusions

Our study demonstrated that in the alpine meadow ecosystem of the Qinghai-Tibet Plateau, the combined effects of warming and grazing could modify the P cycling by significantly increasing the mineralization of organic P and phosphatase activities. The effects of warming and grazing on soil P cycling were additive, and there could be a gradual decrease in soil-available and total P in the long term under future warming and grazing conditions. These results, together with the previously reported profound effects of warming and grazing on C and N cycling, imply that the P may be in short supply for the system in the long run so the long-term productivity and sustainability of the system may face a challenge.

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Chapter 5    The effects of warming and grazing on soil bacterial
diversity and N-cycling gene abundance in alpine
meadow ecosystem of the Tibet Plateau

5.1 Abstract

How soil microorganisms, especially key functional groups involved in nitrogen (N) cycling respond to warming, is pivotal for understanding terrestrial ecosystem functioning under climate change. In order to assess the microbial response to co-occurring warming and grazing in the alpine meadows of the Qinghai-Tibet Plateau, we conducted investigations in a controlled warming-grazing experiment. After three years of treatment, warming significantly reduced bacterial diversity and abundance, based on 1.2-1.7 °C temperature increase, while the effects of warming and grazing on N-cycling microbial gene abundance varied with soil depths and between the drought year of 2009 and the normal precipitation year of 2010. Despite the lack of significant treatment effects in 2009, warming significantly reduced abundance of N-cycling genes at the 0-10 cm soil depth in 2010, while grazing alone markedly increased the abundance of denitrifying genes *narG*, *nirS* and *nosZ* by 27-46% at 10-20 cm, which effect was surprisingly dampened by warming. Plant species diversity explained 54.5% of the variation of bacterial diversity over the two years, while soil moisture and nitrate concentration explained 39-53% of the variation of the abundance of total bacteria and denitrifiers. In conclusion, warming mainly affected soil bacterial diversity and
abundance indirectly through its impact on plant community and soil moisture, and might reduce the positive effects of grazing on denitrifiers’ abundance and N₂O production in wet conditions. This study provides a unique insight into the response of soil microorganisms to co-occurring climate change and grazing conditions in the alpine meadows in the future.

5.2 Introduction

The ongoing global climate change is predicted to impact the terrestrial ecosystems substantially (ACIA 2005; IPCC 2007), especially in cold biomes (high-latitude and high-altitude sites) where the effects are thought to manifest the most (Robinson 2002; Aerts 2006). Soil nitrogen (N) cycling is critical in regulating the response of terrestrial ecosystem to climate change (Pastor & Post 1986, Melillo et al. 2002); and the key processes involved in soil N cycle (e.g. ammonium oxidation and denitrification) are mediated by microbial communities (Castro et al. 2010). Therefore, changes in microbial composition or the abundance of special functional groups can alter N availability to plants or N loss from the ecosystem (Lindsay et al. 2010). A number of studies have demonstrated that the diversity and abundance of soil microorganisms are regulated by a wide range of biotic and abiotic factors such as litter inputs, temperature and soil moisture (Fierer & Jackson 2006; Walker et al. 2008; Castro et al. 2010; Sheik et al. 2011; Yergeau et al. 2011); however, the combined effect of climate change and land-use management on soil microorganisms remains largely unknown.
Climate change factors such as warming and altered precipitation regimes can affect soil microbial communities both directly and indirectly, but the direction and magnitude of these responses are uncertain. Rising temperature can increase soil microbial activity to shift in favour of representatives adapted to higher temperatures and faster growth rates (Castro et al. 2010). However, a drier circumstance under warming can also lead to the reduced quantity, quality and turnover rate of litter, while the moisture limitation can often induce adverse physiological conditions and therefore eliminate the positive effects on microbial activity and abundance (Hastings et al. 2000; Allison & Treseder 2008; Castro et al. 2010). Water availability affects the osmotic status of bacterial cells and can indirectly regulate substrate availability, diffusion of oxygen, and soil pH (Griffiths et al. 2003). It has been reported that long-term warming shows complex effects on structure and richness of denitrifier communities in high arctic tundra soils (Walker et al. 2008). Therefore, increasing temperature may have non-linear and disproportional effects on microorganisms, and the indirect effects caused by warming can be more important than its direct effects in some cases (Yergeau & Kowalchuk 2008).

Grazing is one of the key disturbance factors that shape the composition of plant communities (Akiyama & Kawamura 2007) and affect the activity, abundance and structure of soil microbial communities (Le Roux et al. 2008; Abell et al. 2009) in grassland ecosystems. Grazing shifts the allocation of C and N between above- and below-ground through vegetation removal (Holland & Detling 1990), accelerates N cycling by efficiently recycling nutrients through the animal excreta pathway (Kohler et
al. 2005; Ma et al. 2006; Lin et al. 2009), and compacts soil and hence decreases air permeability and hydraulic conductivity by animal trampling. Through its direct and indirect influence on substrates, nutrient supply and soil physicochemical conditions, grazing can increase the abundance and induce changes in community structure of ammonia oxidizers and denitrifiers (Le Roux et al. 2008). Moreover, phospholipid fatty acid (PLFA) analysis shows that warming affects soil microbial community composition differently in the presence and absence of herbivores in a subarctic heath (Rinnan et al. 2009). Yet research focusing on how N-cycling microorganisms respond to grazing in alpine ecosystems is still lacking.

The Qinghai-Tibet Plateau is one of the most sensitive regions to climate change, and is predicted to confront a greater than average increase in surface temperature (Liu & Chen 2000; Giorgi et al. 2001). Concurrently, there is increasing grazing pressure on its alpine meadows where stock raising is the main land-use mode (Wang & French 1994). Previous studies suggested that, in these areas, both climatic factors and grazing were the main drivers that influence plant community structure and aboveground productivity (Klein et al. 2004; Wu et al. 2009). Therefore, understanding how soil microbial communities especially the N-associated functional groups respond to warming and grazing is important for making precise predictions of ecosystem functioning under future climate change scenarios. Based on a controlled warming-grazing experiment in an alpine meadow of this plateau, we aimed to test the hypothesis that warming would reduce the positive effects of grazing on bacterial diversity and the abundance of
denitrifiers by reducing soil moisture. By applying molecular techniques and targeting the specific functional genes which code for the catalytic enzymes involved in key processes of soil N cycling, we are able to link terrestrial ecosystem functioning with climate change (Wallenstein et al. 2006; Jung et al. 2011). The objectives of this study were to examine: (1) the independent and combined effects of warming and grazing on diversity and abundance of soil bacterial community; (2) the independent and combined effects of warming and grazing on N-cycling functional gene abundance; and (3) the factors that regulate microbial diversity and abundance in a warming-grazing regime in the alpine meadows.

5.3 Materials and methods

5.3.1 Experimental site

The experimental site and design were described in Chapter 2. The soil temperature at depths of 10 and 20 cm were measured automatically using type-K thermocouples (Campbell Scientific, Logan, Utah, U.S.A), which were connected to a CR1000 datalogger (Fig. 2.4). Soil moisture at the depths of 10 and 20 cm was manually measured through a tube in the ground down to a 40 cm depth using a frequency domain reflectometer.

The total rainfall of growing season (From 1 May to 20 September) was 283 and 376 mm in 2009 and 2010, respectively. Fig. 5.1a and b depicted the total rainfall of growing season from 2006 to 2010, and the rainfall amount over certain periods before
sampling in 2009 and 2010. 2009 was comparatively a drought year, as its rainfall of growing season was much lower than average level (369 mm from 2006-2010). The aboveground net primary production (ANPP) was estimated by a non-destructive sampling method described by Klein et al. (2007). Species richness was recorded as the occurrence of the number of plant species in a quadrat (1×1 m), and Shannon-Wiener index ($H'$) of plant community was calculated as: $H' = -\sum P_i \ln P_i$, where $P_i$ was the relative coverage of species $i$.

Fig. 5.1 Total rainfall of the growing season (from 1 May to 20 September) from 2006 to 2010 (a), and the rainfall amount over certain period before sampling in 2009 and 2010 (b).

5.3.2 Soil sampling

Soil samples from each plot were collected on 2 August 2009 and 15 August 2010 using a 5 cm-diameter corer. Five soil cores were randomly collected within each plot and bulked as a composite sample. Soil samples from depths of 0-10 and 10-20 cm were taken. All soil samples were sent to the laboratory and sieved through a 2 mm screen and stored in a
refrigerator at 4 °C prior to chemical analyses, while another portion of soil samples were kept at -80 °C prior to molecular investigations. Analyses of basic soil properties including soil gravimetric moisture, pH, NH$_4^+$-N, NO$_3^-$-N, microbial biomass C and N (MBC and MBN), soluble organic C and N (SOC and SON), total N, $\delta^{13}$C and $\delta^{15}$N were performed as reported by Rui et al. (2011, 2012).

