Biogeochemistry (2017) 136:341–352
https://doi.org/10.1007/s10533-017-0399-5

Increase in ammonia-oxidizing microbe abundance during degradation of alpine meadows may lead to greater soil nitrogen loss

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Received: 29 May 2017 / Accepted: 3 November 2017 / Published online: 13 November 2017

Abstract Alpine meadows on the Tibetan Plateau have experienced severe degradation in recent decades. Although the effects of alpine meadow degradation on soil properties have been well documented, there is still a paucity of knowledge regarding the responses of nitrogen-cycling microbes (NCMs) to degradation and their links to the changes in soil properties. Here, we systematically determined the effects of degraded patch formation on soil properties (i.e., total carbon, total nitrogen, ammonium nitrogen, nitrate nitrogen, available phosphorus, dissolved organic carbon, moisture, $\delta^{15}N$, $\delta^{13}C$, and pH) and NCMs (based on *nifH*, *amoA*, *narG*, *nirK*, and *nirS* genes and their transcripts) across three Tibetan alpine meadows at different degradation stages. Results showed that compared to the original grassed patches, the contents of most soil nutrients (e.g., carbon, nitrogen, and phosphorus) were significantly decreased in the degraded patches across the study sites. Degraded patches also tended to have higher soil $\delta^{15}N$ values and nitrate contents. Among the aforementioned NCMs, soil diazotrophs and denitrifiers only showed weak responses to the patch formation, while ammonia-oxidizing microbes showed the highest consistency and sensitivity in response to the patch formation across the study sites. The abundance of

Responsible Editor: Christine Hawkes.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10533-017-0399-5) contains supplementary material, which is available to authorized users.

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amoA gene and archaeal *amoA* mRNA significantly increased in the degraded patches, and they were positively correlated with soil $\delta^{15}N$ values and nitrate nitrogen contents, but negatively correlated with soil total nitrogen and inorganic nitrogen contents. These results suggest that the increased ammonia-oxidizing microbial abundance may be an important driver of soil nitrogen loss during degraded patch formation in alpine meadows.

Keywords Soil biogeochemistry · Metagene expression · Nitrogen cycling microbes · Alpine meadow degradation

Introduction

Approximately 23.5% of global land is experiencing ongoing degradation, causing 9.56×10^{11} kg carbon loss and severely threatening the lives of more than 1.5 billion people (Bai et al. 2008). This situation is even worse in the Tibetan alpine meadows which account for approximately half of the usable Tibetan grasslands, and are extremely sensitive to climate changes and human activities (Cui and Graf 2009; Wang et al. 2016; Zhao et al. 2015). Soil nitrogen contents have been shown to be susceptible to loss under grassland degradation (Dong et al. 2012; Li et al. 2014; Wen et al. 2013a; Zhang et al. 2017), which in turn may affect the resistance and resilience of grassland ecosystems, and determine the extent of further degradation (Dong et al. 2016). In natural ecosystems, soil nitrogen contents are essentially determined by the balance of the different nitrogen cycling processes that are mediated by a series of microbes (i.e., nitrogen cycling microbes, NCMs; Ollivier et al. 2011). Therefore, exploring the responses of NCMs to grassland degradation not only can contribute to predicting grassland development, but also can provide a theoretical basis for the restoration of degraded grasslands.

In recent decades, the soil NCMs, especially diazotrophs, nitrifiers, and denitrifiers, have been extensively investigated using the corresponding molecular markers (Levy-Booth et al. 2014; Thamdrup 2012). Diazotrophs are a group of prokaryotes that can reduce N_2 to NH_3. The *nifH* gene, encoding dinitrogenase reductase, is widely employed to identify diazotrophs by virtue of its ubiquity and phylogeny consistency in diazotrophs (Gaby and Buckley 2011; Raymond et al. 2004). Similarly, the *amoA* gene, encoding subunit A of ammonia monooxygenase, is commonly used as a molecular marker to investigate the ammonia-oxidizing archaea (AOA) and bacteria (AOB) (Stahl and de la Torre 2012a). As ammonia oxidization is often the rate determining step of nitrification, AOA and AOB are the most well studied nitrifiers over the past decades. Denitrification is a sequence of processes in which nitrate is reduced to N_2 through several intermediate products including nitrite, nitric oxide (NO), and nitrous oxide (N_2O). These processes are performed by a series of heterotrophic bacteria (i.e., denitrifiers), among which *narG* (encoding the catalytic subunit of nitrate reductase), *nirS*, and *nirK* (encoding nitrite reductase) can be used to investigate the microbes mediating the reduction process from nitrate to NO (the first gas product of denitrification) via nitrite (Levy-Booth et al. 2014). Nitrogen fixation can increase soil nitrogen contents, while nitrification may increase the risk of soil nitrogen loss, and denitrification can compromise soil nitrogen contents through gas emission (Galloway et al. 2004; Ollivier et al. 2011). Therefore, investigating soil NCMs, based on the aforementioned molecular markers (i.e., *nifH*, *amoA*, *narG*, *nirS* and *nirK* genes) and their transcripts can provide crucial information for understanding the responses of soil nitrogen contents to grassland degradation.

