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# Effects of winter grazing and N addition on soil phosphorus fractions in an alpine grassland on the Qinghai-Tibet Plateau

Zhen-Huan Guan<sup>a,1</sup>, Zuonan Cao<sup>b,c,1</sup>, Xiao Gang Li<sup>a</sup>, Peter Kühn<sup>b</sup>, Guozheng Hu<sup>c</sup>, Thomas Scholten<sup>b</sup>, Jianxiao Zhu<sup>a</sup>, Jin-Sheng He<sup>a,d,\*</sup>

<sup>a</sup> State Key Laboratory of Herbage Improvement and Grassland Agro-ecosystems, College of Pastoral Agriculture Science and Technology, and College of Ecology, Lanzhou University, Lanzhou 730000, China

<sup>b</sup> Department of Geosciences, Soil Science and Geomorphology, University of Tübingen, Tübingen 72070, Germany

<sup>c</sup> Institute of Environment and Sustainable Development in Agriculture, Chinese Academy of Agricultural Sciences, Beijing 100081, China

<sup>d</sup> Institute of Ecology, College of Urban and Environmental Sciences, Peking University, Beijing 100871, China

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# ABSTRACT

Nutrient cycling in alpine grasslands is susceptible to climate change and anthropogenic activities, which can affect soil phosphorus (P) availability. Despite the crucial role of soil P availability in maintaining stability and productivity of grassland ecosystems, limited research has been conducted on the effects of nitrogen (N) addition and winter grazing on P transformation on the Qinghai-Tibet Plateau. In an 8-year experiment, we applied four different N addition rates (0, 25, 50, and 100 kg urea ha<sup>-1</sup> year<sup>-1</sup>) in combination with winter grazing to investigate the effects of N addition and winter grazing on the soil P fractions. The results reveal that increasing the N addition gradually reduced the resin-Pi and NaOH-Pi contents in the soil by increasing the plant P uptake and promoting the release of carboxylates in the rhizosheath, regardless of grazing. Winter grazing decreased the NaHCO3-Pi and NaOH-Pi contents compared with the no-grazing treatment by increasing the P uptake of the plants. In contrast, neither grazing nor N addition affected the HCl<sub>conc</sub>.-P or residual-P content. In the no-grazing plots, the soil NaHCO3-Po content exhibited a gradual increase in response to N addition, whereas N addition had no discernible effect on the NaOH-Po content. In the grazing plots, the NaHCO3- and NaOH-Po contents gradually decreased with N addition, which was associated with the increased acid phosphatase activity in the rhizosheath and the export of forage. Thus, we conclude that N addition promotes the dissolution of NaOH-Pi to more available inorganic P forms. Under winter grazing conditions only, the transformation of P from inorganic to organic forms gradually decreased with increasing N additions.

# 1. Introduction

The alpine grasslands on the Qinghai-Tibet Plateau (QTP) play a crucial role in maintaining global ecological stability and providing livelihoods for local pastoral communities (Li et al., 2022). The ecological stability of grasslands is a determining factor in the provisioning of ecosystem services, which are under threat from both climate change and intensive management (Li et al., 2020). To facilitate the sustainable development of grassland productivity, the Chinese government has introduced measures such as alternating winter and summer grazing and erecting fences since the 1990 s (Qiu, 2016).

Additionally, limited soil phosphorus (P) availability (less than 3% of the total P) poses a significant threat to grassland productivity (Rui et al., 2011; Mou et al., 2020; Zhou et al., 2021). While numerous studies have suggested that nitrogen (N), rather than P, is the primary factor limiting grass productivity in most areas of the plateau (Gao et al., 2018; Han et al., 2019; Wang et al., 2022a), it appears that plant productivity may have shifted from being limited by N to being limited by P due to increasing N accumulation in the last decade (Li et al., 2016; Zhao and Zeng, 2019; He et al., 2020a; Chen et al., 2021). Hence, the availability of soil P is a pivotal factor that impacts both the stability and productivity of grassland ecosystems.

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<sup>\*</sup> Corresponding author at: State Key Laboratory of Herbage Improvement and Grassland Agro-ecosystems, College of Pastoral Agriculture Science and Technology, and College of Ecology, Lanzhou University, Lanzhou 730000, China.

E-mail address: jshe@pku.edu.cn (J.-S. He).

<sup>&</sup>lt;sup>1</sup> These authors contributed equally to the work and should be considered co-first authors.

Plants acquire available inorganic P (Pi) from the soil solution, while organic P (Po) is reintroduced into the soil via decomposition of plant residues. These organic materials then undergo mineralization to yield Pi, which constitutes the primary process of the biochemical P cycle (Lambers, 2022; Lambers et al., 2006). Both Po and Pi can be easily immobilized by soil minerals, resulting in their availability to plants and microbes being partitioned into labile (extracted using resin and NaHCO<sub>3</sub>), moderately labile (extracted using NaOH and HCl), and stable (extracted using concentrated HCl and H2SO4) forms (Hedley et al., 1982; Hou et al., 2018). Even the plant-available P only accounts for a small fraction of the total P in soil (less than 5%) and it could be continuously supplemented with more stable P fractions to meet plant needs (Niederberger et al., 2019). The mineralization of Po by microorganisms and plant roots that secrete phosphatase to degrade Po provides the primary source of plant-available P (Turner et al., 2013; Shen et al., 2011). In addition, a decrease in the soil pH and biological solubilization via the secretion of carboxylates (e.g., gluconate, citrate, oxalate, and acetate) by plants and microorganisms is an important factor in promoting P mobilization (Bünemann, 2015; Liu et al., 2022). Understanding the different forms of P, particularly the transition from stable to labile P fractions, is essential for regulating soil P dynamics and thus the P availability to plants (Yang and Post, 2011; Fan et al., 2019).