5.3.3 Soil DNA extraction and quantification of total bacteria and functional genes

For each sample, DNA was extracted from 0.3 gram of soil using the MoBio Powersoil DNA Isolation Kit (Carlsbad, USA) according to the manufacturer’s instruction. The extracted DNA was stored at -20 °C. The abundance of total bacteria and specific functional genes involved in soil N cycling (bacteria and archaeal amoA, nifH, narG, nirK, nirS, and nosZ) was investigated using quantitative PCR (qPCR). These genes encode the catalytic subunit of the key enzymes of certain pathways in N cycling. Fragments of all the functional genes were amplified using primers and thermal conditions described in Table 5.1. For quantification of the environmental samples, standards of known amounts of templates were created. A cloned fragment of gene from an environmental sample was chosen to create the standard curve. Standard curves were obtained using serial dilutions (10$^{-1}$-10$^{-8}$ times) of linearized plasmids containing cloned bacteria and archaeal amoA, nifH, narG, nirK, nirS, and nosZ genes. The total bacterial community was quantified using 16s rRNA as a molecular marker. Reactions were carried out in an Effendorf Masterpiece realplex sequence detection system (Applied
Biosystems). Quantification was based on the fluorescence intensity of the SYBR green dye, which bound to double-stranded DNA. The 20 µL PCR mixture contained 10 µL of
Table 5.1 Enzymes encoded by functional genes, thermal condition of reaction and primers’ sequences of qPCR reactions used in this study.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Enzymes</th>
<th>Annealing time and temperature</th>
<th>Elongation time and temperature</th>
<th>Primers</th>
<th>Primers’ sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria</td>
<td>N/A</td>
<td>55°C, 15s</td>
<td>72°C, 30s</td>
<td>16S rRNA 338F, 518R</td>
<td>ACTCCTACGGGGAGGCAG/ ATTACCGGGCTGCTGG</td>
<td>Fierer et al. (2005)</td>
</tr>
<tr>
<td>Bacterial amoA</td>
<td>Ammonia monooxygenase</td>
<td>55°C, 30s</td>
<td>72°C, 45s</td>
<td>amoA1F, amoA2R</td>
<td>GGG GTT TCT ACT GGT GGT/ CCC CTC KGS AAA GCC TTC TTC</td>
<td>Rotthauwe et al. (1997)</td>
</tr>
<tr>
<td>Archaeal amoA</td>
<td>Ammonia monooxygenase</td>
<td>55°C, 30s</td>
<td>72°C, 45s</td>
<td>CrenamoA23F, CrenamoA616R</td>
<td>ATGGTCTGGCTWAGACG/ GGCATCCATCTGTATGTCCA</td>
<td>Tourna et al. (2008)</td>
</tr>
<tr>
<td>nifH&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Nitrogenase</td>
<td>54°C, 30s</td>
<td>72°C, 30s</td>
<td>POI-F, POI-R</td>
<td>TGC GAY CCS AAR GCB GAC TC/ ATS GCC ATC ATY TCR CCG GA</td>
<td>Poly et al. (2001)</td>
</tr>
<tr>
<td>narG&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Nitrate reductase</td>
<td>58°C, 30s</td>
<td>72°C, 30s</td>
<td>narGG-F, narGG-R</td>
<td>TCG CCS ATY CCG GCS ATG TC/ GAG TTG TAC CAG TCR GCS GAY TCS G</td>
<td>Bru et al. (2007)</td>
</tr>
<tr>
<td>nirK&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Nitrite reductase</td>
<td>58°C, 30s</td>
<td>72°C, 30s</td>
<td>nirK876, nirK1040</td>
<td>ATY GGC GGV CAY GGC GA/ GCC TCG ATC AGR TTR TGG TT</td>
<td>Henry et al. (2004)</td>
</tr>
<tr>
<td>nirS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Nitrite reductase</td>
<td>58°C, 30s</td>
<td>72°C, 30s</td>
<td>nirS4QF, nirS6QR</td>
<td>GTS AAC GYS AAG GAR ACS GG/ GAS TTC GGR TGS GTC TTS AYG AA</td>
<td>Kendeler et al. (2006)</td>
</tr>
<tr>
<td>nosZ&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Nitrous oxide reductase</td>
<td>60°C, 30s</td>
<td>72°C, 30s</td>
<td>nosZ2F, nosZ2R</td>
<td>CGC RAC GGC AAS AAG GTS MSS GT/ CAK RTG CAK SGC RTG GCA GAA</td>
<td>Henry et al. (2006)</td>
</tr>
</tbody>
</table>

All target genes were amplified using Takara SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> (Perfect Real Time).

<sup>a</sup>Touch down starting at 59°C temperature decrease of 1°C per cycle for 6 cycles.

<sup>b</sup>Touch down starting at 63°C temperature decrease of 1°C per cycle for 6 cycles.

<sup>c</sup>Touch down starting at 65°C temperature decrease of 1°C per cycle for 6 cycles.
SYBR green PCR Master Mix, 0.2 μM of each primer and 12.5 ng of DNA. No-template controls gave null or negligible values. The presence of PCR inhibitors in DNA extracted from soil was estimated by 1:10 diluting soil DNA. No inhibition was detected. After each run, the PCR products were checked via 1% agarose gel electrophoresis to confirm specific product bands of the expected size.

5.3.4 PCR - Denaturing gradient gel electrophoresis (DGGE)

The diversity of bacterial community was studied using DGGE. The 16S rRNA gene was PCR amplified using the extracted DNA (0-10 cm soil) as template and universal bacterial primers 968f (AACGCGAAGAACCTTAC) with a GC clamp and 1401r (CGGTGTGTACAAGACCC) (Heuer et al. 1997). Amplicons (about 200 ng) were analyzed by DGGE using 8% (w/v) acrylamide/bisacrylamide (37.5:1) gels containing a 40–60% linear gradient of formamide and urea (100% denaturing solution contained 40% (v/v) formamide and 7 M urea). The electrophoresis was run for 7 h at 150 V and a constant temperature of 60 °C, using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The gels were stained with SYBR Gold Nucleic Acid Gel Stain (1:10 000; Invitrogen-Molecular Probes, Eugene, OR, USA) for 30 min, scanned by the Gel Documentation System (Syngene, Frederick, MD, USA) and analyzed using the software Quantity One (Bio-Rad Laboratories). The Shannon-Wiener diversity index (\(H'\)) of bacterial community reflected by DGGE gel profiles was calculated as Ge et al. (2010) had described.
5.3.5 Statistical analyses

The effects of warming and grazing on soil temperature, soil moisture, diversity index of bacterial community, and the abundance of total bacteria and functional genes were determined by repeated-measures ANOVA using Statistix for Windows version 8.0 (Analytical Software, Tallahassee, FL), with warming and grazing as between-subject factors and soil depth and sampling year as within-subject factors. The main effects of warming and grazing in each depth and each year were assessed by two-way ANOVA with warming and grazing as the main factors. Multi-comparison of each treatment at each soil depth was conducted using one-way ANOVA. Least Significant Difference (LSD) was used to separate the means when differences were significant at the \( p<0.05 \) level. Linear regression was used to assess the relationships between environmental parameters and microbial abundance and diversity. Mean soil temperature and moisture of the growing season were used in this study.

5.4 Results

5.4.1 Soil temperature, soil moisture and precipitation

Both warming and grazing significantly increased soil temperature \((p<0.01, \text{Table 5.2 and 5.3})\). WNG significantly increased average soil temperature of the growing season by 1.9 and 1.6 °C at 0-10 and 10-20 cm in both years (Fig. 5.2a and b), compared with
Table 5.2 Results (P-value) from three-way ANOVA for the effects of warming (W), grazing (G), depth (D) and their interactions on soil temperature (T), moisture (M), and the microbial abundance of total bacteria and functional genes in 2009.

<table>
<thead>
<tr>
<th></th>
<th>Soil T</th>
<th>Soil M</th>
<th>Bacteria</th>
<th>Bacterial amoA</th>
<th>Archaeal amoA</th>
<th>nifH</th>
<th>narG</th>
<th>nirK</th>
<th>nirS</th>
<th>nosZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>0.562</td>
<td>0.014*</td>
<td>0.993</td>
<td>0.077</td>
<td>0.387</td>
<td>&lt;0.01*</td>
<td>0.282</td>
<td>0.274</td>
</tr>
<tr>
<td>G</td>
<td>&lt;0.01*</td>
<td>0.551</td>
<td>0.442</td>
<td>0.096</td>
<td>0.798</td>
<td>0.935</td>
<td>0.681</td>
<td>0.818</td>
<td>0.786</td>
<td>0.714</td>
</tr>
<tr>
<td>D</td>
<td>&lt;0.01*</td>
<td>0.040*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>W×G</td>
<td>0.366</td>
<td>0.996</td>
<td>0.735</td>
<td>0.562</td>
<td>0.742</td>
<td>0.632</td>
<td>0.718</td>
<td>0.937</td>
<td>0.517</td>
<td>0.755</td>
</tr>
<tr>
<td>W×D</td>
<td>0.753</td>
<td>0.678</td>
<td>0.287</td>
<td>0.313</td>
<td>0.473</td>
<td>0.155</td>
<td>0.463</td>
<td>&lt;0.01*</td>
<td>1.000</td>
<td>0.736</td>
</tr>
<tr>
<td>G×D</td>
<td>0.921</td>
<td>0.662</td>
<td>0.989</td>
<td>0.404</td>
<td>0.295</td>
<td>0.642</td>
<td>0.743</td>
<td>0.964</td>
<td>0.502</td>
<td>0.604</td>
</tr>
<tr>
<td>W×G×D</td>
<td>0.434</td>
<td>0.215</td>
<td>0.933</td>
<td>0.754</td>
<td>0.899</td>
<td>0.928</td>
<td>0.986</td>
<td>0.921</td>
<td>0.582</td>
<td>0.915</td>
</tr>
</tbody>
</table>

"*" indicates significance at P < 0.05.

Table 5.3 Results (P-value) from three-way ANOVA for the effects of warming (W), grazing (G), depth (D) and their interactions on soil temperature (T), moisture (M), and the microbial abundance of total bacteria and functional genes in 2010.

<table>
<thead>
<tr>
<th></th>
<th>Soil T</th>
<th>Soil M</th>
<th>Bacteria</th>
<th>Bacterial amoA</th>
<th>Archaeal amoA</th>
<th>nifH</th>
<th>narG</th>
<th>nirK</th>
<th>nirS</th>
<th>nosZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>0.018*</td>
<td>0.085</td>
<td>0.163</td>
<td>0.106</td>
<td>0.035*</td>
<td>0.226</td>
<td>0.021*</td>
<td>0.041*</td>
</tr>
<tr>
<td>G</td>
<td>&lt;0.01*</td>
<td>0.511</td>
<td>0.055</td>
<td>0.597</td>
<td>0.827</td>
<td>0.522</td>
<td>0.296</td>
<td>0.826</td>
<td>0.646</td>
<td>0.413</td>
</tr>
<tr>
<td>D</td>
<td>&lt;0.01*</td>
<td>0.019*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>W×G</td>
<td>0.856</td>
<td>0.854</td>
<td>0.691</td>
<td>0.031*</td>
<td>0.790</td>
<td>0.255</td>
<td>0.060</td>
<td>0.634</td>
<td>0.084</td>
<td>0.089</td>
</tr>
<tr>
<td>W×D</td>
<td>0.841</td>
<td>0.528</td>
<td>0.085</td>
<td>0.611</td>
<td>0.251</td>
<td>0.856</td>
<td>0.960</td>
<td>0.110</td>
<td>0.524</td>
<td>0.052</td>
</tr>
<tr>
<td>G×D</td>
<td>0.856</td>
<td>0.576</td>
<td>0.964</td>
<td>0.178</td>
<td>0.642</td>
<td>0.341</td>
<td>0.272</td>
<td>0.179</td>
<td>0.899</td>
<td>0.678</td>
</tr>
<tr>
<td>W×G×D</td>
<td>0.369</td>
<td>0.200</td>
<td>0.263</td>
<td>0.166</td>
<td>0.823</td>
<td>0.198</td>
<td>0.123</td>
<td>0.300</td>
<td>0.273</td>
<td>0.045*</td>
</tr>
</tbody>
</table>

"*" indicates significance at P < 0.05.
NWNG. NWG also increased soil temperature by 1.3 °C at 0-10 cm and 1.0 °C at 10-20 cm in 2009, and 1.1 °C at 0-10 cm and 0.8 °C at 10-20 cm in 2010 compared with NWNG (Fig. 5.2a and b), mainly due to the decrease in vegetation canopy height by grazing which might enhance solar radiation. WG caused the largest increase in soil temperature by 2.7 °C at 0-10 cm and 2.6 °C at 10-20 cm in 2009, and 2.7 °C at 0-10 cm and 2.8 °C at 10-20 cm in 2010 compared with NWNG (Fig. 5.2a and b). No significant interaction between warming and grazing on soil temperature was found (Tables 5.2 and 5.3).