Soil NCMs are sensitive to grassland degradation and the reasons can be multifaceted. First, degradation usually decreases plant biomass and soil organic carbon (Lal 2003; Wen et al. 2013b). Typically, diazotrophs can be either heterotrophic or autotrophic, whereas most nitrifiers and denitrifiers are autotrophic and heterotrophic, respectively (Levy-Booth et al. 2014; Raymond et al. 2004; Stahl and de la Torre 2012a). Thus, the lower input of organic carbon under degradation should be less critical to soil nitrifiers and autotrophic diazotrophs, and more detrimental to denitrifiers and heterotrophic diazotrophs. Second, soil nitrogen has been shown to decrease under degradation (Dong et al. 2012; Li et al. 2014), which should promote the activities of diazotrophs but not nitrifiers and denitrifiers. Third, degradation also can decrease other soil nutrients (Ren et al. 2013; Wu et al. 2014; Wu and Tiessen 2002), altering plant

community composition and soil physical properties (Tang et al. 2015; Wu and Tiessen 2002), which may exert dramatic effects on the soil NCMs.

However, there is still a paucity of data to elucidate the responses of soil NCMs to grassland degradation, especially in the Tibetan alpine meadows. To date, no publications have documented the responses of NCMs to degradation in this region. Moreover, alpine meadow degradation can be divided into six stages, among which stages three to five are critical and characterized by the formation and expansion of degraded patches (Fig. 1; Lin et al. 2015). At these three degradation stages, the characteristic mattic epipedons of alpine meadow are first eroded to form bare soil patches (Fig. 1, Site 1 and Site 2), which then expand and interconnect to dominate over the isolated meadow patches (Fig. 1, Site 3). Most existing studies focused only on the vegetation and soil property variations along the degradation gradients (e.g. Li et al. 2015, 2016), whereas very few studies have examined the effects of degraded patch formation on soil nitrogen contents and NCMs (e.g., Zhang et al. 2017).

To address the aforementioned knowledge gaps, we initiated a study across three degraded Tibetan alpine meadows corresponding to the three aforementioned critical degradation stages to investigate: (i) whether and how soil physicochemical properties and NCMs (based on the *nifH*, *amoA*, *narG*, *nirS*, and *nirK* genes and their transcripts) were affected by degraded patch formation of alpine meadows; (ii) whether the responses of soil physicochemical properties and NCMs to the patch formation were consistent across different degradation stages; and (iii) whether the changes in NCMs were correlated with the variations in soil physicochemical properties.

Materials and methods

Study sites and soil sampling

We collected soil samples from three degraded alpine meadows (Fig. 1), Site 1 (32°52.39′N, 96°54.78′E; asl 3 982 m), Site 2 (33°14.96′N, 97°27.62′E; asl 4332 m), and Site 3 (34°34.06′N, 97°5.88′E; asl 4314 m), in August 2014. The degradation extents of the alpine meadows increased from Site 1 to Site 3, corresponding to the third to fifth degradation stages of alpine meadows (Lin et al. 2015), respectively. At Site 1 and Site 2, around 20% of the grassland was degraded to bare patches, but the original mattic epipedons at Site 1 remained intact. At Site 2, the

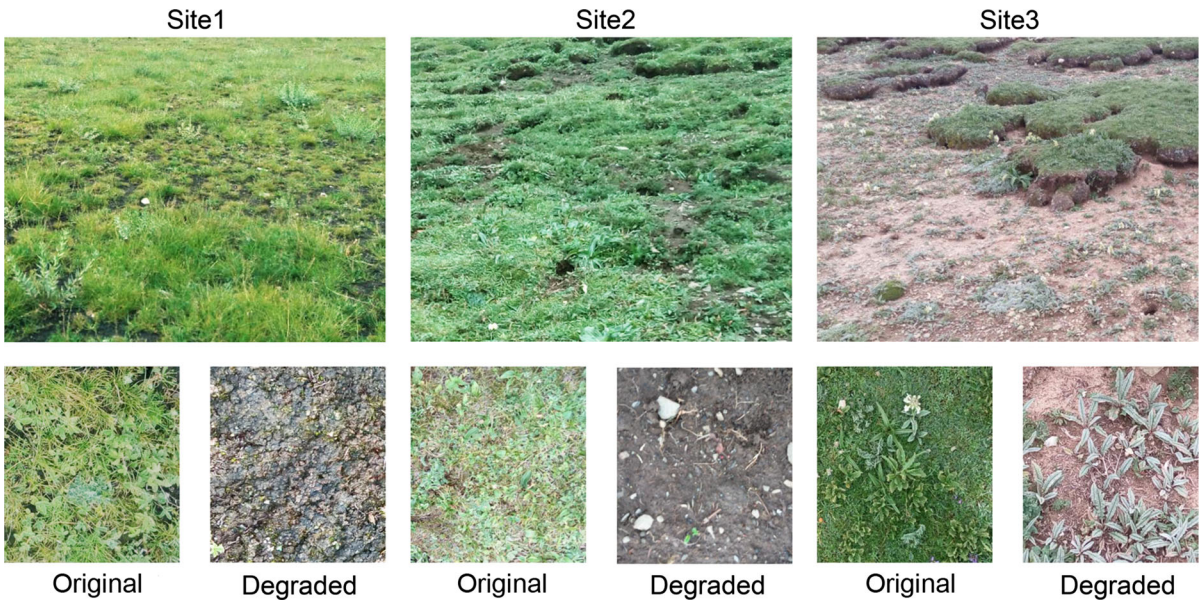

Fig. 1 Photographs of the three degraded alpine meadows investigated in this study

mattic epipedons were partly destroyed and eroded. At Site 3, about 60% of the mattic epipedons were eroded, and the bared patches were re-covered by secondary vegetation. Herein, the original meadow patches and bare soil patches are depicted as original patches and degraded patches, respectively (Fig. 1). The effect of degraded patch formation refers to the difference in soil physicochemical properties and NCM indices between the original and degraded patches.