Furthermore, N enrichment, such as N deposition, has been shown to enhance P mobilization by facilitating plant P acquisition from soil P pools (Schleuss et al., 2020; Wang et al., 2022a). N also stimulates the production of extracellular phosphatase enzymes, which in turn facilitate the liberation of Pi from organic reservoirs (Lu et al., 2012; Schaap et al., 2021). Moreover, N accumulation in ammonium compounds can indirectly lead to soil acidification, which enhances the dissolution of refractory mineral-bound P into moderately labile and labile P fractions (Fu and Shen, 2017; Fan et al., 2019). Therefore, the enrichment of N, plays a supporting role in all aspects of the P cycle. Despite decades of the N supply being sufficient for plant production, as recent meta-analyses have shown, many ecosystems remain limited by N without a noticeable shift toward P limitation (Chen et al., 2020; Wang et al., 2022a). This observation could be explained by the fact that the amount of P mobilized by the N supply is greater than the amount of immobilized P (Wang et al., 2022a; Xiao et al., 2022). The alpine grasslands on the QTP, which have a low mean annual temperature, have a very low rate of Po mineralization in the soil (Rui et al., 2011; Zhou et al., 2021). Additionally, soils in alpine grasslands that are rich in base cations ( $Ca^{2+}$ ,  $Mg^{2+}$ , and  $K^+$ ) may limit P mobilization by stabilizing the soil pH (Tian and Niu, 2015). Therefore, N enrichment in alpine grasslands is likely to promote P immobilization rather than P mobilization.

Continuous year-round grazing increase P export from the soil through forage and livestock products (Chaneton et al., 1996; Wu et al., 2009), and decreases the P return to the soil by reducing the amount of litter and inhibiting decomposition of aboveground litter (Bardgett and Wardle, 2003), resulting in depletion of the soil P pool (Yao et al., 2019). There are also mate-analyses suggesting that grazing can promote rock weathering to increase the soil P pool (He et al., 2020b), but negative effect on soil P mobilization (Yu et al., 2021). Although returning dung and urine to the soil may increase soil P mobilization in the short term, the available P readily accumulates in the topsoil layer due to soil adsorption, and the increase in the soil bulk density caused by trampling causes poor migration to the deeper soil layers (Baron et al., 2001; Wu et al., 2020), which also contributes to the loss of these soil nutrients through run-off, leaching, and transformation by livestock (Vadas et al., 2015). Studies have also shown that grazing negatively affects both the aboveground and belowground biomass (Bai et al., 2012; Bai et al., 2015), alters the structure and composition of the plant community, and reduces the plant's P stock (Li et al., 2010; Bai et al., 2012; Davidson et al., 2017). In contrast to year-round grazing, winter grazing does not interrupt plant growth during the growing season, and has little negative impact on grassland ecosystems (Shi et al., 2017), while avoiding

changes in the plant community composition and degradation of dominant plant species caused by the selective foraging behavior of livestock (Johnson and Sandercock, 2010). In addition, the consumption of aboveground litter by livestock provides abundant solar radiation and spatial resources for plant growth the following year, resulting in an increase in the aboveground plant biomass (Chen et al., 2007; Giese et al., 2013; Shi et al., 2022), which in turn may contribute to the transfer of P from the soil to the plants. In winter, especially on the QTP—which is famous for its cold and windy conditions—the grazing duration is short, and the grazing intensity is high, which is not conducive to the return of dung and urine, and the frozen soil can effectively prevent an increase in the capacity caused by trampling. The inference can be drawn that winter grazing may be less favorable for plant P to return to the soil compared to the year-round grazing.

In alpine grasslands, both N addition and winter grazing may enhance P uptake by plants and increase opportunities for P limitation of plants. Overall, plants may intensify their own P-acquisition strategies in response to P limitation, such as changes in the root morphology, increases in root exudates, and increased levels of symbiosis with arbuscular mycorrhizal fungi (AMF) to mobilize stable P fractions into more available forms (He et al., 2020a; Lambers et al., 2022; Luo et al., 2022). However, the independent effects of gradual N enrichment and winter grazing on the soil P fractions and their interactions are still unclear. As such, it is unclear how alpine grassland plant communities on the QTP adjust their P-acquisition strategies to mobilize and take up P in response to the N supply and winter grazing. To better understand the effects of the N supply and grazing on soil P fractions, we make the following hypotheses:

- The plant P demand gradually increases as the level of the N supply increases, leading to an adjustment of the plant's P-acquisition strategy and promoting the transformation of occluded P to moderately labile and labile P pools, ultimately resulting in an increase in Po accumulation in the soil.
- 2) Winter grazing could facilitate P uptake by plants, resulting in more labile Pi in the soil being converted to plant P, less P returning to the soil, and plant P-acquisition strategies adapting to promote P mobilization and uptake.
- 3) Compared to the no-grazing plots, in the grazing plots, Po accumulation in the soil decreases as the N addition rate increases.

The present study was based on an 8-year field experiment that involved N fertilization and a winter grazing treatment. This experiment was conducted in an alpine grassland on the QTP (Ren et al., 2017). Using a modified Hedley P fractionation procedure (Tissen and Moir, 2007), in conjunction with analyses of plant and microbial P and plant P-acquisition strategies, our objective was to characterize the P content and P transformation within the uppermost 30 cm of the soil.