![Mean soil temperature at the depth of 0-10 and 10-20 cm of growing season of 2009 (a) and 2010 (b) and mean soil moisture (V/V %) of growing season at 0-10 and 10-20 cm of 2009 (c) and 2010 (d) under different warming and grazing regimes. NWNG: no warming with no grazing; NWG: no warming with grazing; WNG: warming with no grazing; and WG: warming with grazing. Different letters mean significant differences between treatments at p<0.05 for each soil depth. Mean ± SE is shown in the figure.](image)

Fig. 5.2
WNG decreased soil moisture significantly by 23.8% at 0-10 cm and 18.7% at 10-20 cm in 2009, and 21.4% at 0-10 cm and 18.3% at 10-20 cm in 2010, while WG also decreased soil moisture by 19.2% at 0-10 cm and 29.5% at 10-20 cm in 2009, and 17.8% at 0-10 cm and 28.9% at 10-20 cm in 2010 (Fig. 5.2c and d). Neither grazing nor the interaction between warming and grazing was found to affect moisture significantly (Tables 5.2 and 5.3).

The average rainfall amount of growing season (From 1 May to 20 September) varied greatly between the two years, which increased from 282 mm in 2009, lower than the average of five years (369 mm), to 376 mm in 2010 (Fig. 5.1a), indicating that the year of 2009 was comparatively a drought year. Fig 5.1b depicts the difference of rainfall amount during certain periods between the two years. However, compared with 2009, average soil temperature decreased by 17.8% and 21.7% while average soil moisture increased by 16.0% and 16.3% at 0-10 and 10-20 cm in growing season of 2010, respectively (Fig. 5.2).

5.4.2 Diversity and abundance of bacteria

According to the Shannon-Wiener diversity index calculated from the DGGE profiles, warming decreased the diversity of bacterial community in both 2009 and 2010 ($p<0.01$), and WNG caused the largest decrease in bacterial diversity, by 10.4% and 19.6% compared with NWNG, in 2009 and 2010 respectively (Fig. 5.3a). However, according to factorial analysis of variance, grazing increased bacterial diversity in 2009 ($p=0.015$) but
did not affect it in 2010 ($p=0.33$) (Fig. 5.3a). ANPP and plant diversity explained 19.0% and 54.5% of the variation in bacterial community diversity over the two years, respectively ($p=0.01$ and $p<0.001$) (Table 5.4; Fig. 5.3b), while soil temperature and soil moisture together explained 19.9% of it ($p=0.02$) (Table 5.4). The plant species data are yet to be published by our collaborators.

Fig. 5.3 Shannon-wiener diversity index ($H'$) of bacterial community under different warming and grazing treatments in 2009 and 2010 (a), and the relationship between plant species diversity and bacterial diversity over the two years (b). NWNG: no warming with no grazing; NWG: no warming with grazing; WNG: warming with no grazing; and WG: warming with grazing. Different letters mean significant differences between treatments at $p<0.05$ for each soil depth. Mean ± SE is shown in the figure.

Table 5.4 Relationships between Shannon-wiener diversity index ($H'$) of bacterial community and aboveground net primary production (ANPP), diversity of plant community and soil temperature (T) and soil moisture (M) over 2009 and 2010.

<table>
<thead>
<tr>
<th>Model</th>
<th>p-value</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H' = 4.03 - 0.003*ANPP$</td>
<td>0.01</td>
<td>0.190</td>
</tr>
<tr>
<td>$H' = -1.11 + 3.39*Plant diversity$</td>
<td>$&lt;0.001$</td>
<td>0.545</td>
</tr>
<tr>
<td>$H' = -0.64 + 0.17<em>Soil T + 0.06</em>Soil M$</td>
<td>0.02</td>
<td>0.229</td>
</tr>
</tbody>
</table>
The abundance of total bacteria among all treatments was much larger in 2010 than in 2009, rising from $8.17 \times 10^7$ to $1.13 \times 10^9$ copies g$^{-1}$ at 0-10 cm and from $1.52 \times 10^8$ to $3.62 \times 10^9$ copies g$^{-1}$ at 10-20 cm (Fig. 5.4a and b). Repeated-measures ANOVA showed that, over the two years, warming reduced bacterial abundance ($p=0.034$, Table 5.5), while the interaction between warming and sampling year was significant ($p=0.018$). Despite the lack of significant treatment effects in drought year of 2009, warming reduced bacterial abundance in 2010 ($p=0.018$), as WNG and WG reduced it by 64.5% and 80.1% at 0-10 cm compared with NWNG, respectively. However, grazing reduced it at 10-20 cm compared with no grazing ($p=0.04$, Table 5.6).
Table 5.5 Results (P-value) from repeated-measures ANOVA for the effects of warming (W), grazing (G), depth (D), Year (Y) and their interaction on the microbial abundance of total bacteria and functional genes.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Bacteria</th>
<th>Bacterial amoA</th>
<th>Archaeal amoA</th>
<th>nifH</th>
<th>narG</th>
<th>nirK</th>
<th>nirS</th>
<th>nosZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>0.034*</td>
<td>0.056</td>
<td>0.62</td>
<td>0.658</td>
<td>0.479</td>
<td>0.807</td>
<td>0.34</td>
<td>0.605</td>
</tr>
<tr>
<td>G</td>
<td>0.068</td>
<td>0.161</td>
<td>0.924</td>
<td>0.688</td>
<td>0.733</td>
<td>0.913</td>
<td>0.886</td>
<td>0.553</td>
</tr>
<tr>
<td>W×G</td>
<td>0.686</td>
<td>0.924</td>
<td>0.756</td>
<td>0.592</td>
<td>0.216</td>
<td>0.689</td>
<td>0.147</td>
<td>0.31</td>
</tr>
<tr>
<td>D</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Y</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>0.924</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>W×Y</td>
<td>0.018*</td>
<td>&lt;0.01*</td>
<td>0.391</td>
<td>0.030*</td>
<td>0.013*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>G×Y</td>
<td>0.063</td>
<td>0.07</td>
<td>0.661</td>
<td>0.577</td>
<td>0.193</td>
<td>0.664</td>
<td>0.429</td>
<td>0.638</td>
</tr>
<tr>
<td>D×Y</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>0.306</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>&lt;0.01*</td>
<td>0.024*</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>W×G×Y</td>
<td>0.703</td>
<td>0.273</td>
<td>0.820</td>
<td>0.210</td>
<td>0.167</td>
<td>0.490</td>
<td>0.174</td>
<td>0.152</td>
</tr>
<tr>
<td>W×D×Y</td>
<td>0.066</td>
<td>0.359</td>
<td>0.237</td>
<td>0.440</td>
<td>0.645</td>
<td>0.739</td>
<td>0.688</td>
<td>0.108</td>
</tr>
<tr>
<td>G×D×Y</td>
<td>0.961</td>
<td>0.262</td>
<td>0.457</td>
<td>0.304</td>
<td>0.359</td>
<td>0.218</td>
<td>0.773</td>
<td>0.939</td>
</tr>
<tr>
<td>W×G×D×Y</td>
<td>0.248</td>
<td>0.986</td>
<td>0.823</td>
<td>0.069</td>
<td>0.325</td>
<td>0.328</td>
<td>0.691</td>
<td>0.169</td>
</tr>
</tbody>
</table>

“*” indicates significance at P < 0.05;
Table 5.6 Results ($P$-value) from two-way ANOVA for the effects of warming (W), grazing (G) and their interaction on the microbial abundance of total bacteria and functional genes at the depths of 0-10 cm and 10-20 cm in 2009 and 2010.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Bacteria</th>
<th>Bacterial amoA</th>
<th>Archaeal amoA</th>
<th>nifH</th>
<th>narG</th>
<th>nirK</th>
<th>nirS</th>
<th>nosZ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2009 0-10 cm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.264</td>
<td>0.141</td>
<td>0.487</td>
<td>0.676</td>
<td>0.876</td>
<td>0.201</td>
<td>0.297</td>
<td>0.280</td>
</tr>
<tr>
<td>G</td>
<td>0.585</td>
<td>0.408</td>
<td>0.213</td>
<td>0.673</td>
<td>0.922</td>
<td>0.631</td>
<td>0.362</td>
<td>0.499</td>
</tr>
<tr>
<td>W×G</td>
<td>0.859</td>
<td>0.400</td>
<td>0.653</td>
<td>0.669</td>
<td>0.690</td>
<td>0.960</td>
<td>0.922</td>
<td>0.874</td>
</tr>
<tr>
<td><strong>2009 10-20 cm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.742</td>
<td>0.066</td>
<td>0.697</td>
<td>0.121</td>
<td>0.429</td>
<td>0.011*</td>
<td>0.578</td>
<td>0.659</td>
</tr>
<tr>
<td>G</td>
<td>0.616</td>
<td>0.178</td>
<td>0.669</td>
<td>0.781</td>
<td>0.712</td>
<td>0.894</td>
<td>0.836</td>
<td>0.930</td>
</tr>
<tr>
<td>W×G</td>
<td>0.779</td>
<td>0.882</td>
<td>0.914</td>
<td>0.774</td>
<td>0.850</td>
<td>0.931</td>
<td>0.540</td>
<td>0.810</td>
</tr>
<tr>
<td><strong>2010 0-10 cm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.016*</td>
<td>0.049*</td>
<td>0.084</td>
<td>0.033*</td>
<td>0.031*</td>
<td>0.023*</td>
<td>0.066</td>
<td>0.028*</td>
</tr>
<tr>
<td>G</td>
<td>0.214</td>
<td>0.424</td>
<td>0.857</td>
<td>0.676</td>
<td>0.950</td>
<td>0.303</td>
<td>0.827</td>
<td>0.459</td>
</tr>
<tr>
<td>W×G</td>
<td>0.626</td>
<td>0.412</td>
<td>0.975</td>
<td>0.835</td>
<td>0.681</td>
<td>0.601</td>
<td>0.656</td>
<td>0.832</td>
</tr>
<tr>
<td><strong>2010 10-20 cm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.412</td>
<td>0.507</td>
<td>0.867</td>
<td>0.462</td>
<td>0.285</td>
<td>0.750</td>
<td>0.162</td>
<td>0.896</td>
</tr>
<tr>
<td>G</td>
<td>0.040*</td>
<td>0.329</td>
<td>0.663</td>
<td>0.429</td>
<td>0.285</td>
<td>0.237</td>
<td>0.635</td>
<td>0.663</td>
</tr>
<tr>
<td>W×G</td>
<td>0.107</td>
<td>0.076</td>
<td>0.754</td>
<td>0.235</td>
<td>0.096</td>
<td>0.256</td>
<td>0.038*</td>
<td>&lt;0.01*</td>
</tr>
</tbody>
</table>

“*” indicates significance at $P < 0.05$. 

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Fig. 5.4 Population size of total bacteria (a and b), bacterial amoA (c and d), archaeal amoA (e and f) and nifH (g and h) at the depth of 0-10 and 10-20 cm in 2009 and 2010 under different warming and grazing regimes. NWNG: no warming with no grazing; NWG: no warming with grazing; WNG: warming with no grazing; and WG: warming with grazing. Different letters mean significant differences between treatments at $p<0.05$ for each soil depth. Mean ± SE is shown in the figure.
**Fig. 5.5** Population size of denitrifiers including *narG* (a and b), *nirK* (c and d), *nirS* (e and f), and *nosZ* (g and h) at the depth of 0-10 and 10-20 cm in 2009 and 2010 under different warming and grazing regimes. NWNG: no warming with no grazing; NWG: no warming with grazing; WNG: warming with no grazing; and WG: warming with grazing. Different letters mean significant differences between treatments at $p<0.05$ for each soil depth. Mean ± SE is shown in the figure.

