Variations in soil properties rather than functional gene abundances dominate soil phosphorus dynamics under short-term nitrogen input

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Abstract

Background and aims Microorganisms play a vital role in regulating soil phosphorus (P) dynamics in terrestrial ecosystems. Here, we investigated the response of soil microbial P cycling potential traits to nitrogen (N) addition via metagenomics and the relationship between microbial potentials and soil P dynamics.

Methods Topsoil (0–10 cm) samples were collected from experimental soil that had been maintained for 3 years with low and high level of N addition in an alpine meadow of the Qinghai-Tibet Plateau. Soil microbial functional genes and P fractions were determined.

Results The soil available P and microbial biomass P were significantly affected by N inputs and significantly associated with soil properties (including soil pH, alkaline phosphatase activity, and soil total N and NO3−-N contents). Meanwhile, high N input decreased the relative abundance of the pstS gene, and low N input reduced the relative abundances of phoB, ugpQ and C-P lyase genes. We further found that the pstS gene was a determinant of soil microbial biomass P and significantly correlated with soil pH. Moreover, Alphaproteobacteria with C-P lyase and Actinobacteria related to alkaline phosphatases and phosphate-specific transport were the most abundant taxa but not affected by N input.

Conclusions Short-term high N input could alter soil P dynamics and microbial functional genes. Although there were relationships between the pstS gene, microbial biomass P and soil pH, the microbial functional gene abundance was less important than soil properties in regulating soil P dynamics.

Keywords Nitrogen input · Microbial functional genes · Soil phosphorus dynamics · Soil properties

Introduction

Phosphorus (P) is a critical macronutrient for plants. However, a large proportion of P is fixed or immobilized by nonlabile compounds in terrestrial ecosystems, and only a limited amount of available P can be
taken and utilized (Shen et al. 2011). Plant roots can acidify the soil through the release of protons, resulting in the dissolution of non-soluble phosphorus (Liu et al. 2021; Zhang et al. 2010). The secretion of phosphatases by plants and microorganisms can also mobilize organic P to available P (Richardson and Simpson 2011). The growth of soil microorganisms also requires P uptake, which results in them competing with plants for soil available P (Lupwayi et al. 2007). Therefore, soil available P is generally as a good indicator of the P bioavailability in soil (Bai et al. 2013; Shen et al. 2014).

Many factors influence the availability of P for plants and microorganisms Cross and Schlesinger 2001; Daly et al. 2015; Baumann et al. 2018). Among them, the dynamics of nitrogen (N) deposition are key in influencing P dynamics through their effects on other factors (e.g. plant P demand, soil N concentration and soil pH). Previous studies demonstrated that N deposition stimulated the P demand of plants by increasing plant production, which caused P limitation in terrestrial ecosystems (Braun et al. 2010; Deng et al. 2017). The changes in soil properties under N deposition determined soil P dynamics (Heuck et al. 2018). For example, Li et al. (2019a) reported that soil NO$_3^-$-N was the most important driver of labile organic P change. Long-term N input induces soil acidification through the accumulation of nitrate (Rustad et al. 1993), which promotes the dissolution of phosphate and increases soil P availability in calcareous soil (Robles-Aguilar et al. 2019). In contrast, decreases in the soil pH mobilizes soil metal cations, including aluminum and iron, thus reducing available P and the mineralization of organic matter in acidic soil (Carreira et al. 2000). Furthermore, changes in the soil pH resulted in N input indirectly affecting the bacterial community (Ling et al. 2017), which is the main source of alkaline phosphatase (Fraser et al. 2015).

Microbial biomass P accounts for a large proportion of the total P in soil (Achat et al. 2010). Under N addition conditions, increases in microbial biomass require immobilization of additional available P to maintain a stable N:P ratio, thus reducing soil available P (Lupwayi et al. 2007). Nevertheless, other studies also revealed that exogenous N addition may limit microbial biomass and activity due to C restriction and reduce microbial P fixation ability and microbial biomass P concentration (Demoling et al. 2008). However, soil microbial biomass P was not consistently affected by N inputs in meta-analysis. These inconsistencies indicated that moderating variables (e.g. climate, latitude and experimental factors) also had significantly affected on soil P pools in terrestrial ecosystems, and different response also found in variable P pools such as plant, soil and microbial biomass (Yue et al. 2018).