# 2. Materials and methods

# 2.1. Site description and experimental design

The field experiment was carried out at the Haibei National Field Research Station of Alpine Grassland Ecosystem (at  $37^{\circ}37'$  N,  $101^{\circ}19'$  E) of the Chinese Academy of Sciences, located on the northeast part of the QTP at 3250 m above sea level. This region has a monsoon continental climate, with a mean annual temperature of -1.7 °C, a January mean monthly temperature of -14.8 °C, a July mean temperature of 9.8 °C, and an average annual precipitation of 489.0 mm, with most of the precipitation falling from May to September (Liu et al., 2018). The soil is classified as *Mat-Cryic Cambisol* according to the Chinese Soil Taxonomy Research Group (1995) or *Gelic Cambisol* according to the World Reference Base for Soil Resources classification (2015), and the site is dominated by *Kobresia humilis, Stipa aliena, Elymus nutans*, and *Festuca ovina*. Starting in 2011, N fertilizers were applied to  $6 \times 6$  m plots at the soil surface after sunset during the growing season at the beginning of June, July, and August. Four different N-addition rates (control, N25, N50, and N100, corresponding to 25, 50, and 100 kg (NH<sub>2</sub>)<sub>2</sub>CO ha<sup>-1</sup> yr<sup>-1</sup>) were studied. A fully randomized experimental design was adopted with six replicates of each treatment (Fig. S1).

The winter grazing experiment was conducted based on local conditions since 2011. Starting with the fertilized plots mentioned above, the  $6 \times 6$  m plots were divided into two subplots, the first of which was grazed in winter ( $3 \times 6$  m) and the second of which was fenced ( $3 \times 6$ m). Tibetan sheep (*Ovis aries*) grazed in the subplots continuously from December to April each year after plant dieback, and the grazing intensity was two sheep per subplot. Depending on the local conditions, winter grazing completed for the year, when 75% of the surface litter and standing dead material had been eaten. The no-grazing subplots had no sheep grazing for 8 years.

# 2.1.1. Soil sampling

Soil samples were collected at depth of 0–10 cm (topsoil, covering the A horizon) and 10–20 and 20–30 cm depths (subsoil). Five soil samples from each subplot and depth were collected at random from six replicate blocks using a soil auger (Ø 50 mm) and were combined into one composite sample. The sampling was conducted in August 2018, and the samples were packed in polyethylene bags and stored in an ice box prior to shipment to the laboratory. The soils were then sieved (< 2 mm), and all of the visible roots, residues, and stones were removed. An aliquot of each soil sample (10 g) was removed aseptically and stored at – 80 °C for the purpose of microbial analysis (phospholipid fatty acids [PLFA]). An additional portion was stored at 4 °C for analysis of the soil microbial biomass P (MBP), carbon (MBC), and nitrogen (MBN). The remaining material was air dried at 60 °C for further analyses of the soil properties.

# 2.1.2. Vegetation sampling

The vegetation communities were surveyed in mid-August 2018, the time of the peak biomass. Three species richness surveys, covering 1 m<sup>2</sup> were conducted in each plot. Plants were harvested from a  $0.5 \times 0.5$  m square chosen at random within each plot. Shoots (leaves and stems) were cut, and litter was collected with scissors at the base and sorted to measure the aboveground biomass. To measure the plant C, N, and P concentrations, we pooled all the plant species within the square and combined them into a mixed community sample.

# 2.1.3. Rhizosheath soil and root sampling

The study of root systems was particularly susceptible to undersampling errors due to the high spatial heterogeneity and non-normal distribution of root diameters in soil (Taylor et al., 2013). Rhizosheath soil and soil cores from root samples (0-10 cm) were collected from each plot using a root auger (Ø 70 mm; Mou et al., 2020). The roots in the soil were carefully separated from the soil and transferred to a beaker containing 0.2 mM CaCl<sub>2</sub> (Pearse et al., 2007). The roots were repeatedly immersed in the solution until all the rhizosheath soil was removed. For the measurements of the activities of the acid phosphatase (ACP) and alkaline phosphatase (ALP), two 0.5 mL aliquots of the soil suspension (sample and control) were transferred to 2 mL centrifuge tubes for analysis (Neumann and Römheld, 1999). Two drops of concentrated phosphoric acid were added to 7-8 mL subsamples before storage at -20 °C until analysis of the carboxylates via high-performance liquid chromatography (Wen et al., 2019). After analysis of the rhizosheath exudates, the soaked roots were harvested and washed with tap water, placed in paper bags, and kept at 4 °C before assessing the C, N, and P contents, the root biomass and morphology, and the colonization by AMF.

# 2.2. Laboratory measurements

# 2.2.1. Soil properties and plant nutrients

The soil pH was measured in deionized water in a 1:5 soil: water suspension using air-dried water after shaking for 30 min, and the soil bulk density was determined using the mass per volume method (with 100 cm<sup>3</sup> for the entire core). The soil organic carbon (SOC), total nitrogen (TN), and plant C and N contents were measured using an elemental analyzer (Elementar Vario EL III, Elementar Analysensysteme GmbH, Langenselbold, Germany). The plant P concentrations (shoot, aboveground litter, and roots) were determined colorimetrically after digestion in an HClO<sub>4</sub>-H<sub>2</sub>SO<sub>4</sub> mixture, and the P stocks were calculated by multiplying the biomass by the P concentrations. The amount of soil available N (KCl-extractable ammonium  $[NH_4^+]$  and nitrate  $[NO_3^-]$ ) was determined using a continuous flow analyzer (Skalar San, Breda, Netherlands). The exchangeable cations ( $Ca^{2+}$  and  $Mg^{2+}$ ) were extracted using 1 M NH4OAc and were measured via atomic absorption spectroscopy (Ngewoh et al., 1989). Consistent with the regular Hedley sequential extraction of P (Hedley et al., 1982), which was later modified by Tissen and Moir (2007), Pätzold et al. (2013), and Niederberger et al., 2015, 0.5 g of each soil sample, which was air-dried and sieved, was dissolved. An anion-exchange resin bag containing 0.5 M HCl was used to measure the labile resin-Pi directly from the soil solution, followed by extraction with 0.5 M NaHCO<sub>3</sub> (pH 8.5) to obtain the relatively labile plant-available Pi and readily mineralized Po. Furthermore, 0.1 M NaOH was used to extract the soil residue for the moderately labile Pi, which covered the Pi associated with Fe and Al oxides as well as the Po involved in the slow transformation processes. However, in the presence of concentrated HCl, the HClconc. -P fraction of the highly stable Pi in the primary minerals at 85 °C was applied instead of the fraction extracted using 1 M HCl in the traditional Hedley method, which was found to be negligibly small (Tissen and Moir, 2007; Alt et al., 2011). For the highly resistant and occluded Pi forms, 0.5 M H<sub>2</sub>SO<sub>4</sub> was used to extract the residual P (Table S1). All of the Pi fractions were determined as described above using continuous-flow-analysis (SEAL Auto Analyzer AA3, Analytical GmbH, Norderstedt, Germany), and the total NaHCO3-P and NaOH-P fractions were determined via inductively coupled plasma-optical emission spectrometry (Optima 5300 DV, PerkinElmer, Waltham, MA, USA). The concentrations of the Po fractions were calculated as the difference between the total fractions (NaHCO3- and NaOH-P) and the Pi fractions. The concentrations of different P pools in the soil were calculated on a soil dry weight basis (mg kg<sup>-1</sup>) and then converted to an area basis (g  $m^{-2}$ ) based on the bulk densities.