### 5.4.3 Abundance of N-associated functional genes

The abundance of N-cycling groups responds differently to warming and grazing, and the response varies with each year. Repeated-measures ANOVA showed that the effect of soil
depth was significant for all of the functional microbial groups \((p<0.001)\), while the effect of sampling year was significant for all \((p<0.001)\) except the archaeal \(amoA\) genes (Table 5.5). Average abundance of N-fixing gene \(nifH\) and denitrifying genes (including \(narG\), \(nirK\), \(nirS\) and \(nosZ\)) increased from 2009 to 2010 and the increase was more pronounced at 10-20 cm (Figs. 5.4 and 5.5). The abundance of archaeal \(amoA\) remained constant over the two years (Fig. 5.4e and f), while that of bacterial \(amoA\) decreased greatly in 2010 compared with 2009, from \(9.64 \times 10^5\) to \(1.87 \times 10^5\) copies g\(^{-1}\) at 0-10 cm and \(3.04 \times 10^6\) to \(7.20 \times 10^5\) copies g\(^{-1}\) at 10-20 cm (Fig. 5.4c and d). Significant interaction between warming and sampling year was also found on all but the archaeal \(amoA\) abundance (Table 5.5).

In 2009, the abundance of most functional genes measured showed little response to warming and grazing (Figs. 5.4 and 5.5; Table 5.6). Exceptionally, WG increased the abundance of bacterial \(amoA\) \((p=0.037)\) while warming treatments increased the abundance of \(nirK\) compared with no warming treatments at 10-20 cm \((p=0.011)\) (Figs. 5.4 and 5.5; Table 5.6). In 2010, warming significantly decreased the abundance of most functional genes except archaeal \(amoA\) and \(nirS\) compared with no warming treatments at 0-10 cm (Figs. 5.4 and 5.5; Table 5.6), whereas at 10-20 cm, NWG significantly increased the abundance of \(narG\), \(nirS\) and \(nosZ\) by 46.2%, 31.4% and 27.2% compared with NWNG and WG, respectively (Fig. 5.5). The lack of significant difference between the mean of NWNG and WG indicated that the effects of grazing on these functional genes were dampened by warming. WNG also significantly increased abundance of \(nosZ\)
by 29.4% compared with NWNG at 10-20 cm, and the interaction between warming and grazing was significant on abundance of nirS and nosZ at 10-20 cm ($p=0.038$ and $p<0.01$ respectively) (Table 5.6). Meanwhile, compared with WNG, the abundance of bacterial amoA and nosZ were significantly lower under WG (Fig. 5.5).

5.4.4 Relationships between microbial abundance and environmental parameters

In 2009, soil temperature and moisture explained 31.7% and 26.1% of the variation in bacterial amoA abundance ($p=0.023$ and 0.043) at 10-20 cm, respectively (Table 5.7). Soil temperature explained 32.7% of the variation in nirK abundance ($p<0.05$), while ANPP and NO$_3^-$-N together explained 67.5% of it ($p<0.05$, Table 5.7). The abundance of bacterial amoA and nirK was positively correlated with soil temperature but negatively correlated with soil moisture (Table 5.7).

In 2010, soil moisture was positively correlated with the abundance of total bacteria, bacterial amoA, nifH, narG, nirK, nosZ at 0-10 cm and narG and nirS at 10-20 cm (Table 5.7), implying that soil moisture was a critical factor controlling microbial abundance in the normal precipitation year. Soil moisture and NO$_3^-$-N together explained 53%, 51.3% and 47.7% of the variation in abundance of total bacteria, nirK and nosZ at 0-10 cm, and 38.8% of that of narG at 10-20 cm ($p<0.05$) (Table 5.7). ANPP and MBC explained 46.9% of the variation of total bacteria abundance at 10-20 cm ($p<0.05$) (Table 5.7). Soil moisture alone explained 24.3% of the variation of narG’s abundance at 0-10 cm and 36.8% of that of nirS at 10-20 cm ($p<0.05$) (Table 5.7).
Table 5.7 Relationships between population size of total bacteria and functional genes and environmental parameters in 2009 and 2010. ANPP: aboveground net primary production; $\delta^{15}$N: N isotope composition; and MBC: microbial biomass C.

<table>
<thead>
<tr>
<th>Model</th>
<th>p-value</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2009, 10-20 cm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial <em>amoA</em> = -9.2E6 + 1.0E6*Soil T</td>
<td>&lt;0.05</td>
<td>0.317</td>
</tr>
<tr>
<td>Bacterial <em>amoA</em> = 7.3E6 – 1.9E5*Soil M</td>
<td>&lt;0.05</td>
<td>0.261</td>
</tr>
<tr>
<td><em>nirK</em>=5.2E7 + 5E6*Soil T</td>
<td>&lt;0.05</td>
<td>0.327</td>
</tr>
<tr>
<td><em>nirK</em>= -3.7E7 + 85259<em>ANPP +1.2E6</em>NO$_3$-N</td>
<td>&lt;0.05</td>
<td>0.675</td>
</tr>
<tr>
<td><strong>2010, 0-10 cm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria = -2.3E9 + 1.5E8<em>Soil M – 1.2E7</em>NO$_3$-N</td>
<td>&lt;0.05</td>
<td>0.530</td>
</tr>
<tr>
<td>Bacterial <em>amoA</em> = -2.1E6+3.0E4<em>Soil M +4.1E5</em>$\delta^{15}$N</td>
<td>&lt;0.05</td>
<td>0.550</td>
</tr>
<tr>
<td><em>nifH</em>= -1.4E6 + 5.5E4<em>Soil M + 3305</em>NH$_4$+-N</td>
<td>&lt;0.05</td>
<td>0.463</td>
</tr>
<tr>
<td><em>narG</em>= -1.3E7 + 6E5* Soil M</td>
<td>&lt;0.05</td>
<td>0.243</td>
</tr>
<tr>
<td><em>nirK</em>= -2.7E7 + 1.6E6<em>Soil M – 1.5E5</em> NO$_3$-N</td>
<td>&lt;0.05</td>
<td>0.513</td>
</tr>
<tr>
<td><em>nosZ</em>= -3.5E6 + 2.5E5<em>Soil M – 2.0E4</em> NO$_3$-N</td>
<td>&lt;0.05</td>
<td>0.477</td>
</tr>
<tr>
<td><strong>2010, 10-20 cm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria = 5.3E9 – 6.9E6<em>ANPP + 2.3E6</em>MBC</td>
<td>&lt;0.05</td>
<td>0.469</td>
</tr>
<tr>
<td>Bacterial <em>amoA</em> = 3.0E6 - 5.4E5*$\delta^{15}$N</td>
<td>0.05&lt; $p&lt;$0.10</td>
<td>0.190</td>
</tr>
<tr>
<td><em>narG</em>= 9.2E6 + 5.4E5<em>Soil M + 1.4E5</em> NO$_3$-N</td>
<td>0.05&lt; $p&lt;$0.10</td>
<td>0.388</td>
</tr>
<tr>
<td><em>nirS</em>= 35863 + 5448*Soil M</td>
<td>&lt;0.05</td>
<td>0.368</td>
</tr>
</tbody>
</table>
5.5 Discussion

5.5.1 Effects of warming and grazing on bacterial diversity and abundance

The response of bacterial community to experimental warming in alpine ecosystems has rarely been investigated. Although many studies have observed a decline in soil bacterial diversity accompanied with warming in various ecosystems. It is reported that soil microbial community is resistant to warming in the arctic tundra ecosystem where more than a decade is required to detect any change in microbial biomass or structure (Rinnan et al. 2007; Lamb et al. 2011). In our study, warming of 1.2–1.7 °C significantly reduced bacterial diversity after three years of manipulation, and tended to dampen the positive effects of grazing on bacterial diversity. Nevertheless, the direct contribution of soil temperature and moisture to the variation of bacterial diversity was small, while plant species diversity explained 54.5% of its variation, reflecting the strong link between plant and microbial communities, primarily through root exudates and litter (Stephan et al. 2000; Brimecombe et al. 2001; Bardgett 2011). In this meadow, warming has been reported to reduce plant species diversity as a result of heat stress and litter accumulation (Klein et al. 2004); and also has accelerated litter mass loss and increased the concentration of dissolved organic carbon in our experiment (Luo et al. 2009, 2010); while grazing can increase plant species diversity and fencing has the opposite effect (Klein et al. 2004; Wu et al. 2009). Our study confirms that the plant-mediated indirect effects of climate change and land-use practice are likely to have a significant role in the response of the soil microbiome (Bardgett et al. 2008). A similar result has been reported by Sheik et al.
(2011) that soil water budgets strongly influence bacterial diversity under warming conditions in tall grass prairies. Although in the arctic tundra, soil bacterial community shows little response to warming despite the significant shifts in plant community (Lamb et al. 2011), the accordant changes in plant and bacterial communities under warming in our study demonstrate that the alpine meadow ecosystem of the Qinghai-Tibet Plateau is highly sensitive to climate change. Although DGGE has some limitations, it is effective for detecting changes in microbial communities occurring in response to experimental conditions (Flury & Gessner 2011). Soil microbial diversity plays an essential role in sustaining ecosystem function and the loss of biodiversity is one of the major threats to terrestrial ecosystems (Peters & Lovejoy, 1992). The reduction of microbial diversity can be an important indicator of the loss of resilience and, consequently, soil quality. Therefore, in the alpine meadow of the Qinghai-Tibet Plateau, a moderate intensity of grazing can be considered as a useful management practice to maintain diversity of plant species and the soil microbiome (Wu et al. 2009).

While microbial diversity reveals the balance between the number of microorganisms and the functional domains in the soil, the abundance of bacteria reflects more immediately the short-term microbial fluctuation (Kennedy 1999; Lavelle 2000). Despite the significant effect of the sampling year in our study, warming generally reduced the total bacterial abundance over the two years. The significant positive relationship between soil moisture and bacterial abundance, as well as the sharp rise in bacterial abundance accompanied with the increase in rainfall
amount from 2009 to 2010, demonstrate the critical role of soil water availability in controlling soil microbial abundance in this meadow. Microbial communities respond to soil moisture directly, because they require water for physiological activities, and indirectly, owing to the effect of changing soil moisture on gas diffusion rates and oxygen availability (Singh et al. 2010).