At each site, we collected soil samples (0–10 cm) from four paired original-degraded patches (Fig. 1). After being sieved to 2 mm, we treated the soils (2.0 g) used for RNA extraction with 4 mL LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Carlsbad, CA, USA) following the instruction manual. The treated and 100 g nontreated fresh soil (used for DNA extraction and soil property analysis) was immediately placed into the − 20 °C freezer, transported to the laboratory within 2 weeks, and then preserved at − 80 °C. We also air dried some soil subsamples (100 g) for the analysis of soil pH values and available phosphorus contents.

Analysis of soil physicochemical properties

Soil gravimetric moisture content was determined by drying at 105 °C for 24 h. Soil pH was determined with the soil mass to water ratio of 1:5. Soil dissolved organic carbon (DOC) content was analyzed using the cold water method (soil mass to water ratio of 1:3), and measured using a TOC Analyzer (Liqui TOC II; Elementar Analysensysteme GmbH, Hanau, Germany). Soil ammonium and nitrate nitrogen (AN and NN) contents were determined using 2 M KCl extraction (soil mass to extractant ratio of 1:5) and an auto flow analyzer (Auto Analyzer 3 System; SEAL Analytical GmbH, Norderstedt, Germany). Soil available phosphorus (AP) content was analyzed using the method depicted by Olsen et al. (1954). Soil total carbon (TC) contents, total nitrogen (TN) contents, and values of $\delta^{13}C$ and $\delta^{15}N$ were determined using an isotope ratio mass spectrometer coupled with an auto elemental analyzer (NA1500, Fisons Instruments, Milano, Italy). The soil gravimetric moisture, DOC, AN, and NN contents were determined using the fresh soils, while the values of soil pH, $\delta^{13}C$ and $\delta^{15}N$ values, and the contents of AP, TC, and TN were analyzed using the air-dried soils.

Nucleic acid extraction and cDNA synthesis

We used 0.30 and 2.0 g soil to extract DNA and RNA, respectively. The extractions were separately conducted using a PowerSoil™ DNA Isolation Kit and PowerSoil® Total RNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), as described by suppliers. RNA extracts were treated with DNase I (MO BIO Laboratories, Carlsbad, CA, USA) to remove DNA residuals. The RNA was then reverse-transcribed into cDNA using a PrimeScript™ II 1st Strand cDNA Synthesis Kit with random hexamers (Takara Bio Inc., Shiga, Japan). Next, the DNA solutions were diluted 1:3 with water for the subsequent polymerase chain reaction (PCR) and real-time PCR amplifications, to attenuate the potential effects of PCR inhibitors.

Real-time PCR

We quantified the copy numbers of *nifH*, archaeal *amoA*, bacterial *amoA*, *narG*, *nirK*, and *nirS* genes and mRNAs using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), with the degenerated primer sets as described in Table S1. The 20 μL reaction mixtures contained the following: 1 μL template DNA (DNA, cDNA, or serially diluted standards), 10 μL Maxima™ SYBR Green or ROX (2×, Thermo Fisher Scientific, Waltham, MA, USA), 0.5 μL forward primer (20 μmol L^{-1}), 0.5 μL reverse primer (20 μmol L^{-1}), and 8.0 μL nuclease-free water. Standard curves were constructed using plasmids harboring the corresponding DNA fragments. We analyzed all DNAs and cDNAs in triplicate and utilized three no template controls to check for reagent contamination. PCR runs started with an initial denaturation and enzyme activation step for 10 min at 95 °C, which was followed by 40 cycles of 15 s at 95 °C, 30 s at the annealing temperatures depicted in Table S1, 40 s at 72 °C, and 30 s at 80 °C. We recorded the fluorescence signals at 80 °C to attenuate influences of primer dimers. We tested the specificities of the PCR products using melting curve analysis. The amplification efficiencies and R^2 values of the real-time PCRs are detailed in Table S2.

Terminal restriction fragment length polymorphism (T-RFLP)

The six nitrogen-cycling genes were separately amplified using PCR with labeled degenerate primer sets (Table S1). The forward primers were labeled with 6-carboxyfluorescein (FAM) at the 5′ ends. The 50 μL reaction mixture contained: 25 μL Premix Taq^{TM} Hot Start Version (Takara Bio Inc., Shiga, Japan), 1 μL template DNA, 1 μL forward primer (20 μmol L^{-1}), 1 μL reverse primer (20 μmol L^{-1}), and 22 μL nuclease-free water. The PCR programs consisted of the initial denaturation and enzyme activation at 95 °C for 10 min, followed by 38 cycles of 45 s at 95 °C, 30 s at the annealing temperatures depicted in Table S1, and 45 s at 72 °C, and then with a final extension at 72 °C for 10 min. We conducted three technical replicates for each sample and then separately pooled the technical replicates into one tube. Subsequently, the PCR products were purified using a GeneJET Gel Extraction Kit (Thermo Scientific, Lithuania), after which the purified PCR products were completely digested with the restriction endonucleases listed in Table S1 (New England Biolabs Inc. Beverly, MA, USA). Terminal restriction fragments (T-RFs) were size-separated by Beijing Ruibo Bio-Tech Co. Ltd (Beijing, China) using an ABI 3730 system (Applied Biosystems, Foster City, CA, USA). Electropherograms of T-RFLPs were retrieved using PeakScanner 1.0 (Applied Biosystems, Foster City, CA, USA). The threshold for peak assignment was ± 2 bp. After normalization, we removed unqualified peaks with a size less than the length of forward primers, more than the theoretical length, or a peak height lower than 50. Proportions of the qualified peaks were calculated based on their peak areas.