# 2.2.2. Root morphology

Fresh root samples were washed under running water, spread in water with minimal overlap, and digitized on an Epson Expression 10,000 XL desktop scanner (resolution, 300 dpi). The root images were analyzed using the WinRHIZO software (Regent Instruments, Quebec, QC, Canada) to determine the average diameter and total length of the roots.

### 2.2.3. Root exudates

The inorganic fractions in all of the extracts were determined after filtration using the ammonium molybdate spectrophotometric method at 700 nm. These contents were calculated based on the differences in the inorganic fractions extracted from fumigated and non-fumigated samples (Khan and Joergensen, 2012). Analysis of the amount of rhizosheath carboxylates was carried out using a Waters 1525 HPLC equipped with a Waters 2489 detector and a Waters Symmetry C<sup>18</sup> reverse-phase column (Waters, Milford, MA, USA). The mobile phase was prepared by adjusting 20 M KH<sub>2</sub>PO<sub>4</sub> to a pH of 2.5 using concentrated H<sub>3</sub>PO<sub>4</sub> at a flow rate of 0.6 mL min<sup>-1</sup> and 100% methanol at a concentration of 0.01 mL min<sup>-1</sup>. The following work standards were applied to identify the carboxylates at 210 nm: oxalate, tartrate, formate, malate, malonate, lactate, acetate, citrate, succinate, and propionate were used (Cawthray, 2003). The carboxylates content was given as total content per unit root dry mass (rdm).

The phosphatase activity was determined via colorimetric analysis based on the amount of p-nitrophenol liberated from the soil suspension during incubation with p-nitrophenyl phosphate (PNPP) in a buffer of either pH 6.5 (ACP) or pH 11 (ALP) for 1 h at 37 °C (Hopkins et al., 2008). The 0.5 mL aliquots of soil suspension with an oven-dried weight of 1 g were transferred into 2 mL centrifuge tubes, treated with 0.4 mL of Na-Ac buffer and 0.1 mL of substrate solution (PNPP), and incubated. At the end of the incubation, 0.5 mL of 0.5 M NaOH was added to the slurries to halt the enzymatic reactions. The absorbance of the enzyme extracts was monitored at 410 nm (UV-1800, MAPADA, Shanghai, China) immediately after the filtration process was completed. The carboxylate content of the rhizosheath and phosphatase activity was expressed in mmol per unit of dry root weight.

# 2.2.4. AMF colonization

The root samples were cleaned with a 10% (w/v) solution of KOH in a water bath at 90 °C for 20 min, rinsed with water, and then acidified with 2% (v/v) HCl for 5 min at 18 °C (Zhang et al., 2023). Samples were collected from the roots, placed in a water bath, and stained with Trypan blue at 0.05% (w/v) nonvital in a water bath at 90 °C for 30 min (Phillips and Hayman, 1970). The stained root fragments were placed in an acid-glycerol-water lactide solution (v/v/v, 1: 1: 1) overnight to remove the excess stain. For each sample, 30 stained root fragments from first and second order roots with an average length of 1 cm were randomly selected from each sample and were mounted on two slides for visualization under a light microscope (BX63, Olympus Corporation, Tokyo, Japan). The colonization by AMF (%) was evaluated according to the method outlined by Trouvelot et al. (1986).

# 2.2.5. Microbial biomass nutrients and community structure

The MBP, MBC, and MBN were analyzed using a chloroform fumigation-extraction procedure (Vance et al., 1987; Morel et al., 1996). The soil microbial PLFAs were determined using a modified Bligh-Dyer method (Bligh and Dyer, 1959; Frostegård et al., 1991). The fatty acids extracted from a 10 g soil sample using were chloroform-methanol-citric acid buffer (Kourtev et al., 2002). The lipid in the concentrated extract was separated on solid-phase extraction cartridges and fractionated into neutral, glycolipid, and polar fractions. After elution with methanol, the polar lipid fraction was dried under nitrogen gas, saponified and methylated. Specific fatty acids were used as a proxy for the biomass of the AMF (Olsson et al., 1998). The individual fatty acid methyl esters were identified and quantified using an MIDI Sherlock Microbial Identification System (MIDI, Newark, DE, USA). The 16:105c content represents the AMF fatty acid fraction. Finally, the contents of the samples were converted to units of nmol per g of soil sample based on the water content.

# 2.3. Statistical analysis

Two-way analysis of variance (ANOVA) with the least significant difference (LSD) test for post hoc means comparison was used to test the effects of N addition, grazing, and their interactions on the soil parameters, P fractions, plant growth parameters, and P-acquisition strategy indicators in the field experiment. The SPSS 25.0 software (IBM SPSS, Chicago, IL, USA) was used to conduct the above analyses, and differences were significant at p < 0.05.