In recent years, the methods of studying microorganisms have been continuously updated (Hill et al. 2000; Roesch et al. 2007; Ouyang et al. 2018), with metagenomic sequencing providing detailed information on multiple genes involved in soil nutrient cycling (Ranjan et al. 2016). Metagenomics can be used to verify the genetic mechanisms by which soil microbial genes manipulate soil P transformations and to enhance the understanding of the functional potential of microbial communities (Neal et al. 2017). In arable, forest and grassland soils, the changes in soil pH caused by N input were a key driver of a phoX-harbouring communities (Ragot et al. 2016). In response to long-term N input, the potential activity of alkaline phosphatase and the abundance of the bacterial phoD gene were significantly reduced, and the ability of bacteria to mineralize nonlabile organic P was enhanced in agricultural ecosystems (Chen et al. 2019). In addition, long-term N input reduced the relative abundances of total microbial genes related to P solubilization and the abundance of some microorganisms (e.g. Actinobacteria, Gammaproteobacteria and Alphaproteobacteria) containing alkaline phosphatase genes in four experimental agroecosystems (Dai et al. 2020). However, in other agricultural soils in Germany, the addition of fertilizers had no significant effect on the relative abundances of genes related to P turnover (Grafe et al. 2018).

Scientists have reported that the functional genes are connection with the variations in rates of soil carbon mineralization (Zhang et al. 2019), however, whether N input can alter the abundances of soil microbial P transformation genes and whether these genes are linked to changes in soil P availability remain unclear, especially in alpine regions. The Qinghai-Tibetan Plateau is sensitive to climate change and human activities owing to its high altitude and geographical characteristics (Sun et al. 2020). Exploring the changes in microbial functional genes associated with P cycling under N inputs could further explain P cycling in alpine meadows. Our objectives...
were to (1) investigate the response of the functional genes linked to soil P dynamics to short-term N addition; (2) explore the relationships between the soil P dynamics, soil properties and functional genes under N addition. Soil P availability and microbial functional profiles were changed under N input in Chinese agroecosystems (see Dai et al. 2020). We hypothesized that (1) N input increased the relative abundances of genes coding for inorganic P solubilization or organic P mineralization to facilitate plant utilization and (2) N input changed the soil P dynamics genes via decreasing soil pH and there was positive linkage between the gene abundance and availability of soil P.

**Materials and methods**

**Study site and experimental design**

The experiment was conducted in the N deposition simulation platform located in Haibei, Qinghai Province, China (36° 55′N, 100° 57′E, 3040 m elevation). This region has a continental plateau climate with −0.45 °C mean annual temperature that is characterized by a long, cold winter with a minimum monthly mean air temperature of -29 °C and a short, cool summer with an extreme maximum temperature of 27 °C in July. The mean annual precipitation is 400 mm, which mainly occurs from June to August. The soil is classified as Mat-Gryic Cambisol with a clay loam texture under the Chinese soil classification system (Ma et al. 2017), and the initial plant species are dominantly *Stipa capillata*, *Potentilla chinensis Poo pratensis*, *Agropyron cristatum*, *Elymus dahuricus*, *Artemisia scoparia*, *Ajuga lupulina* Maxim, and *Potentilla anserine*. As previously reported, the soil background properties were a total N of 3.53 g kg⁻¹, a total P of 0.29 g kg⁻¹, an inorganic N of 15.68 mg kg⁻¹ and an available P of 5.94 mg kg⁻¹ (Li et al. 2019b).

The experiment plots were established in May 2017. Grasses in this region began to green up and uptake a relatively large amount of N to support their growth in May. Therefore, N application was selected in this month every year. Three levels of N addition (CK, control without N input; LN, low N addition with 50 Kg N ha⁻¹ yr⁻¹; HN, high N addition with 100 Kg N ha⁻¹ yr⁻¹) were used with three replications. These levels of N addition were based on the study of Zhang et al. (2017). Briefly, nine plots were included in the experiment with a randomized block design, and each plot area was 3 m×3 m. Buffer strips 1-m wide were set between plots. Ammonium nitrate was used as the N source.