Statistical analysis

The effects of degraded patch formation (differences between the original and degraded patches) and study site on the soil physicochemical properties and copies of nitrogen-cycling functional genes and transcripts were determined using two-way nested ANOVA and Duncan's post hoc tests. The effects of degraded patch formation and study site on the NCM community structures were revealed using non-metric multidimensional scaling (NMDS) and permutational multivariate analysis of variance (PERMANOVA). We also conducted Pearson's correlation, redundancy analysis (RDA), and the envfit based on NMDS to analyze the relationships between NCM indices and soil physicochemical properties. We transformed some data to meet the requirements of analysis. All statistical analysis was conducted using R (R Development Core Team 2017) with the vegan package.

Results

Soil physicochemical properties

All the measured soil properties (i.e., TN, TC, C:N, inorganic nitrogen (IN), AN, NN, DOC, $\delta^{15}N$, $\delta^{13}C$, moisture, AP, and pH) differed significantly among the three study sites ($P < 0.006$, Fig. 2; Table S3). The highest soil TN, TC, IN, AN, DOC, AP and moisture contents all were recorded in the original patches of Site 1 and showed a decreasing trend from Sites 1 to 3 (Fig. 2). However, the soil NN contents, $\delta^{15}N$ and $\delta^{13}C$ values tended to increase with the degradation extent (Fig. 2). The soil C:N ratios were similar in the original patches across the three study sites, but they were significantly lower in the degraded patches at Sites 2 and 3 (Fig. 2h). Soil pH values were highest at Site 3 and lowest at Site 2 (Fig. 2l).

Although the soil properties were significantly different among the study sites, the effects of degraded patch formation on most soil properties were consistent. When compared with the original patches, soil TN, TC, IN, AN, DOC, AP, and moisture contents were significantly lower in the degraded patches ($P < 0.001$, Fig. 2; Table S3). However, there were also some inconsistent responses of soil properties to the patch formation across the study sites. The patch formation significantly increased the $\delta^{15}N$ values only at Sites 1 and 2, whereas it significantly decreased soil C:N ratios only at Sites 2 and 3. Additionally, soil NN contents in the degraded patches were significantly lower than those in the original patches at Site 1, but this trend was reversed at Sites 2 and 3 (Fig. 2d).

Copies of nitrogen-cycling genes and transcripts

The copies of most nitrogen-cycling genes and transcripts under examination also significantly differed among the study sites (Table S3; Figs. 3, 4). The most obvious variation was that copies of archaeal

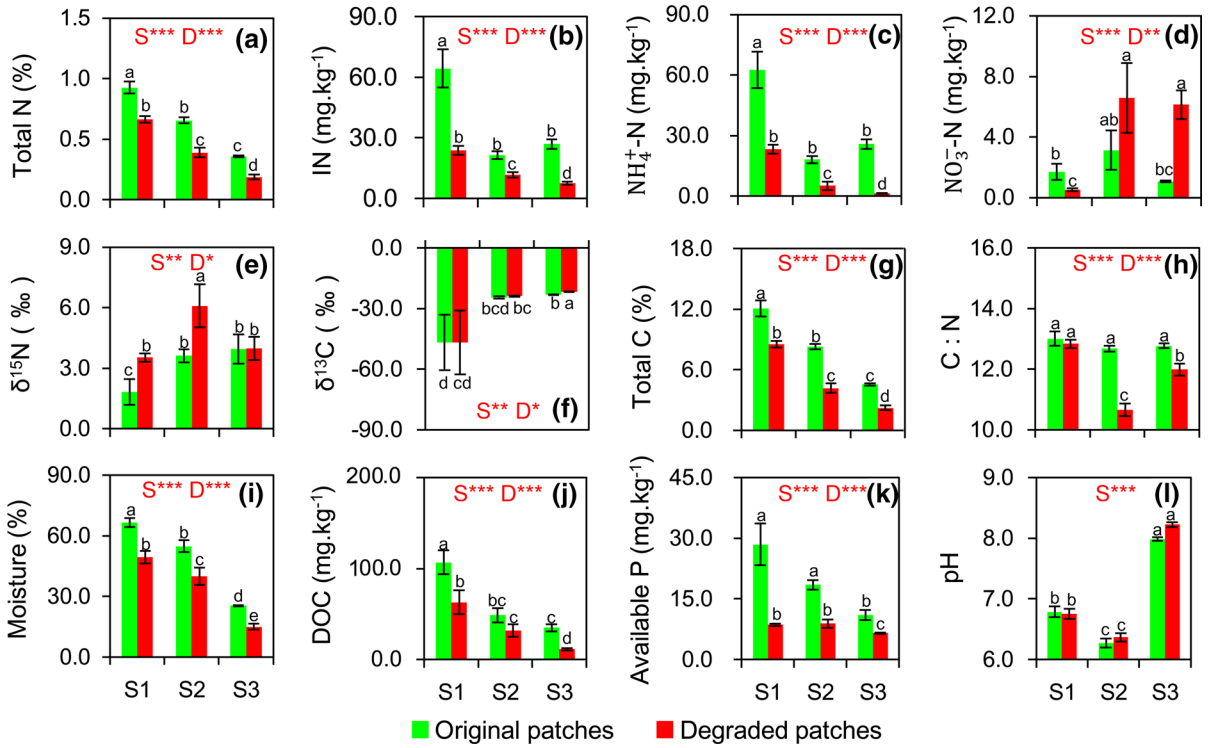

Fig. 2 Effects of the study site and degraded patch formation on soil properties at 0–10 cm depth. *IN* inorganic nitrogen, *DOC* dissolved organic carbon, *S1* Site 1, *S2* Site 2, *S3* Site 3, *S* effect of study site, *D* effect of degraded patch formation; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. All data were presented as the mean ± SE (n = 4); bars with different letters indicate significant differences across the study sites