# 3. Results

# 3.1. Soil biochemical properties and plant communities

No significant differences in the soil properties were observed within the depth range of 10-30 cm; thus, the subsequent findings exclusively focus on the 0-10 cm soil depth interval. After 8 years of treatment, the

N application increased the NO<sub>3</sub>-N content but reduced the MBC and MBN contents compared with the no N addition treatment, regardless of whether the plots were grazed (Tables 1 and 2). Furthermore, neither N addition nor grazing altered the plant community composition and richness (Table S2).

# 3.2. Soil phosphorus fractions

The resin-Pi content decreased with increasing N addition, regardless of the grazing condition (Fig. 1a; Tables S3 and S4), and the NaHCO<sub>3</sub>-Pi content decreased with grazing and was unaffected by N addition (Fig. 1b; Tables S3 and S4). N addition or grazing decreased the NaOH-Pi content (Fig. 1c; Tables S3 and S4). The NaHCO<sub>3</sub>-Po and NaOH-Po contents decreased with increasing N addition in combination with grazing; whereas, the NaHCO<sub>3</sub>-Po content increased with N addition without grazing (Fig. 1e, f; Tables S3 and S4). The proportion of the Po content in the total P decreased with increasing N addition in the grazing plots (Fig. 2a; Table S3), and the changes in the Po pool exhibited the same trend (Fig. 2b). Grazing decreased the soil total P content compared with no grazing (Fig. 1h; Table S3). Neither grazing nor N addition affected the contents of HCl<sub>conc</sub>-P and residual-P and all the P fractions at depths of 10–30 cm (Fig. 1d and g; Table S4).

# 3.3. Plant and microbial phosphorus content

N addition or grazing increased the shoot P stock (p < 0.05; Fig. 3a; Table S3). Grazing reduced the aboveground litter biomass and litter P stock (Fig. 3b; Table S3). The P stock in the plant roots remained stable in all the treatments (Fig. 3c), and N addition elevated the N: P ratio of the shoots compared to the treatment with no N supply (Fig. 3d; Table S3). In addition, in the plots with N addition, a decrease in the MBP pool was observed in the soil regardless of the grazing condition (Fig. 4a; Table S3); whereas, the ALP and ACP activities did not change in the soil (Fig. 4b and c; Table S3).

#### 3.4. Plant P acquisition strategy in the rhizosheath

The ACP activity in the rhizosheath and the carboxylate release increased with increasing N supply (Fig. 5a, b; Table S3). The significant interaction between the N application and grazing affected the ACP activity (Fig. 5a; Table S3). Notably, the AMF colonization decreased with increasing N addition, except in the N100 treatment, where the AMF colonization was the highest among the treatments (Fig. 5c; Table S3). Neither N addition nor grazing affected the root length density, root diameter, or PLFA content (Table S5).

# 3.5. Relationships between plant and microbial factors and soil phosphorus fractions

Principal component analysis (PCA) revealed that in the no-grazing plots, the two principal components explained 72.73% of the total data variance (Fig. 6a). The resin-Pi and residual-P pools were strongly correlated with PC 1 (42.87%), while the NaHCO<sub>3</sub>-Po, HCl<sub>conc.</sub> -P and NaOH-Pi fractions were strongly correlated with PC 2 (29.89%). The plant shoot and litter P pools were positively correlated with the rhizosheath ACP activity and carboxylate release. For the grazing treatments, the components explained 83.07% of the variance (Fig. 6b). Positive correlations were found between the shoot P pool and plant P acquisition strategies (rhizosheath ACP, carboxylate, and AMF colonization), and the root P pool was positively correlated with the soil Pi pools.

# 4. Discussion

Winter grazing is a common livestock management practice on the QTP, and N enrichment is a prominent environmental issue, but their

Table 1

Effects of nitrogen (N) addition rate and grazing (G) on soil biochemical properties in the 0-10 cm depth interval.