**Soil sampling and analyses**

After three years of N input, the dominant plant species did not change among treatments compared the initial stage. Some rare species, such as *Gentiana scabra* Bunge, *Taraxacum mongolicum* and Heteropappus hispidus, were vanished in LN and HN. Five cylindrical soil cores (0–10 cm in depth and 5 cm in diameter) were randomly sampled from each plot in August 2019. In order to ensure the consistency of sampling condition as much as possible, we selected a sunny day to complete all the sampling. Soil samples from the same plot were composted into one sample, and plant roots and litter within the soil core were removed. Each mixed soil sample was then divided into two parts: one part was stored at 4 °C for biogeochemical analysis, and one part was frozen at -80 °C for DNA extraction and metagenome sequencing.

Biogeochemical analysis included determination of pH, ammonium (NH₄⁺-N), nitrate (NO₃⁻-N), total C, total N, total P, available P, microbial biomass P and alkaline phosphatase activity. Soil pH was determined by a pH meter (Orion Star A215, Thermo Fisher Scientific, USA). The concentrations of NH₄⁺-N and NO₃⁻-N were determined by a flow-solution analyser (Flowsys, Ecotech, Germany). Soil total C and total N were measured by a carbon-nitrogen element analyser (Elementar, Hanau, Germany). Soil total P was obtained using the HClO₄-H₂SO₄ digestion-molybdenum antimony colorimetric method (Murphy and Riley 1962). Soil available P was extracted by 0.5 M NaHCO₃ solution (pH 8.5) from1.5 g dry soil for 30 min and determined by colorimetric measurement (Bao 2008). Microbial biomass P was measured by the fumigation-extraction method and calculated from the difference between the P contents of fumigated and nonfumigated soils (Brookes et al. 1985). The activity of alkaline phosphatase was measured after extraction by culturing 5 g of dry soil for 2 h at 37 °C in an Erlenmeyer flask with methylbenzene and disodium phenyl phosphate. The suspensions
were analysed by measuring the absorbance at 510 nm using a spectrophotometer with buffer solution, amino antipyrine solution and potassium ferricyanide (Guan 1986).

DNA extraction and sequencing

Commercial kits were used to extract genomic DNA according to the manufacturer’s instructions (Guangdong Magigene Biotechnology Co., Ltd.), and 1% agarose gels were used to monitor the integrity and purity of DNA. Qubit 2.0 (Thermo Fisher Scientific, USA) and Nanodrop One (Thermo Fisher Scientific, Weihao, USA) instruments were used to determine the DNA concentration and purity. Sequencing libraries were generated using the NEBNext Ultra DNA Library Prepkit for Illumina (New England BioLabs, MA, USA), and index codes were added as recommended by the manufacturer. Library quality was assessed using a Qubit 3.0 fluorometer (Life Technologies, Grand Island, NY) and an Agilent 4200 (Agilent, Santa Clara, CA) system. Finally, a library was generated on the Illumina HiSeq X Ten platform, and 150-bp paired-end reads were obtained. Scaffigs (≥ 500 bp) assembled from both single and mixed combinations of predicted ORFs by MetaGeneMark (Version 3.38, http://exon.gatech.edu/GeneMark/metagenome/Prediction) using default parameters to remove information for sequences shorter than 90 nt from the prediction results. CD-HIT (version: 4.7, http://www.bioinformatics.org/ cd-HIT/) deletes redundant genes to obtain a unique initial catalogue (here, the gene nucleotide sequence was encoded by unique and contiguous genes) that exhibits an identity of 95%, a coverage of 90%, clusters, and the longest single selection sequence. The clean data for each sample were mapped to the initial gene directory using BBMAP software (http://jgi.doe.gov/data-and-tools/bbtools/) to obtain the gene-mapped readings for each sample. According to the number of labelled reads and the lengths of genes, the abundance of each gene in each sample was calculated. Basic statistics, core-pan gene analysis, sample correlation analysis and Venn diagram analysis of gene numbers were all based on the abundances of each gene in each sample in the gene catalogue. DIAMOND software was used to inject unigenes into the functional database. The functional databases include the KEGG database (http://www.kegg.jp/kegg/) and the eggNOG database (http://eggnogdb.embl.de/). Analysis was carried out with the optimal BLAST results. The genes involved in the P transformation of soil microbes were sought in datasets based on previous publications (Dai et al. 2020; Liang et al. 2020). A total of 41 genes related to P transformation and their corresponding KEGG orthology (KO) numbers were collected. According to their functional roles in soil P cycling (Dai et al. 2020), we classified these genes into four groups: the P-starvation response regulation gene group (group 1), organic P mineralization gene group (group 2), inorganic P solubilization gene group (group 3), and P transport system gene group (group 4). The names, functions, and classifications of the genes associated with P cycling are listed in Table S1.