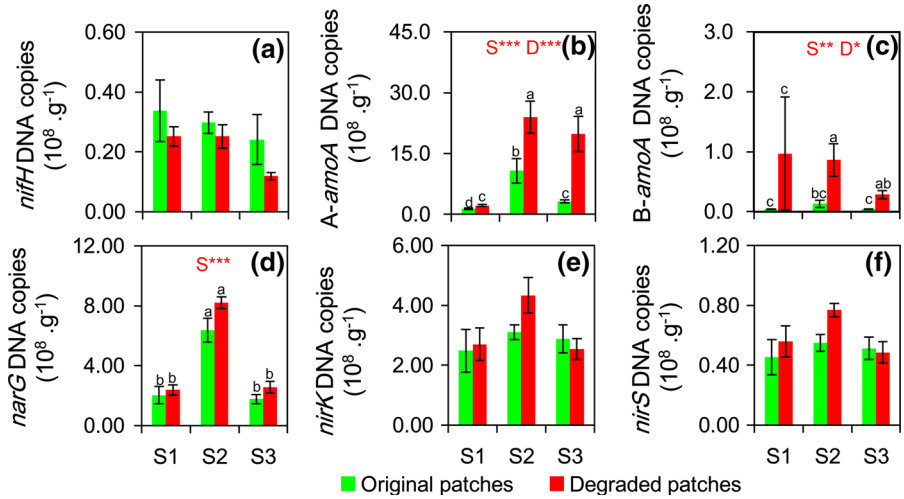

Fig. 3 Effects of the study site and degraded patch formation on soil nitrogen-cycling gene copies. *S1* Site 1, *S2* Site 2, *S3* Site 3, *S* effect of study site, *D* effect of degraded patch formation, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. All data were presented as the mean ± SE (n = 4); bars with different letters indicate significant differences across the study sites

amoA DNA at Site 1 were significantly lower than at Sites 2 and 3 (Fig. 3). Interestingly, the *amoA* gene also showed the highest sensitivity in response to the degraded patch formation. The copies of archaeal and bacterial *amoA* genes increased significantly in the degraded patches (Table S3; Fig. 3b). At Site 3, the *amoA* gene copies in the degraded patches were more than five times higher than in the original patches (Fig. 3b). The transcription of archaeal *amoA* genes was also greatly enhanced in the degraded patches

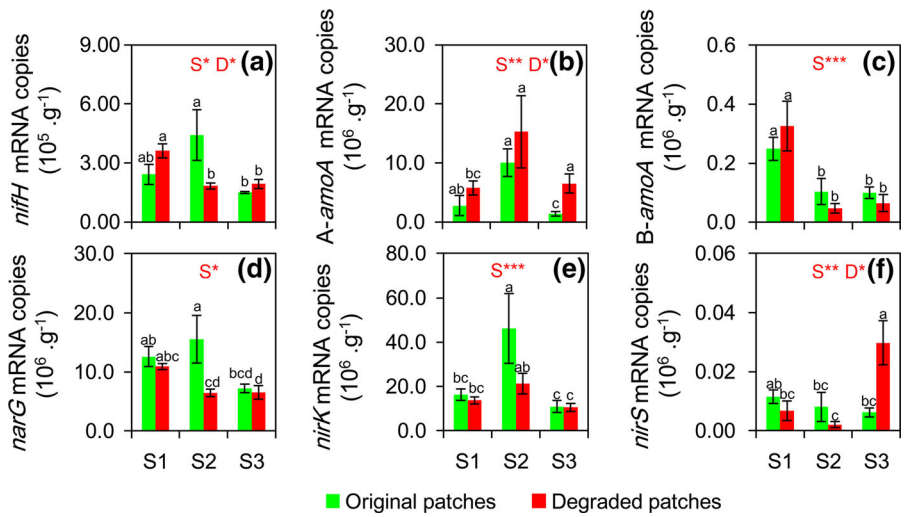

Fig. 4 Effects of the study site and degraded patch formation on soil nitrogen-cycling gene mRNA copies. *S1* Site 1, *S2* Site 2, *S3* Site 3, *S* effect of study site, *D* effect of degraded patch formation, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. All data were presented as the mean ± SE (n = 4); bars with different letters indicate significant differences across the study sites