It is reported that warming can result in an up to 50% decrease in bacterial abundance in high-latitude ecosystems (Allison & Treseder 2008). Castro et al. (2010) also reported that bacterial abundance significantly decreased under 3 °C degrees temperature increase as a result of water stress. Microorganisms require an optimal point of soil temperature and water content. The higher abundance of bacteria at the 10-20 cm soil depth compared with 0-10 cm suggests that, the larger temperature and moisture variation at surface soil between day and night, which can occasionally result in night freezing of soil in this unique ecosystem, causes negative conditions for microbial growth. In addition, the effect of warming in our study is more noticeable in the normal precipitation year 2010 than in 2009, an extremely drought year when bacterial abundance can be largely suppressed in all plots.

Grazing alone also significantly increased soil temperature in our experiment but did not affect the soil moisture, hence had no influence on bacterial abundance, reconfirming the direct relationship between soil moisture and soil microbial abundance. We therefore draw the conclusion that soil water availability in warming and grazing regimes may be the driving factor for the shift in soil microbial abundance, while dramatic sudden changes in temperature and moisture can harm
microbial growth in the alpine meadows.

5.5.2 Effects of warming and grazing on the abundance of N-cycling related genes

In soil, N$_2$O is produced primarily as an intermediate product in the process of denitrification and also as a by-product of nitrification (Regan et al. 2011). Changes in temperature and other environmental factors may modify the net N balance of terrestrial ecosystems, causing feedback on atmospheric N$_2$O and climate (Cao & Woodward 1998). As microbial populations control natural production and consumption of N$_2$O, the abundance of the associated functional groups represents a potential method for predicting gas emissions from soil (Morales et al. 2010). In addition, as we have discovered that warming can reduce the effects of grazing in increasing annual N$_2$O flux and soil $^{15}$N in previous experiments (Hu et al. 2010; Rui et al. 2011), there is a necessity to investigate the microbial mechanisms of these responses.

Ammonia-oxidizing bacteria (AOB) play a fundamental role in nitrification and the abundance of AOB is affected by temperature, ammonium availability, pH and soil moisture (Avrahami et al. 2003; Horz et al. 2004; He et al.; 2007). Horz et al. (2004) found that abundance of AOB decreased in response to combined elevated CO$_2$ and increased precipitation, but these effects appeared to be buffered under elevated temperature conditions. The sharp decrease of the AOB abundance in our study accompanied by an increase in rainfall from 2009 to 2010, confirmed that the AOB preferred aerobic conditions with more oxygen and less water. However, at the
10-20 cm depth, WG significantly increased the abundance of AOB compared with NWNG in 2009, but reduced it compared with WNG in 2010, suggesting that grazing interacted with warming oppositely between dry and wet conditions. The change in AOB abundance in response to multi-factorial climate change factors was probably a result of indirect effects, most likely mediated by the plant community and soil moisture (Horz et al. 2004). We have previously reported that grazing significantly increases nitrate concentration but does not affect ammonium concentration in our experiment (Rui et al. 2011). However, the interactive effect of warming and grazing is further complicated by soil moisture variations. When soil water availability exceeds the optimum, a net negative effect on AOB abundance can be caused by animal trampling because of a reduction in oxygen availability in the compacted soils (Horz et al. 2004). Thus, it is plausible that grazing negates the positive effects of warming on AOB abundance in wet conditions. The novel finding that grazing augments the positive effect of warming on AOB abundance in drought years, but negates it in wet years has profound implications for predicting nitrification rates and N₂O production in the future. Although more abundant than its bacterial counterpart, the ammonia oxidizing archaea (AOA) showed little response to changes in temperature, rainfall and grazing treatments, indicating that AOA might be stable and resistant to climate change and land-use management in the alpine meadows. However, the direct contribution of AOA to nitrification is yet to be identified (Lindsay et al. 2010).

Denitrification is an important process in producing N₂O in grasslands, while the
abundance of denitrifiers can be used to predict potential N$_2$O emissions (Attard et al. 2011). The abundance of denitrifiers is reported to be positively related to soil moisture (Philippot et al. 2009). Despite the lack of treatment effects in the drought year of 2009, warming reduced the abundance of most denitrifiers at 0-10 cm depth in 2010, and dampened the positive effects of grazing of denitrifiers at 10-20 cm. Soil moisture and nitrate concentration are main drivers for changes in denitrifiers’ abundance. According to Regan et al. (2011), denitrification may be the major process contributing to N$_2$O emissions in wet conditions in grasslands. Abundant rainfall may therefore result in anoxic soil conditions which may promote N$_2$O production through enhanced denitrification (Parsons et al. 1991). Moreover, the increased soil nitrate under grazing in our study can indirectly stimulate denitrification and higher N$_2$O production in wet conditions, due to the more abundant and recent input of labile N from dung and urine in grazed plots (Lindsay et al. 2010). However, the significant positive relationship between soil moisture and denitrifiers’ abundance suggests that warming may repress denitrifiers indirectly by reducing soil moisture. The most striking finding of this study is, therefore, that the positive effect of grazing on denitrifiers is dampened by warming, implying that there is a potential role of warming in mitigating N$_2$O in the alpine meadows under grazing. We have previously reported that warming can reduce the effects of grazing, which increases the average annual N$_2$O flux by 57.8% (Hu et al. 2010). In addition, soil $\delta^{15}$N, an index of N cycling and gaseous loss rates, is more enriched under grazing treatments, but warming counteracts its effect (Rui et al. 2011). Taken together, evidence is mounting
to suggest that, in the alpine meadow of the Qinghai-Tibet Plateau, which is reported to experience a slightly increase in precipitation during the past decades, a possible increase of N\textsubscript{2}O emission as a result of increased grazing practice can potentially be counteracted by warming.

A number of studies suggest that the abundance of \textit{nifH} can be affected by warming (Walker et al. 2008; Jung et al. 2011) or grazing (Lindsay et al. 2010), but we failed to detect any significant effect of warming or grazing on \textit{nifH} abundance over the two years. However, the measured gene abundance patterns only reflect a microbial potential for nitrogen transformation and do not describe actual turnover rates in soils or N\textsubscript{2}O emission. Investigations of gene expression and enzyme activity are yet to be performed to compare the presence of functional groups with their activities and actual turnover rates. However, changes in N functional gene abundance are related to bacterial population size and substrate availability, and can be considered as useful ecological tools for predicting the capacity of the ecosystem to carry out the processes of the soil N cycle (Lindsay et al. 2010; Petersen et al. 2012).

This is the first study to simultaneously examine the influence of experimental warming and grazing on soil bacterial diversity and abundance of N associated microorganisms in the alpine meadow ecosystem of the Qinghai-Tibet Plateau. The results suggest that warming may affect soil bacterial diversity and abundance indirectly, mainly through its impact on plant community and soil moisture. In addition, this study highlights the potential role of warming in mitigating higher N\textsubscript{2}O production under grazing by decreasing soil moisture.
5.6 Conclusion

In conclusion, warming mainly affected soil bacterial diversity and abundance indirectly through its impact on plant community and soil moisture, and might reduce the positive effects of grazing on denitrifiers’ abundance and N₂O production in wet conditions. This study provides a unique insight into the response of soil microorganisms to co-occurring climate change and grazing conditions in the alpine meadows in the future.

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Chapter 6  Warming effect in other ecosystems - A study of the effects of warming and altered precipitation on soil functional gene abundance in semiarid grassland ecosystems

6.1 Abstract

In order to compare the difference in effects of warming on soil N-cycling gene abundance in different ecosystems, we conducted a separate study in a semiarid steppe of Inner Mongolia Plateau. In this study, the independent and combined effects of experimental warming and increased precipitation on N-cycling gene abundance were investigated in a 6 years’ multi-factorial experiment in a semiarid steppe in Inner Mongolia, China. Soil samples were taken in early August of 2010 and 2011, and quantitative PCR was used to examine the population size of total bacteria and functional genes including nifH, bacterial amoA, narG, nirK, nirS, and nosZ. Large inter-annual variation was observed, as the N-cycling gene abundance surged from 2010 to 2011 along with the elevated seasonal precipitation and ammonium concentration. However, abundance of total bacteria and N-cycling genes generally showed little response to warming over the two years, while increased precipitation markedly elevated abundance of total bacteria, nifH, narG and nosZ at 0-10 cm in the relatively dry year of 2010, but reduced the abundance of nifH, nirK, nirS and nosZ at 10-20 cm in 2011. The significant interaction between warming and increased precipitation in 2010 demonstrated that warming could counteract the positive effects
of increased precipitation. Although the population size of bacteria and N-associated functional genes were positively correlated with and best explained by $\text{NH}_4^+$-N over the two years, 48-77% of the variation of microbial abundance could be explained by $\text{NO}_3^-$-N in 2010. These results suggested that in the semiarid steppe, increased precipitation could exert a positive impact on microbial abundance under xeric conditions in dry years; while the functional microbial abundance and $\text{NH}_4^+$-N were driven by and could respond rapidly to seasonal precipitation variation. It highlighted the predominant role of precipitation in controlling microbial abundance in semiarid grassland ecosystems and proved that soil water availability is a driving factor controlling microbial response to warming in both ecosystems (alpine meadow and semi-arid steppe).

6.2 Introduction

The ongoing global climate change, including rising atmospheric temperature and changing precipitation regime, has been predicted to impact on the terrestrial ecosystems profoundly (IPCC 2007). Soil nitrogen (N) cycling, however, plays a critical role in controlling the responses of terrestrial ecosystems to climate change (Melillo et al. 2002). It has been well documented that the key processes involved in soil N cycle (e.g. ammonium oxidation and denitrification) are carried out by specific functional microbial groups, and changes in the abundance of these microorganisms can alter N supply to plants or gaseous N loss, consequently causing repercussions for ecosystem structure and feedbacks to the climate (Wardle et al. 1998). With relatively short generation times and rapid growth under favorable conditions, microbial
communities could be among the fastest components of an ecosystem to respond to changing environmental conditions (Nicol et al., 2007). As the abundance, community structure, and activity of soil microbial communities are often directly influenced by abiotic factors such as temperature and precipitation (Castro et al. 2010; Sheik et al. 2011; Yergeau et al. 2011), understanding how warming and changing precipitation regime shape N-cycling microorganisms is important for predicting ecosystem functioning under global climate change.

Warming as one of the major climate change factors can affect soil microbial communities substantially, but the direction and magnitude of these responses are uncertain. Rising temperature can enhance soil microbial activity and gene expression, and favour representatives adapted to higher temperatures and faster growth rates (Castro et al. 2010). However, a drier circumstance under warming can also lead to the reduced quantity, quality and turnover rate of litter, while the moisture limitation can often induce adverse physiological conditions and therefore eliminate the positive effects on microbial activity and abundance (Hastings et al. 2000; Allison & Treseder 2008; Castro et al. 2010). Therefore, increasing temperature may have non-linear and disproportional effects on microorganisms, and the indirect effects caused by warming can be more important than its direct effects in some cases (Yergeau & Kowalchuk 2008).