Statistical analysis

Multivariate analysis of variance (MANOVA) and least significant difference (LSD) tests were employed to verify the effect of N input on soil properties, soil total P, available P, microbial biomass P, and the relative abundances of functional genes and microorganisms coding for P transformation (SPSS 20.0.0 for Windows). Redundancy analysis (RDA) was used to analyze the relationships among functional microorganisms and soil P dynamics. First, we selected the microorganisms with increased abundance or significant changes under N input. Then, the selected microbial variables and soil P dynamics were analyzed by RDA in the “vegan” package in R (R 4.0.2 for Windows). Pearson correlation analysis was used to explore the relationships between soil P dynamics and soil properties and the relationships between soil properties and microbial genes and microorganisms harbouring functional genes using the package “corrplot” in R (R 4.0.2 for Windows). Random forest analysis was performed to identify the main functional genes influencing soil available P and microbial biomass P.

Results

Responses of main soil properties to N input

The concentration of soil total P was not significantly affected by N input ($P > 0.05$, Fig. 1a). Compared to CK treatment, LN and HN inputs significantly
increased the soil available P by 21.74 and 27.27%, respectively ($P < 0.05$, Fig. 1b). However, the microbial biomass P decreased significantly by 61.92% under HN input ($P < 0.05$, Fig. 1c), LN input decreased the content by 22.49%.

Soil pH decreased significantly in HN compared with CK and LN ($P < 0.05$, Table 1). Soil alkaline phosphatase activity remarkably increased under the N inputs ($P < 0.05$, Table 1). Soil inorganic N and NO$_3^-$-N were different ($P < 0.05$) between CK and HN, whereas total C, total N and NH$_4^+$-N were not different among the three treatments ($P > 0.05$, Table 1). The soil C/ N, C/ P and N/ P ratios did not change with N addition level ($P > 0.05$, Table 1).

**Table 1** Soil properties under different N input levels

| Soil properties  | CK         | LN         | HN         | P value |
|------------------|------------|------------|------------|---------|
| Moisture (%)     | 16.92 ± 0.72a | 14.49 ± 0.05b | 15.35 ± 0.42b | < 0.05  |
| pH               | 8.16 ± 0.01a  | 8.16 ± 0.01a  | 8.09 ± 0.01b  | < 0.05  |
| Total C (g Kg$^{-1}$) | 40.787 ± 0.655a | 41.913 ± 0.912a | 41.230 ± 0.324a | 0.297   |
| Total N (g Kg$^{-1}$) | 3.083 ± 0.072a  | 3.350 ± 0.142a  | 3.240 ± 0.092a  | 0.136   |
| Inorganic N (mg Kg$^{-1}$) | 4.771 ± 0.202b  | 5.709 ± 0.207ab  | 6.050 ± 0.538a  | < 0.05  |
| NH$_4^+$-N (mg Kg$^{-1}$) | 1.560 ± 0.056a  | 1.596 ± 0.066a  | 1.593 ± 0.121a  | 0.785   |
| NO$_3^-$-N (mg Kg$^{-1}$) | 3.211 ± 0.157b  | 4.113 ± 0.203ab  | 4.457 ± 0.417a  | < 0.05  |
| Aalp (mg g$^{-1}$ 24 h$^{-1}$) | 1.202 ± 0.178b  | 1.532 ± 0.979a  | 1.640 ± 0.650a  | < 0.05  |
| Soil total C: N  | 13.23 ± 0.10a  | 12.53 ± 0.26a  | 12.74 ± 0.28a  | 0.083   |
| Soil total C: P  | 66.43 ± 1.14a  | 68.80 ± 3.14a  | 67.90 ± 0.35a  | 0.434   |
| Soil total N: P  | 5.02 ± 0.21a  | 5.50 ± 0.37a  | 5.34 ± 0.15a  | 0.219   |