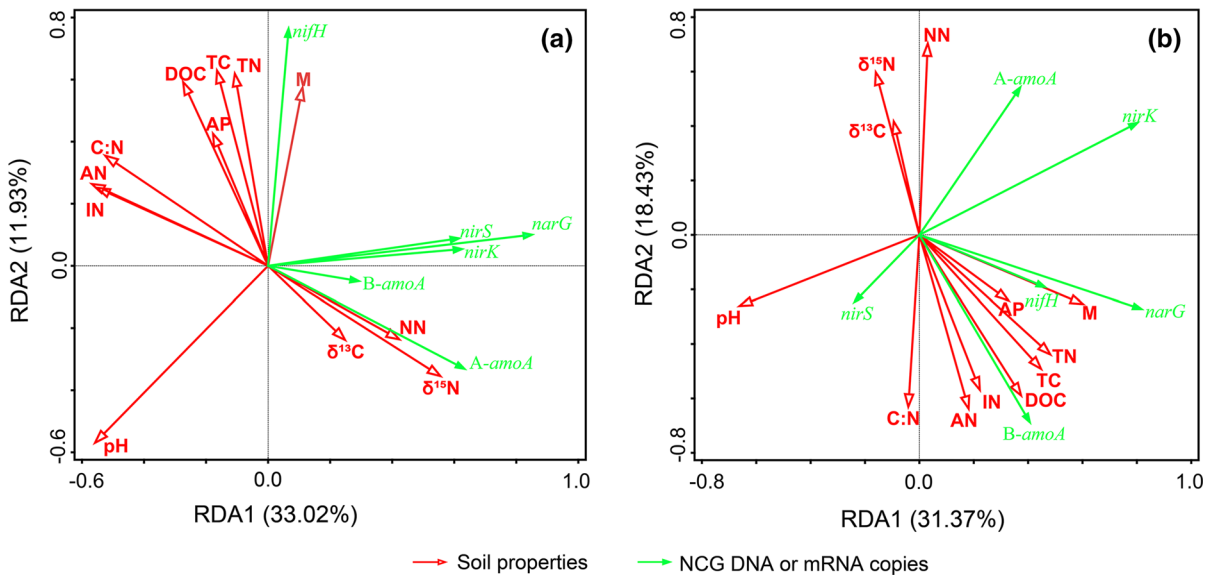

Fig. 5 Relationships between soil properties and DNA (**a**) or mRNA (**b**) copies of the nitrogen cycling genes. *TC* total carbon contents, *TN* total nitrogen contents, *C:N* ratio of total carbon to total nitrogen contents, *M* soil moisture contents, *AP* available phosphorus contents, *IN* inorganic nitrogen contents, *DOC* dissolved organic carbon contents, *AN* ammonium nitrogen contents, *NN* nitrate nitrogen contents, *A-amoA* archaeal *amoA*, *B-amoA* bacterial *amoA*. The ordinations were based on redundancy analysis

($P = 0.032$, Fig. 4b), especially at Site 3. However, the bacterial *amoA* mRNA copies remained unaffected by the patch formation, and showed a decreasing trend from Sites 1 to 3.

The degraded patch formation also tended to decrease *nifH* DNA copies, but this effect was not statistically significant ($P = 0.094$, Fig. 3a). In addition, transcriptions of the *nifH* and *narG* genes were significantly suppressed in the degraded patches at

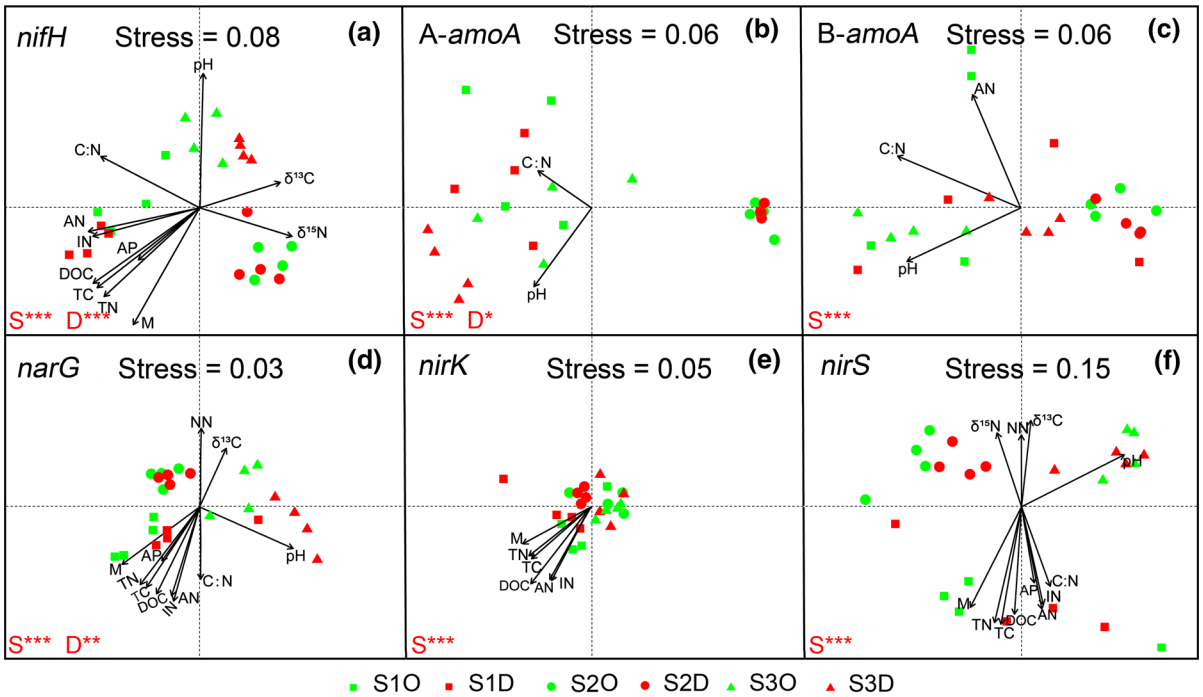

Fig. 6 NMDS ordinations of the DNA-based community structures of nitrogen cycling microbes. *S1O* original patches at Site 1, *S1D* degraded patches at Site 1, *S2O* original patches at Site 2, *S2D* degraded patches at Site 2, *S3O* original patches at Site 3, *S3D* degraded patches at Site 3, *TC* total carbon contents, *TN* total nitrogen contents, *C:N* the ratios of total carbon to total nitrogen, *M* soil moisture, *AP* available phosphorus contents, *IN* inorganic nitrogen contents, *DOC* dissolved organic carbon contents, *AN* ammonium nitrogen contents, *NN* nitrate nitrogen contents, *S* effect of study site, *D* effect of degraded patch formation, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Vectors represent the soil properties that were significantly correlated with the corresponding NCG community structures ($P < 0.05$); the directions of the vectors represent the increase in gradient of each soil property. All vectors were drawn using the envfit function in the vegan package

Site 2 (Fig. 4a, d), and the *nirS* transcription was significantly enhanced at Site 3 (Fig. 4f).