Treatment pH		рН	SOC (g kg <sup>-1</sup> )	TN (g kg <sup>-1</sup> )	MBC (mg kg <sup>-1</sup> )	MBN (mg kg <sup>-1</sup> )	NH <sub>4</sub> <sup>+</sup> -N (mg kg <sup>-1</sup> )	NO <sub>3</sub> -N (mg kg <sup>-1</sup> )	EX. $Mg^{2+}$ (mg $g^{-1}$ )	EX. $Ca^{2+}$ (mg g <sup>-1</sup> )	Soil bulk density (g cm <sup>-3</sup> )
NG	Ctrl	$\begin{array}{c} \textbf{7.47} \pm \\ \textbf{0.08} \end{array}$	$\begin{array}{c} 61.9 \pm \\ 5.21 \end{array}$	$\begin{array}{c} \textbf{8.80} \pm \\ \textbf{0.26} \end{array}$	$\begin{array}{c} 1366 \pm \\ 3.24 \end{array}$	$257 \pm 4.04$	$\textbf{0.63} \pm \textbf{0.04}$	$2.01\pm0.42$	$0.58\pm0.02$	$16.82\pm0.80$	$0.71\pm0.02$
	N <sub>25</sub>	$\begin{array}{c} \textbf{7.40} \pm \\ \textbf{0.10} \end{array}$	$\begin{array}{c} 61.4 \pm \\ 4.92 \end{array}$	$\begin{array}{c} \textbf{8.44} \pm \\ \textbf{0.19} \end{array}$	$\begin{array}{c} 1172 \pm \\ 35.8 \end{array}$	$253 \pm 11.7$	$\textbf{0.80} \pm \textbf{0.15}$	$\textbf{5.16} \pm \textbf{1.87}$	$0.53\pm0.03$	$14.36\pm1.22$	$0.71\pm0.02$
	N <sub>50</sub>	$\begin{array}{c} \textbf{7.46} \pm \\ \textbf{0.10} \end{array}$	$\begin{array}{c} 66.1 \pm \\ 1.36 \end{array}$	$\begin{array}{c} 8.65 \pm \\ 0.54 \end{array}$	$\textbf{998} \pm \textbf{12.3}$	$227 \pm 1.34$	$\textbf{0.97} \pm \textbf{0.20}$	$\textbf{2.63} \pm \textbf{0.52}$	$0.47 \pm 0.02$	$13.46\pm0.24$	$0.71\pm0.02$
	N100	$7.53~\pm$ 0.14	$\begin{array}{c} 67.2 \pm \\ 0.95 \end{array}$	$\begin{array}{c}\textbf{8.74} \pm \\ \textbf{0.48} \end{array}$	$925\pm3.94$	$172\pm3.22$	$\textbf{0.80} \pm \textbf{0.15}$	$\textbf{2.88} \pm \textbf{0.86}$	$0.41\pm0.03$	$12.03\pm0.28$	$0.70\pm0.03$
G	Ctrl	$\begin{array}{c} \textbf{7.37} \pm \\ \textbf{0.12} \end{array}$	$\begin{array}{c} \textbf{67.4} \pm \\ \textbf{0.87} \end{array}$	$\begin{array}{c} \textbf{8.18} \pm \\ \textbf{0.50} \end{array}$	$1361 \pm 8.66$	$\textbf{263} \pm \textbf{8.98}$	$\textbf{0.67} \pm \textbf{0.04}$	$1.16\pm0.14$	$0.58\pm0.04$	$16.15\pm0.61$	$0.71\pm0.01$
	N <sub>25</sub>	$\begin{array}{c} \textbf{7.35} \pm \\ \textbf{0.05} \end{array}$	$\begin{array}{c} 66.6 \pm \\ 0.42 \end{array}$	$9.01 \pm 0.87$	$\begin{array}{c} 1109 \pm \\ 83.0 \end{array}$	$214\pm4.44$	$\textbf{0.74} \pm \textbf{0.05}$	$\textbf{4.04} \pm \textbf{0.94}$	$0.56\pm0.04$	$13.80\pm0.83$	$\textbf{0.70} \pm \textbf{0.04}$
	N <sub>50</sub>	$\begin{array}{c} \textbf{7.42} \pm \\ \textbf{0.02} \end{array}$	$\begin{array}{c} 65.9 \pm \\ 0.99 \end{array}$	$8.26 \pm 0.47$	$1106 \pm 22.7$	$228 \pm 17.3$	$\textbf{0.79} \pm \textbf{0.06}$	$\textbf{3.14} \pm \textbf{0.39}$	$0.45\pm0.03$	$15.10\pm0.86$	$0.72\pm0.03$
	N100	$\begin{array}{c} \textbf{7.61} \pm \\ \textbf{0.05} \end{array}$	$\begin{array}{c} 66.4 \pm \\ 1.35 \end{array}$	$\begin{array}{c} 8.63 \pm \\ 0.36 \end{array}$	$939 \pm 27.2$	$168\pm4.63$	$\textbf{0.73} \pm \textbf{0.04}$	$\textbf{3.20} \pm \textbf{0.37}$	$\textbf{0.36} \pm \textbf{0.04}$	$11.40\pm0.89$	$0.71 \pm 0.01$

The pH, soil organic carbon (SOC), total nitrogen (TN), microbial biomass carbon (MBC), microbial biomass nitrogen (MBN), soil nitrate and ammonium (NO3 -\_N and NH4 +\_N), soil exchangeable Mg2 + and Ca2 +, and soil bulk density were recorded. Ctrl indicates the treatment with no N addition, and N addition indicates the treatments with three levels of N fertilizer; G: treatment with grazing; NG: treatment with no grazing. The data are presented as the mean ( $\pm$  SE) of six replicates.

Table 2 Summary of two-way ANOVA results for the effects of N addition and grazing on soil factors.

Variable	Two-way A	ANOVA	Effect of N addition				
	N addition	Grazing	$\frac{N\times}{Grazing}$	Ctrl	N <sub>25</sub>	N <sub>50</sub>	N <sub>100</sub>
pН	n.s	n.s	n.s	а	а	а	а
SOC	n.s	n.s	n.s	а	а	а	а
TN	n.s	n.s	n.s	а	а	а	а
MBC	< 0.001	n.s	n.s	а	b	с	d
MBN	< 0.001	n.s	n.s	а	b	b	с
NH <sub>4</sub> _N	n.s	n.s	n.s	а	а	а	а
NO <sub>3</sub> _N	0.017	n.s	n.s	b	а	ab	ab
EX. Mg <sup>2+</sup>	< 0.001	n.s	n.s	а	а	b	b
EX. Ca <sup>2+</sup>	< 0.001	n.s	n.s	а	b	b	с
Soil bulk density	n.s	n.s	n.s	а	а	а	а

Two-way ANOVA results for the effects of N addition and grazing on soil factors. The pH, soil organic carbon (SOC), total nitrogen (TN), microbial biomass carbon (MBC), microbial biomass nitrogen (MBN), soil nitrate and ammonium (NO3 -N and NH4 +N), soil exchangeable Mg2 + and Ca2 +, and soil bulk density were recorded. The different lowercase letters indicate significant differences among the N addition rates (p < 0.05). n.s. = no significance (p values).

effects and the effects of their interactions on the soil P transformation are poorly understood. Our experiment demonstrated that N addition alone increased plant P uptake by adjusting the P-acquisition strategies (i.e., increased rhizosheath ACP activity, carboxylate release, AMF colonization), leading to the dissolution of NaOH-Pi into more available forms of Pi and an increase in P transformation from inorganic to organic pools in the soil. These results support our first hypothesis that N inputs may result in an increase in Po accumulation in the soil. In agreement with our second hypothesis, winter grazing increased the plant P stock and rhizosheath ACP activity but decreased the NaHCO<sub>3</sub>-Pi, NaOH-Pi, Po and total P contents of the soil. With increasing N application, there was a greater accumulation of Po in the soil of the no-grazing plots; whereas in the grazing plots, the soil Po pool decreased with increasing N application, which is consistent with our third hypothesis (Fig. 7).

