



# Litter amendment rather than phosphorus can dramatically change inorganic nitrogen pools in a degraded grassland soil by affecting nitrogen-cycling microbes

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## ABSTRACT

Phosphorus fertilisation and increasing litter input are widely employed to restore the degraded grasslands. Despite the key roles of nitrogen-cycling microbes in determining the soil nitrogen dynamics and development of grassland degradation, little is known about their responses to these restoration efforts. Here, a microcosm experiment, with soils collected from a degraded Tibetan alpine meadow, was conducted to investigate the responses of nitrogen-cycling microbes to litter and phosphorus amendments, and their links with the changes in soil properties. Copies of the corresponding nitrogen-cycling genes (*nifH*, *amoA*, *narG*, *nirK*, and *nirS* genes) and their mRNAs were determined using real-time PCR. The results showed that the litter amendment significantly stimulated the transcription of *nifH* and *nirS* genes, but reduced the copies of *amoA* gene and bacterial *amoA* mRNA. It also significantly increased soil dissolved organic carbon, available phosphorus, ammonium nitrogen, and microbial biomass concentrations, but decreased soil inorganic and nitrate nitrogen concentrations. The phosphorus amendment exerted little effects on soil properties and nitrogen-cycling microbes, while the litter-phosphorus interactions significantly offset the individual negative effects of the litter and phosphorus amendments on the denitrifier abundance. The soil nitrate and inorganic nitrogen concentrations were positively correlated with the *amoA* genes and bacterial *amoA* mRNA copies, but negatively correlated with *nirS* mRNA copies. These results indicate that litter addition may decrease soil nitrate and inorganic nitrogen concentrations by suppressing nitrifiers and stimulating *nirS* gene expression, highlighting the vital roles of nitrogen-cycling microbes in determining the soil nitrogen dynamics during the restoration of degraded grasslands.

## 1. Introduction

Due to climate change and human activities, 49.25% of the global grasslands have experienced degradation (Gang et al., 2014), which has dramatically decreased soil nutrient storage, pasture productivity, and livestock husbandry (Bai et al., 2008; Li et al., 2016b; Liu et al., 2018). Meanwhile, phosphorus (P) fertilisation (Reed et al., 2007; Smits et al., 2008) and measures of increasing organic matter input, such as fencing (Harris et al., 2015; Yang et al., 2017), reseeding (Feng et al., 2010), and organic matter amendments (Averett et al., 2004), have been widely employed to restore the degraded grasslands. Nitrogen (N), as the predominantly limiting factor for the primary productivity of terrestrial ecosystem (LeBauer and Treseder, 2008), is also one of the determinative factors for the restoration of degraded grassland. Although the changes in soil N contents during the aforementioned

restoration efforts have been observed in a number of studies, the underlying mechanisms are still poorly understood (Xiong et al., 2014; Dong et al., 2015; Qi et al., 2015). In natural ecosystems, soil N dynamics are mainly driven by a series of microbes (e.g., diazotrophs, nitrifiers, and denitrifiers) which are collectively described as N-cycling microbes (Levy-Booth et al., 2014). Therefore, examining the responses of N-cycling microbes to litter and P amendments can not only contribute to deepening the insights into the interactions among carbon (C), N, and P, but also provide essential guidelines for grassland management.

Currently, soil N-cycling microbes are extensively investigated using the molecular markers, such as *nifH*, *amoA*, *narG*, *nirK*, and *nirS* genes (Long et al., 2012b; Shen et al., 2014; Zhong et al., 2014). These genes code the enzyme subunits which are essential for the corresponding N-cycling processes (*nifH* gene for N-fixation; *amoA* gene for nitrification;