Values are presented as mean± SE of three replicates per treatment

Total C total carbon, Total N total nitrogen, Inorganic N inorganic nitrogen, NO$_3^-$-N nitrate nitrogen, NH$_4^+$-N ammonium nitrogen, Aalp Alkaline phosphatase activity

a, b Treatments not sharing the same letter are significantly different from each other (MANOVA, $P < 0.05$)
These results indicated that N input notably influenced the soil phosphorus dynamics and some soil properties.

Responses of genes involved in soil P cycling to N input

The genes in functional gene group 4 were the most abundant genes (Fig. 2). Nitrogen input had no significant impact on the total relative abundances of the genes coding for P-starvation response regulation, inorganic P solubilization, organic P mineralization and P uptake and transport (P > 0.05, Table S2).

The relative abundances of genes involved in P-starvation response regulation (i.e. phoB, phoR and phoU) showed a decreasing trend under N input, in which only phoB gene abundance showed significant change under LN input (P < 0.05, Fig. 2). Within group 2, the relative abundance of the ugpQ gene significantly decreased by 10.5% in LN compared to CK and HN (P < 0.05), and genes coding for the C-P lyase subunit (phn FGHJKLMNOP) showed lower abundances in LN than in CK and HN (P < 0.05, Fig. 2). Nitrogen input had no significant effect on the relative abundances of some specific genes coding for acid phosphatase (i.e. phoN, olpA and aphA) and alkaline phosphatase (i.e. phoD, phoA and phoX) (P > 0.05, Fig. 2). Within group 4, pstA, pstB, pstC and pstS showed relatively higher abundances. The relative abundances of the pstA, pstB and pstC genes were not different among treatments, but the pstS gene in HN was 8.7% less abundant than that other (n=3, MANOVA, P < 0.05). The relative abundance of ugp transporter systems was calculated as the average abundances of gene ugpA, ugpB, ugpC, and ugpE; the phn transporter systems was calculated as the average abundances of gene phnC, phnE, and phnD; the C-P lyase subunit was calculated as the average abundances of gene phnF, phnG, phnH, phnI, phnJ, phnK, phnL, phnM, phnN, phnO, and phnP.
in CK ($P<0.05$, Fig. 2). The relative abundances of some specific genes coding for glycerol-3-phosphate transporter systems (ugpABCE) and phosphonate transporter systems (phnCED) showed no difference among treatments.

**Taxonomic assignments of genes involved in P transport and mineralization**

The taxonomic assignment of investigated genes was based on KEGG database results. The results are shown at the class level and reflect the overall abundance of taxa in the metagenomic data sets. Within taxa containing genes coding for alkaline phosphatase, N input reduced the relative abundance of Gammaproteobacteria in the short term ($P<0.05$, Fig. S1a). Actinobacteria were dominant but exhibited no significant response to N input ($P>0.05$, Fig. 3 and S1a). Among the genes involved in C-P lyase, those in Alphaproteobacteria were most abundant, but they had no significant change under N input ($P>0.05$, Fig. 3 and S1b); the relative abundance of Betaproteobacteria significantly decreased under N input ($P<0.05$, Fig. S1b). Among P-cycle genes, genes corresponding to phosphate-specific transport systems were the most abundant (Fig. 2). For genes involved in phosphate-specific transport systems, those in Actinobacteria and Deltaproteobacteria were most abundant, but they had no significant change under N input ($P>0.05$, Fig. 3 and S1d), and those of Thermomicrobia significantly decreased under N input ($P<0.05$, Fig. S1d).

**Relationships among soil P, soil properties and functional genes**

Soil available P had a significant positive correlation with inorganic N, NO$_3$-N and alkaline phosphatase activity ($P<0.05$, Fig. 4). Soil microbial biomass P showed a positive correlation with soil pH and a negative relationship with alkaline phosphatase activity ($P<0.05$, Fig. 4).