Most climate change scenarios predict not only a general warming trend, but also the alteration in the variability of precipitation which is another important factor affecting terrestrial ecosystems both globally and regionally (IPCC, 2007). Soil
microorganisms are subject to large variations in water availability due to changes at various scales, ranging from seasonal fluctuations to changes in soil water availability at the microhabitat level (Torsvik and Avreas, 2002). Water availability affects microbial gene expression in soil directly by controlling all physiological functions of the cell, and indirectly through regulating substrate availability, diffusion of oxygen, and soil pH. However, predicting the response of microbial communities to climate change is highly dependent on seasonal dynamics, background climatic variability, and the composition of the associated aboveground community. Seasonal and temporal shifts in rainfall, especially in arid and semiarid ecosystems where organisms may be at or near their physiological tolerance limits, can have a large impact on the diversity, abundance, and responsiveness of soil microbial communities. Nevertheless, the combined effects of warming and increased precipitation on N-cycling functional microorganisms remain largely unknown in semiarid grassland ecosystems.

The semiarid steppe of Inner Mongolia grassland in northern China is a part of the Eurasian grassland, which is the largest grassland biome in the world (Zhou et al. 2012). Previous studies suggested that, in these areas, soil water availability is of great importance for ecosystem function. Global climate change predictions state that the variability and magnitude of rain events are likely to occur resulting in extended droughts and periods of increased rainfall in the southwestern USA.

Soil microbial communities may be more resilient to environmental change relative to their aboveground plant counterparts, and changes to soil microbial
communities may only occur when abiotic variables are outside the range normally experienced by the communities. Large-scale manipulation of climate variables can inform scientists how ecosystems, and their associated communities, will respond in the future. Therefore, understanding how soil N-associated functional groups respond to warming and increased precipitation is important for making precise predictions of ecosystem functioning under future climate change scenarios. Based on a controlled warming-increased precipitation experiment in the semiarid steppe in Inner Mongolia, we aimed to test the hypothesis that warming would reduce while increased precipitation would increase the abundance of N-cycling genes, and similar with the warming experiment on the Tibet Plateau, soil water could control the microbial response to warming. The objectives of this study were to examine: (1) the independent and combined effects of warming and increased precipitation on abundance of soil bacteria; (2) the independent and combined effects of warming and increased precipitation on N-cycling functional gene abundance; and (3) the factors that regulate microbial abundance on semi-arid grasslands.

6.3 Materials and methods

6.3.1 Experimental site

The experimental site is located in a semiarid temperate steppe in Duolun County (42°02′N, 116°17′E, 1324 m a.s.l.), Inner Mongolia, China. The mean annual temperature of this area is 2.1 °C with monthly mean temperature ranging from -17.5 °C in January to 18.9 °C in July, while the mean annual precipitation is 382.3 mm with approximately 90% occurring in the growing season from May to October.
The soil in this area is classified as chestnut soil according to Chinese classification or *Haplic Calcisols* according to the FAO classification, with 62.75 ± 0.04 % sand, 20.30 ± 0.01 % silt, and 16.95 ± 0.01 % clay, respectively. The soil bulk density is 1.31 ± 0.02 g cm$^{-3}$ at the 0-10 cm depth, and the soil organic C and total N contents are 20.86 ± 3.53 and 1.87 ± 0.31 g kg$^{-1}$, respectively. The plant community at our experimental site is dominated by *Stipa krylovii*, *Artemisia frigida*, *Potentilla acaulis*, *Cleistogenes squarrosa*, *Allium bidentatum*, and *Agropyron cristatum* (Yang et al. 2011).

The experiment was established in April 2005. Basically, we used a nested design, with increased precipitation manipulated at the plot level and warming manipulated at the subplot level. There were three blocks with a 44×28 m area. In each block, there were two 10×15 m plots. One plot was assigned as the increased precipitation treatment and the other as the control. Within each plot, four 3×4 m subplots with two warmed subplots and two control subplots were arranged randomly. Six sprinklers were evenly arranged into two rows in each of the increased precipitation treatment plots. In July and August, 15 mm of water was added weekly to the increased precipitation treatment plots. Thus, a total of 120 mm precipitation was supplied each year. In the warmed subplot, a 1.65×0.15 m MSR-2420 infrared radiant heater (Kalglo Electronics Inc., Bethlehem, PA, USA) that was suspended 2.5 m above the ground had heated the subplot continuously since April 28, 2005. Experimental warming elevated soil temperature at 10 cm depth by 1.17 °C. In the control subplot, a “dummy” heater with the same shape and size as the infrared radiator was suspended...
at the same height to simulate the shading effect of the heater. Therefore, the experimental design consisted of 24 subplots with six replicates for four treatments [control (C), warming (W), increased precipitation (P), and warming plus increased precipitation (WP)].

Soil temperature at the 10 cm and 20 cm soil depth was recorded automatically using a CR1000 datalogger (Campbell Scientific, Logan, Utah, U.S.A), while the soil moisture (0-10 cm and 10-20 cm) was measured using a portable soil moisture device Diviner 2000 (Sentek Pty Ltd, Balmain, Australia). Although it is not exactly accurate, in this thesis they are used to represent them as temperature and moisture for the layer of 0-10 and 10-20 cm to make easy correlations.

6.3.2 Soil sampling

Soil samples from each plot were collected on 5 August 2010 and 11 August 2011 using an auger. Five soil cores were randomly collected within each plot and bulked as a single sample. Soil samples from soil depths of 0–10 and 10–20 cm were taken. All soil samples were sent to the laboratory and sieved through a 2-mm screen and stored in a refrigerator at 4 °C prior to chemical analyses, while another portion of soil samples were kept at -80 °C before molecular investigations. Soil NH$_4^+$-N and NO$_3^-$-N concentrations were determined in 2M KCl extracts.

6.3.3 Soil DNA extraction and quantification of total bacteria and functional genes

The methods used were described in Chapter 5.
6.3.4 Statistical analyses

Statistical significances of the effects of warming and increased precipitation on soil temperature, soil moisture, ammonium and nitrate concentration, and microbial abundance at each soil depth were determined by two-way analysis of variance (ANOVA) for a blocked split-plot design using Statistix for Windows version 8.0 (Analytical Software, Tallahassee, FL). Multi-comparison of each treatment was conducted using one-way ANOVA. Turkey HSD was used to separate the means when differences were significant at the $p=0.05$ level. Linear regression was used to assess the relationships between environmental parameters and microbial abundance. Mean soil temperature and moisture of the growing season were used in this study.

6.4 Results

6.4.1 Seasonal precipitation, soil temperature and soil moisture

The seasonal precipitation amount (from 1st May to 31st July) of the experimental site generally increased from 2007 to 2011, as 2011 peaked over the five years with 183 mm, 31.5% higher than average (Fig. 6.1).

Fig. 6.1 Total precipitation (mm) over May to July from 2007 to 2011.
Warming increased soil temperature at both soil depths over the two years ($p<0.01$, Table 6.1 and Fig. 6.2), while no significant effect of increased precipitation or the interaction between warming and increased precipitation on soil temperature was detected (Table 6.1 and Fig. 6.2). Notably, the treatment of warming with no increased precipitation (W), with the highest soil temperature across all treatments, elevated the soil temperature by 6.2-7.8% compared with the control over 2010 and 2011.

Table 6.1 Results ($P$-value) from two-way ANOVA on the effects of warming (W), increased precipitation (P) and their interactions on soil temperature, soil moisture, and ammonium and nitrate concentration at two depths of 0-10 and 10-20 cm.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Soil temperature ($^\circ$C)</th>
<th>Soil moisture (V/V %)</th>
<th>NH$_4^+$-N (mg kg$^{-1}$)</th>
<th>NO$_3^-$-N (mg kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010 0-10 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.001</td>
<td>0.160</td>
<td>0.020</td>
<td>0.187</td>
</tr>
<tr>
<td>P</td>
<td>0.104</td>
<td>0.157</td>
<td>0.051</td>
<td>0.031</td>
</tr>
<tr>
<td>W×P</td>
<td>0.352</td>
<td>0.616</td>
<td>0.041</td>
<td>0.355</td>
</tr>
<tr>
<td>2010 10-20 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.001</td>
<td>0.703</td>
<td>0.046</td>
<td>0.568</td>
</tr>
<tr>
<td>P</td>
<td>0.150</td>
<td>0.096</td>
<td>0.086</td>
<td>0.406</td>
</tr>
<tr>
<td>W×P</td>
<td>0.446</td>
<td>0.490</td>
<td>0.011</td>
<td>0.608</td>
</tr>
<tr>
<td>2011 0-10 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.003</td>
<td>0.136</td>
<td>0.201</td>
<td>0.004</td>
</tr>
<tr>
<td>P</td>
<td>0.136</td>
<td>0.054</td>
<td>0.548</td>
<td>0.781</td>
</tr>
<tr>
<td>W×P</td>
<td>0.293</td>
<td>0.887</td>
<td>0.737</td>
<td>0.932</td>
</tr>
<tr>
<td>2011 10-20 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.001</td>
<td>0.418</td>
<td>0.054</td>
<td>0.025</td>
</tr>
<tr>
<td>P</td>
<td>0.202</td>
<td>0.010</td>
<td>0.849</td>
<td>0.114</td>
</tr>
<tr>
<td>W×P</td>
<td>0.374</td>
<td>0.458</td>
<td>0.330</td>
<td>0.594</td>
</tr>
</tbody>
</table>
Increased precipitation increased soil moisture at the 10-20 cm soil depth in 2011 ($p<0.01$, Table 6.1 and Fig. 6.2) and marginally increased it at 10-20 cm in 2010 ($p=0.096$) and 0-10 cm in 2011 ($p=0.054$). However, no significant effect of warming or the interaction between warming and increased precipitation on soil moisture was detected (Table 6.1 and Fig. 6.2). Meanwhile, with the elevated seasonal precipitation amount, soil moisture also increased over the two years, rising from 7.0-9.8% at 0-10 cm and 12.9-14.9% at 10-20 cm in 2010 to 8.7-12.2% at 0-10 cm and 14.3-17.8% at 10-20 cm in 2011, respectively (Fig. 6.2).

6.4.2 Soil ammonium and nitrate concentration

Warming increased NH$_4^+$-N at both soil depths in 2010 ($p<0.05$), while the interaction
between warming and increased precipitation was significant ($p<0.05$). However, neither warming nor increased precipitation affected it significantly in 2011, while the ammonium concentration increased notably from 5.9-12.1 mg kg$^{-1}$ at 0-10 cm and 2.6-4.8 mg kg$^{-1}$ at 10-20 cm in 2010 to 22.2-27.2 mg kg$^{-1}$ at 0-10 cm and 20.7-26.8 mg kg$^{-1}$ at 10-20 cm in 2011, respectively (Fig. 6.2).

Increased precipitation elevated NO$_3^-$-N at 0-10 cm in 2010 ($p=0.03$, table 6.1), while warming increased it at both soil depths in 2011 ($p<0.05$, Table 6.1). However, the nitrate concentration did not vary markedly between the two years (Fig. 6.3).