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and *narG*, *nirK*, and *nirS* genes for denitrification; Fig. S1). Their copy numbers are widely employed to indicate the N-cycling microbial abundance (Long et al., 2012a; Liu et al., 2017; Zhong et al., 2017). However, the measured N-cycling microbial abundances and corresponding N-cycling process rates frequently showed inconsistencies (Philippot et al., 2009; Attard et al., 2011; Liu et al., 2013a), mainly due to the high dormant proportion of microbes in soils (Lennon and Jones, 2011). Fortunately, the mRNA copies of the N-cycling genes can be used to study the transcription activity of the genes, showing strong correlations with the corresponding N-cycling process rates (Shi et al., 2010; Chen et al., 2015; Zhong et al., 2015). Simultaneous quantification of the copies of N-cycling genes and mRNAs, hence, can provide systematic insights into both the N-cycling microbial communities and the activities of corresponding N-cycling processes.

Theoretically, litter and P amendments can affect soil N-cycling microbial abundance and the expression of N-cycling genes in a number of ways. First, biological N fixation is a process with high energy consumption (Simpson and Burris, 1984), and thus increased litter, as an energy source, would increase soil N-fixer abundance and promote the expression of the *nifH* genes. Moreover, a large amount of P, in the form of adenosine triphosphate, is required by diazotrophs for biological N fixation (Hill, 1988; Vitousek et al., 2002; Reed et al., 2011). Therefore, in P-limited ecosystems, soil N-fixers and *nifH* gene expression would benefit from P amendment. Second, some nitrifiers, such as ammonia-oxidising bacteria and archaea (AOB and AOA), have been classified as oligotrophic lineages (Zhalnina et al., 2012; Kits et al., 2017), while most denitrifiers are copiotrophs (Levy-Booth et al., 2014; Morrissey and Franklin, 2015). Thus, litter addition could decrease the abundance of ammonia-oxidising microbes, indirectly affect the *amoA* gene expression, while increase denitrifier abundance. Furthermore, as the litter amendment could result in a more anaerobic environment by stimulating microbial respiration (Huang et al., 2004; Li et al., 2016a), the expression of denitrifying genes may also be stimulated. To date, the effects of organic matter and P applications on soil N-cycling microbes have been documented in a few publications, mainly focusing on the abundance and community structures of N-cycling microbes (Marcos et al., 2016; Tang et al., 2016; Mushinski et al., 2017; Xiang et al., 2017). Nevertheless, the expressions of N-cycling genes are usually overlooked. Therefore, examining the response of N-cycling gene expression to litter and P amendments of degraded soils would dramatically improve our understanding of the effects of the aforementioned restoration efforts on soil N pools.

The Tibetan Plateau, known as the “Third Pole” of the earth, has experienced serious degradation, due to overgrazing, pika population increase, and climate change in recent decades (Cui and Graf, 2009; Wang et al., 2016). The fertilisation and stoichiometry investigations suggested that the primary productivity of Tibetan grasslands is highly limited by soil N and P (Bing et al., 2016; Dong et al., 2016; Zhou et al., 2017). Moreover, the degradation of Tibetan grasslands dramatically decreased soil C, N, and P contents, and caused the erosion and even disappearance of mottic epipedons (Liu et al., 2018). Although N deposition rate has compensated a portion of the lost N, the total annual N deposition rate is still low (Liu et al., 2013b). In response, increasing litter input and fertilisation have been widely employed to restore the degraded Tibetan grassland (Cai et al., 2015; Dong et al., 2016). Our complimentary study has also proposed that increasing litter input and P fertilisation could alleviate soil N loss and improve the soil N fertility, based on the N-cycling microbial responses during Tibetan alpine meadow degradation (Che et al., 2017b). Collectively, exploring the effects of litter and P amendments on the abundance and expression of N-cycling genes in degraded Tibetan grassland soils would provide crucial guidelines for the restoration of the degraded grasslands.

In this study, we conducted a microcosm experiment, using the soils collected from a degraded Tibetan alpine meadow, to determine the effects of grass litter and P amendments on soil N-cycling microbial abundance and the transcription of N-cycling genes (i.e., *nifH*, archaeal

*amoA*, bacterial *amoA*, *narG*, *nirK*, and *nirS*), and to examine their relationships with soil properties under the treatments. Based on the existing findings, we hypothesized that: 1) litter amendment could significantly increase the DNA and mRNA copies of the N-fixing and denitrifying genes, but decrease the DNA and mRNA copies of the nitrifying genes; and 2) P amendment could significantly increase the DNA and mRNA copies of the N-fixing gene.