The random forest analysis showed that 9 of 41 P-related genes were the determinants of available soil P concentration (Fig. 5a). Among these genes, only **ugpQ** and **phoB** showed a significant decrease under LN conditions ($P<0.05$, Fig. 2). In addition, 12 of 41 P-related genes were the determinants of phosphate specific transport systems (pooled subunits) was taxonomically assigned (DIAMOND against NCBI Non-redundant protein sequences (nr) database). Shown are relative numbers of assigned sequences (n=3).
microbial biomass P concentration (Fig. 5b). Among them, the pstS gene was the most important, and it had a significant decrease under HN input ($P < 0.05$, Fig. 2).

The concentration of microbial biomass P showed a positive relationship with Gammaproteobacteria with alkaline phosphatase genes and Deltaproteobacteria involved in phosphate-specific transport systems (Fig. 6). Actinobacteria involved in alkaline phosphatase and phosphate-specific transport systems showed a positive correlation with available P and total P. Alphaproteobacteria with C-P lyase genes had no relationship with available P, total P and microbial biomass P (Fig. 6).

The relative abundances of 11 genes were significantly correlated with soil parameters ($P < 0.05$). Most of the investigated P transformation genes were positively correlated with the soil total N concentration and C/N ratio (Table S3). The relative abundance of Gammaproteobacteria with alkaline phosphatase genes showed significant positive correlations with soil moisture and the soil C/N ratio and negative correlations with total N and alkaline phosphatase activity ($P < 0.05$, Table S4). The relative abundances of Betaproteobacteria responsible for C-P lyase were positively correlated ($P < 0.05$) with soil moisture (Table S5). The relative abundance of Deltaproteobacteria with phosphate-specific transport systems was negatively correlated with alkaline phosphatase activity ($P < 0.05$). Actinobacteria with alkaline phosphatase genes and phosphate-specific transport systems, Alphaproteobacteria with C-P lyase genes and Thermomicrobia with phosphate-specific transport systems

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### Table 1: Pearson correlation matrix for soil properties and microbial biomass P concentration

|          | Total C | Total N | Inorganic N | NO$_3$$^-$-N | NH$_4$$^+$-N | Aalp | Total P | Available P | Mmribial biomass P |
|----------|---------|---------|-------------|--------------|--------------|------|---------|-------------|-------------------|
| Total C  | 0.692   | 0.639   | -0.586      | -0.339       | -0.496       | -0.447| -0.334  | -0.576      |                   |
| Total N  |         |         |             |              |              |      |         |             |                   |
| Inorganic N |       |         |             |              |              |      |         |             |                   |
| NO$_3$$^-$-N |     |         |             |              |              |      |         |             |                   |
| NH$_4$$^+$-N |     |         |             |              |              |      |         |             |                   |
| Aalp     |         |         |             |              |              |      |         |             |                   |
| Total P  |         |         |             |              |              |      |         |             |                   |
| Available P |       |         |             |              |              |      |         |             |                   |
| Mmribial biomass P |     |         |             |              |              |      |         |             |                   |

**Significant differences in the Pearson correlation coefficients are shown (n=3). Significance levels:** $^*P < 0.05$, $^{**}P < 0.01$

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**Fig. 4** Pearson correlation coefficients between soil P dynamics and soil properties. Total C total carbon, Total N total nitrogen, Inorganic N inorganic nitrogen, NO$_3$$^-$-N nitrate nitrogen, NH$_4$$^+$-N ammonium nitrogen, Aalp alkaline phosphatase activity

**Fig. 5** The linkages between genes responsible for soil microbial P-cycling potential and soil P status. Panel shows the gene predictors of soil available P (a) and microbial biomass P (b), identified by random forest analysis.
were not significantly correlated with soil properties ($P > 0.05$, Table S6).