# 4.1. N addition enhanced the transformation from Pi to Po without grazing

Our results revealed that the resin-Pi and NaOH-Pi contents decreased with increasing N addition. The gradual increases in the N:P

ratio of the shoots and the shoot P stock with increasing N addition indicate that the P requirement of the plants increases with increasing N addition. The resin-Pi in the soil is immediately accessible to plants as their P demand increases (Lal and Stewart, 2016). Until the soluble resin-Pi is depleted, the relatively stable Pi fraction can be released through ligand exchange with the bicarbonate ion for only a short period, i.e., one growing season (Hou et al., 2016; Lal and Stewart, 2016). The principal component analysis revealed that there was a strong negative correlation between the carboxylate release and NaOH-Pi content, confirming that the increased release of carboxylates from the rhizosphere with increasing N addition rate is an important physiological strategy for P-mining to mobilize sparingly soluble soil P. This is attributed to the fact that carboxylates exhibit a stronger binding affinity than phosphates (especially moderately labile P) towards binding sites located on the soil surface (Lambers et al., 2006; Kidd et al., 2018).

In alpine grassland soils, phosphate can precipitate with Ca in alkaline soil environments and can be converted to stable forms (Ca-Pi; it was classified as HCl<sub>conc</sub>.-P in this study), which are less available to plants (Lal and Stewart, 2016). N addition promotes carboxylate release in the rhizosphere (Prescott et al., 2020), decreasing the soil pH and increasing the Ca-Pi dissolution (Lal and Stewart, 2016; Wang et al., 2022b). In this study, N addition did not change the soil pH or the HCl<sub>conc</sub>.-P content, which is inconsistent with previous results for the Inner Mongolia grassland (Wang et al., 2022a). This difference is due to the shorter development time of the QTP soils, which leads to less movement of mineral ions in the soil body, combined with the weak biochemical effect and low SOC decomposition under the cold alpine climatic conditions, so the exchangeable Ca<sup>2+</sup> and Mg<sup>2+</sup> contents of the QTP soils are much higher than those in other regions (Jiang et al., 2005; Baumann et al., 2009; Wang et al., 2013; Zhang et al., 2015; Huang et al., 2019; Chen et al., 2021; Niu et al., 2022). Additionally, abundant exchangeable  $Ca^{2+}$  and  $Mg^{2+}$  are an important factor in buffering against N-induced soil acidification (Tian and Niu, 2015). Although the highest N addition rate decreased the soil exchangeable Ca<sup>2+</sup> and Mg<sup>2+</sup> contents by 23.4% and 29.8%, respectively (Table 2), in our experimental fields, they were still high enough to maintain a stable soil pH until these cations were depleted (Bowman et al., 2008). Ultimately, N addition promoted the conversion of the resin-Pi in the soil to plant P, which was subsequently returned to the soil as Po and gradually accumulated.

The soil  $NaHCO_3$ -P content and total P pool increased with increasing N addition, and the P stock in the plants and litter and shoot N:P ratio increased with increasing N addition, indicating that N



**Fig. 1.** Dependences of soil P fractions (a–g) and total P (h) contents at depths of 0–10 cm on grazing and nitrogen addition rates. The different lowercase letters indicate that the means are different at p < 0.05. The N addition rate did not significantly affect the (b) NaHCO<sub>3</sub>-Pi and (h) total P contents and grazing did not significantly affect the (a) resin-Pi. The values are expressed as the mean  $\pm$  95% confidence intervals.



**Fig. 2.** Plots of the (a) proportions of total organic P (Po, sum of NaHCO<sub>3<sup>-</sup></sub> and NaOH-Po) in the total P and (b) Po pool versus the nitrogen addition rates and grazing in the 0–10 cm soil depths interval. The different lowercase letters indicate that the means are different at p < 0.05. The values are expressed as the mean  $\pm$  95% confidence intervals.

addition increased the plant P requirements and the contribution of the plant residual P to the soil Po. In addition to plant synthesis, the weakening of microbial mineralization is another factor contributing to the increase in the proportion of Po in the total P with increasing N input (Bünemann, 2015). This finding contradicts the results of previous studies suggesting that N addition enhances the activities of P-cycling

enzymes (Allison et al., 2008; Schleuss et al., 2020). Our results suggest that the accumulation of NO<sub>3</sub> over the 8-year study period exceeded the N uptake capacity of the plants and microorganisms (Aber et al., 1989; Bell et al., 2010; Jing et al., 2016), which may have contributed to the inconsistency of the results with those of previous studies. Although the ACP activity in the rhizosheath increased with increasing N addition, the



**Fig. 3.** Dependences of the (a) shoot P stock, (b) aboveground litter P stock, (c) root P stock, and (d) shoot N:P ratio on the nitrogen addition rate and grazing. The different lowercase letters indicate that the means are different at p < 0.05. The N addition rate and grazing did not have a significant effect on the (c) root P stock. The values are expressed as the mean  $\pm$  95% confidence intervals.

ACP activity in the soil did not change. Another factor influencing phosphatase in soil could be that N addition induces plants to allocate fewer photosynthetic products belowground, and decreases microbial biomass and soil ALP activity.

# 4.2. Winter grazing increases plant P uptake and reduces Po accumulation in soil

Winter grazing promotes the transfer of P from the soil to plants. This is evidenced by the higher plant P stock and lower soil Pi content, especially the NaHCO<sub>3</sub>-Pi and NaOH-Pi fractions, in the grazing plots compared to the no-grazing plots. The alpine grassland on the QTP has dense plant growth (Chen et al., 2007; Luo et al., 2002), and due to the cold climate all year round, the decomposition of dead material is slow, so under the no-grazing condition, the cover of litter and standing dead matter the reduces photosynthetically active radiation of the plant community (especially the low-growing plants) in the growing season (Klein et al., 2004), which is unfavorable to the growth of the plant community and the absorption of soil nutrients. Winter grazing significantly reduced the amount of aboveground litter through livestock foraging, and the better sunshine radiation condition favored plant growth the next year in the growing season, which in turn promoted plant P uptake compared with the no-grazing plots (Shi et al., 2022).