## 2. Materials and methods

### 2.1. Study sites, soil sampling, and litter collection

Soils and litters were separately sampled from a degraded (Fig. S2a) and a grazing-free (Fig. S2b) Tibetan alpine meadow near the Haibei Alpine Meadow Ecosystem Research Station (37° 37' N, 101° 12' E; 3200 m asl) which has been described in our previous publication (Che et al., 2018). Briefly, this region experiences a typical plateau continental climate, with mean annual temperature and precipitation of  $-1.7^{\circ}\text{C}$  and 570 mm, respectively (Zhao et al., 2006). The soil was identified as Gelic Cambisols (WRB, 1998). According to the criterion proposed by Lin et al. (2015), the degraded alpine meadow was at the fourth stage of degradation, but the erosion of the mottic epipedon was not serious (Fig. S2a). With average coverage of 65%, the plant community at the degraded alpine meadow was dominated by *Kobresia humilis* and *Leontopodium alpinum*. The grazing-free grassland was near the degraded alpine meadow, and was dominated by *Elymus nutans*, *Poa pratensis*, *Kobresia humilis*, *Festuca ovina*, and *Potentilla nivea* (Fig. S2b).

We randomly collected soil samples (0–10 cm; A horizon; C: 6.30%; N: 0.56%; P: 0.033%; and C/N/P atomic ratio of 495:38:1) from the degraded alpine meadow, using a steel auger with a 7 cm diameter, in July 2014. Then, the soils were homogenised, sieved to  $\leq 2\text{mm}$ , transported to the laboratory in an ice kit, and preserved at  $4^{\circ}\text{C}$  until the start of the incubation. The main characteristics of the soils were detailed in Table S1. The grass litter (C: 41.75%; N: 0.687%; P: 0.037%; and C/N/P atomic ratio of 2915:41:1) was collected from the grazing-free grassland, in December 2013. After the collection, the litter was dried at  $65^{\circ}\text{C}$  to constant weight, ball milled, and homogenised for the subsequent operations (Tahmasbian et al., 2017).

### 2.2. Experiment design and soil incubation

This microcosm experiment followed a two-way factorial design with four replicates for each treatment. The two experimental factors were P (0 or  $0.025\text{ g kg}^{-1}$  dry soil) and litter (0 or  $67\text{ g kg}^{-1}$  dry soil) amendments. The P amendment was achieved by applying calcium superphosphate solutions. Thus, there were four treatments (i.e., control, P amendment, litter amendment, and litter and P amendments) and sixteen microcosms in total. The litter amendment rate was based on the peak plant biomass in the grazing-free alpine meadow, and also in line with a previous study employing carbon amendment to restore the tallgrass prairie (Averett et al., 2004). The P application rate was selected according to the general field fertilisation rate (e.g., Wang et al., 2015), which was approximately equal to the total P contents added to soil through the litter amendment. This enabled us to compare the efficiency of applying chemical fertiliser and grass litter in improving soil P availability.

An aliquot of the field moist soil (36.25%, w/w; 37.73% water holding capacity) containing 30 g of dry mass was placed into each of the sixteen 130-ml flasks. They were pre-incubated in darkness, at  $25^{\circ}\text{C}$  for 14 days to stabilise the soil conditions. On the day 15 of the incubation, 3 mL of calcium superphosphate solution and 2 g of ground litter were added to the corresponding soils, respectively. After being homogenised, the flasks were continually incubated in darkness, at  $25^{\circ}\text{C}$  for 21 days. During the incubation, all the flasks were left open, and water was replenished every three days to keep soil moisture close to the field moisture. At the end of the incubation, soil samples were

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