![Fig. 6.3 Ammonium (a and b) and nitrate (c and d) concentration in the 0-10 and 10-20 cm soil layers in 2010 and 2011.](image)

6.4.3 Abundance of total bacteria and functional genes

In 2010, no significant effect of warming on microbial abundance was detected; however, increased precipitation generally increased the abundance of bacteria
$(p=0.09)$, $nifH$ $(p=0.02)$, $narG$ $(p=0.09)$ and $nosZ$ $(p=0.08)$ at 0-10 cm soil depth, while significant interaction between warming and increased precipitation was found on bacteria $(p=0.07)$, bacterial $amoA$ $(p=0.09)$, $nirK$ $(p=0.046)$, $nirS$ $(p=0.02)$ and $nosZ$ $(p=0.07)$ at 0-10 cm and $nifH$ $(p=0.09)$, $nirS$ $(p<0.01)$ and $nosZ$ $(p=0.02)$ at 10-20 cm (Table 6.2). The treatment of increased precipitation with no warming (P) generally increased the abundance of bacteria and functional microbial groups by 142-994% at 0-10 cm and 65-332% at 10-20 cm compared with the control (Fig. 6.4 and 6.5).

Fig. 6.4 Population size of total bacteria (a and b), $nifH$ (c and d) and bacterial $amoA$ (e and f) at the depth of 0-10 and 10-20 cm in 2010 and 2011 under different warming and precipitation regimes.
Table 6.2 Results (P-value) from two-way ANOVA on the effects of warming (W), increased precipitation (P) and their interactions on the population size of total bacteria, \textit{nifH}, bacterial \textit{amoA}, \textit{narG}, \textit{nirK}, \textit{nirS} and \textit{nosZ} at two depths of 0-10 and 10-20 cm.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Total bacteria</th>
<th>\textit{nifH}</th>
<th>Bacterial \textit{amoA}</th>
<th>\textit{narG}</th>
<th>\textit{nirK}</th>
<th>\textit{nirS}</th>
<th>\textit{nosZ}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2010 0-10 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.645</td>
<td>0.642</td>
<td>0.133</td>
<td>0.168</td>
<td>0.142</td>
<td>0.526</td>
<td>0.875</td>
</tr>
<tr>
<td>P</td>
<td>0.091</td>
<td>0.024</td>
<td>0.310</td>
<td>0.090</td>
<td>0.111</td>
<td>0.153</td>
<td>0.077</td>
</tr>
<tr>
<td>W\times P</td>
<td>0.067</td>
<td>0.286</td>
<td>0.088</td>
<td>0.101</td>
<td>0.046</td>
<td>0.019</td>
<td>0.067</td>
</tr>
<tr>
<td></td>
<td>2010 10-20 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.620</td>
<td>0.095</td>
<td>0.287</td>
<td>0.707</td>
<td>0.224</td>
<td>0.095</td>
<td>0.237</td>
</tr>
<tr>
<td>P</td>
<td>0.374</td>
<td>0.525</td>
<td>0.391</td>
<td>0.280</td>
<td>0.277</td>
<td>0.752</td>
<td>0.626</td>
</tr>
<tr>
<td>W\times P</td>
<td>0.268</td>
<td>0.085</td>
<td>0.233</td>
<td>0.286</td>
<td>0.216</td>
<td>0.001</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>2011 0-10 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.344</td>
<td>0.069</td>
<td>0.138</td>
<td>0.794</td>
<td>0.237</td>
<td>0.129</td>
<td>0.008</td>
</tr>
<tr>
<td>P</td>
<td>0.818</td>
<td>0.225</td>
<td>0.490</td>
<td>0.763</td>
<td>0.077</td>
<td>0.788</td>
<td>0.732</td>
</tr>
<tr>
<td>W\times P</td>
<td>0.716</td>
<td>0.687</td>
<td>0.408</td>
<td>0.832</td>
<td>0.229</td>
<td>0.856</td>
<td>0.154</td>
</tr>
<tr>
<td></td>
<td>2011 10-20 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.972</td>
<td>0.987</td>
<td>0.903</td>
<td>0.880</td>
<td>0.617</td>
<td>0.120</td>
<td>0.153</td>
</tr>
<tr>
<td>P</td>
<td>0.189</td>
<td>0.031</td>
<td>0.057</td>
<td>0.465</td>
<td>0.034</td>
<td>0.009</td>
<td>0.061</td>
</tr>
<tr>
<td>W\times P</td>
<td>0.807</td>
<td>0.683</td>
<td>0.906</td>
<td>0.362</td>
<td>0.415</td>
<td>0.628</td>
<td>0.948</td>
</tr>
</tbody>
</table>
Accompanied with the elevated seasonal precipitation amount and ammonium concentration, the population size of bacteria and N-associated functional genes generally increased from 2010 to 2011 (Fig. 6.4 and 6.5), while no consistent treatment effect was found on these microbial groups. However, despite the drastic rise of functional microbial abundance across all treatments at both soil depths in 2011, increased precipitation reduced nirK ($p=0.08$) at 0-10 cm and nifH ($p=0.03$),
nirK \( (p=0.03) \), nirS \( (p<0.01) \) and nosZ \( (p=0.06) \) at 10-20 cm. The treatment of warming with increased precipitation (WP) reduced the abundance of these microbial groups by 28-96% (Fig. 6.4 and 6.5).

6.4.4 Relationships between environmental factors and microbial abundance

Over the two years, the population size of bacteria and N-associated functional genes were best explained by NH\( _4^+ \)-N (Fig. 6.6). The abundance of bacteria, bacterial amoA, nirS and nosZ were highly positively correlated with NH\( _4^+ \)-N, while no significant correlation between microbial abundance and NO\( _3^- \)-N or microbial abundance and soil moisture over the two years.

Nevertheless, in 2010, the microbial abundance was significantly correlated with NO\( _3^- \)-N, which could explain 75%, 48%, 60%, 66% and 77% of the variation of total bacteria, nifH, nirK, nirS and nosZ, respectively (Table 6.3). However, in 2011, soil moisture explained 34%, 20%, 36%, 33% and 27% of the variation of total bacteria, bacterial amoA, narG, nirS and nosZ, respectively (Table 6.3).
Table 6.3 Results (P-value) from two-way ANOVA on the effects of warming (W), increased precipitation (P) and their interactions on the population size of total bacteria, \textit{nifH}, bacterial \textit{amoA}, \textit{narG}, \textit{nirK}, \textit{nirS} and \textit{nosZ} at two depths of 0-10 and 10-20 cm.

<table>
<thead>
<tr>
<th></th>
<th>Total bacteria</th>
<th>\textit{nifH}</th>
<th>Bacterial \textit{amoA}</th>
<th>\textit{narG}</th>
<th>\textit{nirK}</th>
<th>\textit{nirS}</th>
<th>\textit{nosZ}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(R^2)</td>
<td>(P)</td>
<td>(R^2)</td>
<td>(P)</td>
<td>(R^2)</td>
<td>(P)</td>
<td>(R^2)</td>
</tr>
<tr>
<td>2010</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil T</td>
<td>0.01</td>
<td>0.46</td>
<td>&lt;0.01</td>
<td>0.57</td>
<td>&lt;0.01</td>
<td>0.78</td>
<td>0.08</td>
</tr>
<tr>
<td>Soil M</td>
<td>0.16</td>
<td>&lt;0.01</td>
<td>0.15</td>
<td>&lt;0.01</td>
<td>0.05</td>
<td>0.13</td>
<td>0.05</td>
</tr>
<tr>
<td>(\text{NH}_4^+)-N</td>
<td>0.08</td>
<td>0.06</td>
<td>0.01</td>
<td>0.47</td>
<td>&lt;0.01</td>
<td>0.84</td>
<td>0.04</td>
</tr>
<tr>
<td>(\text{NO}_3^-)-N</td>
<td>0.75</td>
<td>&lt;0.01</td>
<td>0.48</td>
<td>&lt;0.01</td>
<td>0.08</td>
<td>0.06</td>
<td>0.17</td>
</tr>
<tr>
<td>SIN</td>
<td>0.47</td>
<td>&lt;0.01</td>
<td>0.24</td>
<td>&lt;0.01</td>
<td>0.04</td>
<td>0.20</td>
<td>0.02</td>
</tr>
<tr>
<td>2011</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil T</td>
<td>&lt;0.01</td>
<td>0.68</td>
<td>0.02</td>
<td>0.30</td>
<td>&lt;0.01</td>
<td>0.62</td>
<td>0.13</td>
</tr>
<tr>
<td>Soil M</td>
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<td>&lt;0.01</td>
<td>0.05</td>
<td>0.12</td>
<td>0.20</td>
<td>&lt;0.01</td>
<td>0.36</td>
</tr>
<tr>
<td>(\text{NH}_4^+)-N</td>
<td>0.08</td>
<td>0.06</td>
<td>0.01</td>
<td>0.58</td>
<td>0.01</td>
<td>0.42</td>
<td>0.01</td>
</tr>
<tr>
<td>(\text{NO}_3^-)-N</td>
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<td>0.11</td>
<td>0.02</td>
<td>0.37</td>
<td>&lt;0.01</td>
<td>0.79</td>
<td>0.08</td>
</tr>
<tr>
<td>SIN</td>
<td>0.13</td>
<td>0.01</td>
<td>&lt;0.01</td>
<td>0.70</td>
<td>&lt;0.01</td>
<td>0.77</td>
<td>0.08</td>
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</tbody>
</table>
Fig. 6.6 Relationships between ammonium and nitrate concentrations, soil moisture and population sizes of total bacteria (a), bacterial amoA (b), nirS (c) and nosZ (d) over the two soil depths and over the two years in 2010 and 2011 under different warming and precipitation regimes.

6.5 Discussion

6.5.1 Effects of Warming on N cycle gene abundance

In Chapter 5, it is concluded that in the alpine meadow of the Qinghai-Tibet Plateau, warming might affect N-cycling gene abundance indirectly through its impact on soil moisture. Grazing as a major disturbance factor which could accelerate N cycling
(Chapter 3) could increase the abundance of denitrifying genes in wet years, while the
effects of grazing was surprisingly dampened by warming. Here in the semiarid
steppe, the N-cycling gene abundance generally showed little response to warming in
both dry and wet years. There are already many warming sites all over the world and
a wider range of sites need to be studied. Compare with open top chambers (OTC),
infrared heaters now have been increasingly used because of better warming effects
especially in nighttime hours (Johnson et al. 2013). Both experiments in this thesis
used infrared heaters which increased soil temperature by 1-2 °C compared with
controlled plots. However, differences among biomes are indispensable when
considering the response of soil respiration and plant productivity to warming. It is
reported that ecosystem response to warming would be strongly affected by initial
conditions, such as stocks and initial turnover rates of labile soil C and N, the soil
water and precipitation regimes, the chemical composition and turnover rates of plant
residues (Xu et al. 2010). Negative effects of warming in drier climates suggest an
important interaction between future changes in temperature and water availability,
whereby warming induced drying will exacerbate water limitation on belowground
populations in arid and semiarid ecosystems. In contrast to aboveground biota,
positive effects of precipitation on soil biota were just as likely to occur in wetter
climates as in drier climates, indicating that water availability generally limits the
abundance of soil organisms across climatic zones.