As revealed by the RDA ordinations, soil properties could be classified into three categories, with the first category including the contents of most soil nutrient indices (i.e., TN, TC, C:N, IN, AN, DOC, and AP) and soil moisture (Fig. 5). The second category included the soil NN contents and the values of $\delta^{13}C$ and $\delta^{15}N$ values, whereas the third category only included the soil pH values (Fig. 5). As determined by the ordinations, the first and second categories showed obviously negative correlations (Fig. 5).

Similarly, nitrogen cycling DNA copies also could be clustered into three groups corresponding to diazotrophs (*nifH*), nitrifiers (*amoA*), and denitrifiers (*narG*, *nirK*, and *nirS*). Diazotrophs were positively correlated with the first category of the soil properties, but they were negatively correlated with the other soil properties (Fig. 4a). Nitrifiers showed almost totally inverse correlations, and were negatively and positively correlated with the first and second categories of the soil properties, respectively (Fig. 5a). The denitrifiers were only negatively correlated with soil pH values and several soil properties in the first category (Fig. 5a).

Regarding the nitrogen cycling mRNAs, there were no obvious clusters in the RDA ordination plot (Fig. 5b). However, when compared with their DNA counterparts, the *nifH* and archaeal *amoA* mRNA copies still showed similar correlations with the soil properties (Fig. 5b). Conversely, the other nitrogen cycling mRNA copies (e.g., bacterial *amoA* mRNA copies) showed different or even completely inverse correlations with the soil properties, when compared with their DNA counterparts (Fig. 5b). The results of RDA also were confirmed by the Pearson correlation between soil NCMs and properties (Table S4).

The community structures of the nitrogen-cycling groups

As suggested by the NMDS and PERMANOVA, the community structures of all the nitrogen-cycling groups differed significantly among the three study sites (Table S5; Fig. 6). However, only the community structures based on the *nifH*, *narG*, and archaeal *amoA* genes were significantly altered by the degraded patch formation (Table S5; Fig. 6). As revealed by the envfit analysis, the variations in the community structures were significantly correlated with a series of environmental factors (Table S6; Fig. 6). In particular, the community structures based on the *nifH*, *narG*, and *nirS* genes showed significant correlations with almost all soil properties included in this study. In addition, the *nirK* community structures were significantly correlated with the soil TN, TC, DOC, AN, IN, and moisture contents. However, the nitrifier community structures showed much weaker correlations with the soil properties. The AOA community structure was only significantly correlated with the soil C:N ratios and pH values, whereas the AOB community structure only showed significant correlations with the soil C:N ratios, pH values, and AN contents.

Discussion

Decreased soil nitrogen contents under grassland degradation have been observed in a number of studies (Dong et al. 2012; Li et al. 2014; Wang et al. 2005, 2015a; Wen et al. 2013a). Our study found that, when compared with the adjacent original patches, the nitrogen contents in the degraded patches declined dramatically, and the nitrogen contents in the original patches also decreased from Sites 1 to 3 (Fig. 2). Thus, the formation of degraded patches might decrease not only the nitrogen contents within the bared patches but also the contents of their adjacent original patches through the "barren patch effect" (Han et al. 2011). The ^{15}N natural abundance is widely used to explore soil nitrogen dynamics (Almaraz and Porder 2016; Chalk et al. 2017; Hall et al. 2016; Wang et al. 2015b). As summarized by Wang et al. (2014), plant absorption and gas emissions from ammonia volatilization, nitrification, and denitrification tend to increase the $\delta^{15}N$ values of the remaining N, whereas nitrogen fixation and deposition usually decrease soil $\delta^{15}N$ values. However, leaching exerts only a slightly negative fractionation effect on soil ^{15}N values (Mnich and Houlton 2016). Thus, at Sites 1 and 2, the relatively high $\delta^{15}N$ values in the degraded patches may indicate an increased gas loss of nitrogen and (or) lowered biological nitrogen fixation in the degraded patches, whereas at Site 3, the almost identical $\delta^{15}N$ values in the degraded and original patches suggest that the lower nitrogen contents in the degraded patches may be mainly attributed to nitrogen leaching.

Another intriguing finding of this study is that although the degraded patch formation significantly decreased the soil nitrogen contents, it tended to increase the soil NN contents (Fig. 2). Both nitrate and soil particles carry negative charges. Because of the "mutual repulsion effect", nitrate is more susceptible to leaching from soil than ammonium (Di and Cameron 2002a; Kowalchuk and Stephen 2001). Furthermore, nitrate is the substrate of denitrification and therefore is prone to loss through gas emissions during denitrification (Levy-Booth et al. 2014). In this study, the soil nitrate contents were shown to be negatively correlated with soil TN, IN, and AN contents, whereas it was positively correlated with soil $\delta^{15}N$ values (Fig. 4). Therefore, the increased soil nitrate contents might also contribute to the decreased nitrogen contents under the degraded patch formation.