We also found that grazing reduced the soil NaHCO<sub>3</sub>-Po and NaOH-Po contents, the total Po pool, and the proportion of Po in the total P. This suggests that winter grazing hinders the transformation of Pi to Po. Winter grazing usually has a high intensity, with Tibetan sheep consuming more than 75% of the litter biomass throughout the grazing season (according to a local survey, data not given). In grazing area, dung decomposition is a key process of returning plant P to the soil (Jin et al., 2022). Alpine grasslands have an extremely cold climate and high wind speeds in winter (Chen et al., 2007). To avoid fat loss from live-stock during grazing, grazing periods are short, making it difficult to return dung and urine to the soil in the grazing area. In fact, it has been observed that Tibetan sheep tend to defecate less at grazing sites. Even

when livestock excreted small amounts of dung, the P content of dried dung was significantly lower than that of fresh dung (McDowell and Stewart, 2005). Therefore, the high export of litter as forage reduces the recycling of Po. We even found that the phosphatase activity increased more rapidly with increasing N addition in the grazing plots compared to the no-grazing plots, which further accelerated the degradation of the Po.

# 4.3. Po fractions response to the combined effect of N addition and winter grazing

Our study further revealed that the soil Po pool and its proportion of the total P decreased with increasing N addition in the grazing plots compared to the no-grazing plots, in which the soil Po pool increased with increasing N addition. Under no-grazing condition, plant P uptake increases with the increasing N addition, and except for an amount in the form of litter blown away by the wind or carried away with surface runoff, the remainder plant P returns to the soil where it is stored in the organic form. Under the grazing condition, both N addition and winter grazing promoted P uptake by plants, so that more soil P was converted to plant P than under grazing condition, but little was returned to the soil caused by forage export, so that the proportion of Po pool in the total P in the soil gradually decreased (Fig. 7). Notably, N supply and winter grazing had an interactive effect on the inter-root phosphatase activity in plant communities, i.e., rhizosheath ACP activity showed a more pronounced upward trend with increasing N addition under the winter grazing condition compared to the no-grazing condition. This was also responsible for the decline in the Po pool in the grazing plots.

These results emphasize the need for sustainable livestock management and consideration of the effects of grazing and N addition on soil P transformation and nutrient limitation. Further research is needed to understand the mechanisms underlying these processes and the development of effective livestock management to prevent Po loss in alpine grassland ecosystems.



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**Fig. 4.** Dependences of the (a) microbial biomass phosphorus pool, (b) alkaline phosphatase (ALP) in soil, and (c) acid phosphatase (ACP) in soil on nitrogen addition rate and grazing. The different lowercase letters indicate that the means are different at p < 0.05. The grazing did not significantly affect these three indicators. The values are expressed as the mean  $\pm$  95% confidence intervals.

# 5. Conclusion

The results of the present study provide valuable insights into the complex interactions of N addition and grazing on the soil P fractions in the alpine grasslands on the QTP. Using a modified Hedley P fractionation method, we observed clear trends in the contents of the various P fractions with increasing N addition in both the grazing and no-grazing plots. In the no-grazing plots, the soil Pi pool decreased while the Po gradually accumulated with increasing N addition. Conversely, in the grazing plots, the Po pools gradually decreased with increasing N addition. In alpine grassland, N addition did not affect the soil pH, but it did reduce the microbial P pool. The results suggest that plants only drive the conversion of Pi to Po in the upper 10 cm of the soil under N addition. Furthermore, plant P-acquisition strategies (release of carboxylate and symbiosis with AMF) resulted in the mobilization of the moderately labile Pi pools in the soil. Therefore, we concluded that

**Fig. 5.** Dependences of the (a) acid phosphatases (ACP), (b) carboxylate release, and (c) AMF colonization rate in the rhizosheath on grazing and nitrogen addition rate. The different lowercase letters indicate that the means are different at p < 0.05. The grazing did not significantly affect the carboxylate release and the AMF colonization rate. The values are expressed as the mean  $\pm$  95% confidence intervals.

grazing may enhance P uptake by plants and remove the litter P pools, resulting in ongoing Po loss from the soil. It should be noted that our results concerned the effects of winter grazing on the soil P fractions, but the soil and plant samples were collected in the period of the peak biomass. The main reason for this was that in alpine grasslands, the P-acquisition strategies of plants have an important influence on the P transformation, and plant growth stops completely in winter and the plant samples cannot be collected whereas the effects of winter grazing on P transformation are long-lasting. Future studies could investigate the responses of the soil P fractions and the dynamics of grazing during different seasons.

# CRediT authorship contribution statement

Z-H.G. and J-S.H. designed the study; Z-H.G. and Z.C. performed the field and laboratory work; Z-H.G. and Z.C. analyzed the data; Z-H.G. and Z.C. wrote the manuscript with discussion with X.L. G.H. and P.K., and



Fig. 6. Principal component analysis (PCA) revealed that the two principal components are a) N addition with no grazing and b) N addition combined with grazing. Green arrows: plant P pools; yellow arrows: plant P-acquisition strategies; cyan arrows: soil P fractions and blue arrows: other soil and microbial factors.



P stocks in soil

Fig. 7. General trends of the stocks of the P pools in the soil with increasing N addition rate under no grazing (left) and grazing (right) conditions. The arrows show the main pathways of the increased net accumulation and transformation of organic P in alpine grassland soils.

all authors contributed to the article and approved the submitted version.

# **Declaration of Competing Interest**

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests Zhen-huan Guan reports financial support was provided by National Natural Science Foundation of China.

# Data Availability

Data will be made available on request.

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#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2023.108700.

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