Length of warming is another factor to be considered when comparing different
warming experiments. Warming tends to affect ecosystem processes and organic
matter pools at different rates. Negative effects of warming were more likely to occur in long-term experiments (Ruess et al. 1999; Convey and Wynn-Williams 2002) followed by long-term water limitation associated with warming-induced drying. The principal long-term mechanisms of regulation of NPP and overall C balance with respect to temperature may be different from the short-term mechanisms.

Meanwhile, the combined effects of warming and other climate change factors (e.g. changing precipitation regimes) should be taken into account. In these two warming experiments, changes in temperature are occurring simultaneously with increased precipitation or land-use management, and both factors affect the effects of warming on microbial community, so it will not be possible to fully understand and predict ecosystem responses to temperature change without taking into account the interactions with the other components of global environmental change. Changes in land use and disturbance regime may greatly influence responses to warming. The variability of precipitation hence soil water availability could be a critical factor.

6.5.2 Effects of Increased precipitation on N cycle gene abundance

The semiarid steppe of Inner Mongolia Plateau has a more variable climate compared with Tibet Plateau, including more extreme weathers, droughts, and larger precipitation events. Changes in precipitation regimes can alter soil microbial communities by causing shifts in community composition through the local extinction of certain operational taxonomic units (OTUs) or by shifting the abundance of bacteria and fungi in favor of one group over those in another group (Cregger et al.
In addition to the direct effect of precipitation change on soil microbial community abundance and diversity, soil microbial communities are influenced by changes in plant community abundance and composition which are subject to precipitation regimes. Here, increased precipitation had different effects on soil N-cycling gene abundance in different years. Seasonal and temporal shifts in rainfall, especially in ecosystems where organisms may be at or near their physiological tolerance limits, can have a large impact on the diversity, abundance, and responsiveness of soil microbial communities. Environments that have greater seasonal variation in rainfall may ameliorate the direct effects of climate change on soil microbial communities because a wide range of physiological tolerances may already exist within the community. Alternatively climate change may increase the severity of this variation resulting in new dynamics within the microbial community such as changes in species richness or composition. Moreover, seasonal variation in rainfall may result in a microbial community that is acclimated to fluctuations in precipitation thus resulting in a diminished response to the precipitation manipulation (Cregger et al. 2012). Consistent with this, in this comparative study it is found that soil microbial community structure and abundance were more responsive to fluctuations in seasonal rainfall than to altered precipitation treatments. It is possible that when microbial communities are acclimated to multiple dry-wet episodes, their response is diminished with each repeated event, and the magnitude of this response is dependent upon precipitation history and the associated aboveground community.
6.6 Conclusion

In conclusion, in the semiarid steppe, increased precipitation could exert a positive impact on microbial abundance under xeric conditions in dry year; while the functional microbial abundance and NH$_4^+$-N were driven by and could respond rapidly to seasonal precipitation variation. In both the alpine meadow of the Tibet Plateau and the semi-arid steppe of the Inner Mongolia Plateau, soil moisture and precipitation are of great importance in controlling microbial abundance.

6.7 Reference


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Chapter 7  General conclusions

7.1 General conclusions

This study was conducted based on a controlled warming-grazing experiment in the Haibei Alpine Meadow Ecosystem Station, Chinese Academy of Sciences. Soil samples were taken from a three years’ warming-grazing experiment. Soil total nitrogen (TN), inorganic N, microbial biomass carbon and N (MBC and MBN), soluble organic C and N (SOC and SON), as well as natural abundances of C and N stable isotopes were examined. A fractionation method was applied to investigate the sizes of different soil inorganic and organic P fractions and the activities of acid and alkaline phosphomonoesterase were studied. The diversity of bacteria was investigated using denaturing gradient gel electrophoresis (DGGE). Abundances of N-cycling functional microbial groups were measured with real-time quantitative PCR. In order to compare the warming effects on soil N-cycling gene abundance in different ecosystems, another comparative study was conducted in the semiarid steppe of Inner Mongolia Plateau. The main conclusions are listed as follows:

( 1 ) Warming and grazing treatments affected soil C and N pools differently and these effects varied with soil depth. Warming increased TN, MBC, MBN and SON and decreased δ\textsubscript{13}C at the 10–20 and 20–30 cm soil depths, while grazing generally decreased SON at 10-20 and 20-30 cm and MBC at 20-30 cm. At the 0–10 cm depth, neither warming nor grazing alone affect these soil parameters significantly, indicating that there could be considerable perturbation on the soil surface. However, grazing alone increased NO\textsubscript{3}−-N, total inorganic N, SOC and δ\textsubscript{15}N at the 0–10 cm depth.
In 2009, both warming and grazing significantly decreased the quantity of organic P extracted by first NaOH (N(I)Po), as well as the total extractable organic P (TPo) at the 0-10 cm depth. Warming also decreased the total P of soil at 0-10 cm. The combined warming and grazing treatment (WG) led to the reduction of major soil organic P fractions (N(I)Po, TPo) by 40-48% and 28-32%, respectively compared with other treatments at 0-10 cm. The activities of acid and alkaline phosphomonoesterase (AcPME and AlPME) were both enhanced by warming and grazing, and their interaction. Decreased concentrations of soil N(I)Po and TPo were accompanied by increased AcPME activity (P < 0.01) and soil temperature (P < 0.05), indicating the enhanced mineralization of organic P under rising temperature. Meanwhile, leaf biomass P of two major species (Potentilla anserine and Gentiana straminea) within these plots were significantly enhanced by either grazing or warming.

Warming significantly reduced bacterial diversity and abundance, based on 1.2-1.7 °C temperature increase, while the effects of warming and grazing on N-cycling microbial gene abundance varied with soil depths and between the drought year of 2009 and the normal precipitation year of 2010. Despite the lack of significant treatment effects in 2009, warming significantly reduced abundance of N-cycling genes at the 0-10 cm soil depth in 2010, while grazing alone markedly increased the abundance of denitrifying genes narG, nirS and nosZ by 27-46% at 10-20 cm, which effect was surprisingly dampened by warming. Plant species diversity explained 54.5% of the variation of bacterial diversity over the two years, while soil moisture and nitrate concentration explained 39-53% of the variation of the abundance of total bacteria and denitrifiers.
In order to compare the warming effects on soil N-cycling gene abundance in different ecosystems, we conducted another comparative study in the semiarid steppe of Inner Mongolia Plateau. In this study, the independent and combined effects of experimental warming and increased precipitation on N-cycling gene abundance were investigated in a 6 years’ multi-factorial experiment in 2010 and 2011. Large inter-annual variation was observed, as the N-cycling gene abundance surged from 2010 to 2011 along with the elevated seasonal precipitation and ammonium concentration. However, abundance of total bacteria and N-cycling genes generally showed little response to warming over the two years, while increased precipitation markedly elevated abundance of total bacteria, nifH, narG and nosZ at 0-10 cm in the relatively dry year of 2010, but reduced the abundance of nifH, nirK, nirS and nosZ at 10-20 cm in 2011. The significant interaction between warming and increased precipitation in 2010 demonstrated that warming could counteract the positive effects of increased precipitation.

In conclusion, warming and grazing affected labile C, N and P fractions significantly but differently: warming tended to increase labile C and N pools through increased litter inputs, while grazing tended to increase inorganic N pools, decrease SON and accelerate N cycling; while the microbial mineralization of soil organic P could be strongly increased under combined warming and grazing conditions as driven by increasing plant demand for P and enhanced microbial activities. As for the microbial communities, warming mainly affected soil bacterial diversity and abundance indirectly through its impact on plant community and soil moisture, and might reduce the positive effects of grazing on denitrifiers’ abundance and N₂O.
production in wet conditions in this alpine meadow. Meanwhile, in the semiarid steppe, increased precipitation could exert a positive impact on microbial abundance under xeric conditions in dry year; while the functional microbial abundance and NH$_4^+$-N were driven by and could respond rapidly to seasonal precipitation variation. In both ecosystems, soil moisture and precipitation are of great importance in controlling microbial abundance.

The snapshot sampling in this study may weaken the conclusion to some extent. However, although not always significant, the results obtained in this study were very indicative: the effects of warming in increasing C, N and P pools were consistent while the effects of grazing were inconsistent. The effects of warming and grazing on functional gene abundance were more dependent on soil water availability. Additionally, a longer term of treatments is required to observe future changes.

7.2 Future studies

It is recognized that the alpine meadow of the Tibetan Plateau is very sensitive to climate change and human disturbance, and temperature is a critical factor controlling ecosystem functioning. The present study showed that combined warming and grazing could potentially increase the size of soil C and N pools. This is contradictory with some studies which claim that warming could reduce the size of soil C pool through enhanced decomposition and soil respiration, while grazing could reduce the sizes of soil C and N pools through the removal of plant biomass. In our study, grazing might have increased soil N availability through elevated N transformation
rate. Soil moisture could be a critical driving factor of microbial communities. The
direct effects of warming on microbial gene abundance were not significant, while the
indirect effects of warming through its impact on soil moisture could affect microbial
gene abundance more pronouncedly. The direct stimulation of grazing on the
abundance denitrifying genes showed that it was likely that larger N\textsubscript{2}O production
under grazing in alpine meadow ecosystem; however, warming might dampen the
effects of grazing through its impact on soil moisture.

Because of some restrictions, there were still some problems and shortcomings of
this study. For instance, the sizes of soil C, N and P fractions were only measured in
summer season, while the freeze-thaw process was neglected. The growing season of
the alpine meadow is relatively short and the winter is long, thus there could be large
production of greenhouse gases and microbial activity within this process. There is a
lack of investigation into this question; The global warming is a gradual process, and
in the next 100 years the temperature of each decade might be just 0.2-0.5℃. Therefore, microbes and plants may gradually develop their own mechanism to adapt
to this gradual temperature increase. The 2℃ temperature increase in this experiment
is literally different from the global warming trend.

The future studies could be conducted accordingly:

(1) Establish a long-term monitoring system of soil C, N and P pools combing
NPP, soil respiratory and the natural abundance of stable isotopes, to study the
long-term impacts of warming and grazing on ecosystem C and N storage.

(2) As soil moisture is found to be a critical driving factor to microbial activity,
and in natural conditions, all soil moisture were not controlled, there is a necessity to study the microbial response under a soil moisture gradient.

(3) Because the rainfall is abundant in the alpine meadow, it is recognized that there is large denitrification potential. In this study, only N$_2$O production from the denitrification process was considered, the N$_2$O production from the nitrification is neglected. As in the future conditions, there is a larger frequency of dry-wet change, there is a necessity to study the N$_2$O production from both nitrification and denitrification under warming and grazing.

These techniques could be employed in future studies:

(1) Using the DNA-stable isotope probing (SIP), and the $^{15}$N labeled soil DNA, to track the soil N process, in order to investigate the production of N$_2$O.

(2) Because qPCR of soil DNA will retrieve the information of all soil DNA, including those inactive DNA, the qPCR of mRNA could be applied to quantitatively study the active microbial genes at a certain time.

(3) Using Nuclear Magnetic Resonance (NMR), study the chemical structure of C, N and P during soil process under warming and grazing conditions.