In the degraded patches, the accumulation of NN could be attributed to the potentially enhanced production and depressed consumption processes of nitrate. First, as soil nitrate is mainly derived from nitrification (Ollivier et al. 2011), the dramatically increased soil nitrifier abundance (Fig. 3b, c) and the upregulated transcription of the archaeal *amoA* gene (Fig. 4b) should play a vital role in the accumulation of NN in the degraded patches. Indeed, this is also supported by the significant correlations between the abundance and transcription of the *amoA* gene and NN contents (Fig. 5). Moreover, the lack of (or reduced number of) plants in the degraded patches could lower the plant assimilation of ammonium, thereby allowing more ammonium to be nitrified to nitrate. Similarly, the decrease in plant nitrate uptake also could contribute to the higher soil NN contents in the degraded patches. Among these potential causes, decreased consumption of nitrate should benefit the retention of soil nitrogen fertility, while the potential enhancement of nitrification could result in increased nitrogen loss. Moreover, because of the low

availability of nitrogen in the degraded patches, AOA should be more responsible for the nitrification than AOB (Prosser and Nicol 2012; Stahl and de la Torre 2012a).

According to the physiological properties of nitrifiers and nitrification, the increased nitrifier abundance and archaeal *amoA* mRNA copies in the degraded patches could be elicited in a number of ways. First, degraded patch formation significantly decreased the soil moisture and led to erosion and coarsening of surface soils (Figs. 1, 2i; He and Richards 2015; Lin et al. 2015), which might indirectly increase the soil aeration. In most cases, ammoxidation is an aerobic process (Zhalnina et al. 2012), and thus the increased soil aeration should promote soil nitrification. Second, as most AOA and AOB are chemoautotrophic (Kowalchuk and Stephen 2001; Zhalnina et al. 2012), the decreased soil DOC under degradation should increase their competitiveness and abundance. This is supported by the significantly negative correlations between soil DOC and the abundance of ammonia-oxidizing microbes in this study (Fig. 2a) and a number of other investigations (Bates et al. 2011; Wessen et al. 2010). Consistently, our unpublished data also showed that plant litter deprivation could significantly increase the abundance of ammonia oxidizing microbes. Third, as reviewed by Subbarao et al. (2015), roots of some plants can produce and release nitrification inhibitors; therefore, the elimination of vegetation in the degraded patches also should promote nitrification. Furthermore, the lack of (or reduced number of) plants in the degraded patches could result in lower plant–microbe competition for ammonium (Kuzyakov and Xu 2013), which supports the higher nitrifier abundance and the potentially enhanced nitrification. Although several other soil properties (e.g., soil available phosphorus contents) also showed significant correlations with AOA and AOB abundance, because of the paucity of theoretical supports, their contributions to the increases in nitrifier abundance are still difficult to justify.

On the basis of these findings, there are several potentially promising management practices to alleviate the accelerated nitrogen loss that occurs under degraded patch formation. First, grazing exclusion, pika control, and the establishment of artificial grassland can increase the carbon input from plants to soil (Chen et al. 2017; Dong et al. 2015; Li et al. 2013, 2014; Lu et al. 2015; Xiong et al. 2014), which can attenuate the competitive ability of nitrifiers, and thus lower nitrification and nitrogen loss risk. Second, application of nitrification inhibitors (e.g., 3, 4-dimethylpyrazole phosphate, dicyandiamide, and nitrapyrin) should be another potential measure, as they can directly inhibit soil nitrification (Di and Cameron 2002b; Zaman et al. 2008). However, the high cost of nitrification inhibitors may impede their current use in the restoration of degraded grassland. Accordingly, increased effort should be made to reduce the cost of nitrification inhibitors or incorporate plants capable of producing biogenic nitrification inhibitors into grassland restoration in the future.

In this study, we also examined the responses of diazotrophs and denitrifiers to degraded patch formation (Figs. 3, 4, and 6). However, the responses of the nitrogen-fixing and denitrifying gene copies and their transcriptions were generally weak and showed high inconsistency across the study sites (Figs. 3, 4; Table S3). This response could be elicited by the contrasting effects of the patch formation-induced variations. On one hand, the decreased soil IN and increased soil nitrate contents should benefit diazotrophs and denitrifiers, respectively (Che et al. 2017; Levy-Booth et al. 2014; Reed et al. 2011). On the other hand, the decrease in soil DOC, phosphorous, and other nutrient contents should negatively affect diazotrophs and denitrifiers (Levy-Booth et al. 2014; Reed et al. 2011). Nevertheless, the community structures based on *nifH* and *narG* genes were much more sensitive to the patch formation, and were significantly correlated with several different soil properties (Fig. 6; Table S5, S6), which deserves further exploration with more precise taxonomy techniques (e.g., MiSeq sequencing).

In conclusion, this study showed that degraded patch formation of the Tibetan alpine meadows significantly decreased most soil nutrient properties, while it tended to increase soil nitrate contents in heavily degraded alpine meadows. Among the NCMs included in this study, nitrifiers showed the highest sensitivity to the degraded patch formation. AOA and AOB abundance and archaeal *amoA* mRNA copies all significantly increased in the degraded patches. Moreover, *amoA* gene and archaeal *amoA* mRNA copies were positively correlated with soil nitrate nitrogen contents and $\delta^{15}N$ values, but negatively correlated with soil total and inorganic nitrogen contents. These results suggest that the dramatically increased

ammonia-oxidizing microbial abundance in the degraded patches may be a potentially important driver for the higher nitrogen loss in these patches. Therefore, grazing reduction, pika control, reseeding, and nitrification inhibitor applications could be potentially promising ways to alleviate nitrogen loss during degraded patch formation.

Acknowledgements This work was supported by the Strategic Priority Research Program (B) of the Chinese Academy of Sciences (XDB15010201), National Natural Science Foundation of China (41230750), National Basic Research Program (2013CB956000), and National Key Research and Development Program of China (2016YFC0501800). We sincerely appreciate the constructive suggestions from Prof. Zhongjun Jia and Dr. Yang Li regarding preparation of the manuscript. Rongxiao Che was also supported by a Griffith University Postgraduate Research Scholarship.

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