**Discussion**

Shifts in individual microbial genes associated with phosphorus dynamics under N input

Notably, metagenomic analysis showed that HN input decreased the relative abundance of the $pstS$ gene, which encodes a high-affinity phosphate-specific transporter (Fig. 2) that allows inorganic P assimilation under P-low conditions (Hsieh and Wanner 2010). The $pstS$ gene is required for the high-affinity acquisition of phosphate and is expressed only when environmental phosphate is limiting (Vuppada et al. 2018). In our results, the soil available P increased under N input, so the environmental phosphate would not be probably limited under this treatment. This change might be a reason to lead to the decrease of the relative abundance of the $pstS$ gene. This finding was also supported by a lower relative abundance of $pstS$ in P-rich soil than in P-depleted soils (Bergkemper et al. 2016). In the restoration of degraded land, the $pstS$ gene was more relatively abundant in an unamended layer of reclaimed tailings with lower concentrations of total P and available P (Liang et al. 2020).

The $phoB$ gene acts as the regulation of P-starvation response had strong connection with the genes related to P-uptake and transport system (i.e. inorganic phosphate transporter), which capacitate microorganisms to utilize soil available P in low P condition (Eder et al. 1996). Those genes in group 4 except $pst$ transport system both decreased under LN input, the abundance of $phoB$ gene decreased accordingly. Our study suggested that the microbial function of hydrolysing glycerol phosphate generally decreased under LN addition since the relative abundances of the $ugpQ$ genes were lower in LN than in CK and HN (Fig. 2). The protein encoded by the $ugpQ$ gene is a glycerophosphoryl diester phosphodiesterase that hydrolyses only diesters (Luo et al. 2009), and glycerol phosphate is taken up with $ugp$ transporter systems ($ugp ABCE$) in P-limiting environments (Brzoska et al. 1994a). It is interesting that the activity of $ugp$ transporter system is restricted by
high concentration of phosphate in cell (Brzoska et al. 1994a, b). On the contrary, the ugpQ as the operon of glycerophosphodiester-utilizing system usually accumulates in the phosphate starvation conditions (Ohshima et al. 2008). In our study, the ugpQ gene was not adopted the environment of increased available P content under LN condition. However, the relative abundance of ugpQ was not changed with the increase of N inputs level. There were little studies to explore the response of microbial genes to different N inputs, we hypothesized this result was attribute to the short term of experimental site.

In contrast to our hypothesis, the total relative abundances of functional gene groups involved in soil P transformation were not significantly influenced by N input in our 3-year experiment. In agricultural soils, the relative abundances of genes related to P turnover were also not significantly affected by more than 20 years mineral N fertilizer application at different sites and seasons (Grafe et al. 2018). However, long-term N input (over 35 years) significantly reduced the relative abundances of total genes coding for microbial P solubilization in agroecosystems (Dai et al. 2020). These contrasting results indicate that different durations of treatment might cause varied changes in microbial functional genes (Dai et al. 2020).

In addition, we found that N input did not significantly increase the relative abundances of genes coding for alkaline phosphatase, while the alkaline phosphatase activity had a positive response to N addition. One possible reason for this difference is that there are significant differences in the presence and expression of genes involved in P turnover among some taxa (Ragot et al. 2016).

Microbial community performing P transformation

Due to the significant change in available P, we performed taxonomic assignments of genes involved in organic P mineralization and microorganisms containing genes related to alkaline phosphatase and C-P lyase. Alkaline phosphatase contributes greatly to the mineralization of soil organic P (Kageyama et al. 2011). The phoD gene, encoding alkaline phosphatase, is mainly found in the bacterial phylum Actinobacteria (Ragot et al. 2015), which is relatively abundant in soil and water (Luo et al. 2017; Tan et al. 2013). It has been shown that Actinobacteria are metabolically active among the total phoD-harboring microbial communities which the community structure is not changed with the increase of soil P pool (Ragot et al. 2016). In our study, we found that the relative abundance of phoD-harboring Actinobacteria was high. This result emphasized the importance of Actinobacteria for the soil microbial organic P mineralization.

The C-P lyase performs C-P cleavage in organic phosphonates (Rodríguez et al. 2006). The genes coding for the C-P lyase are principally from Alphaproteobacteria and from Betaproteobacteria in small part (Liu et al. 2018a, b). Our results also find most C-P lyases are harboured by Alphaproteobacteria and Betaproteobacteria, which emphasizes the significance of Proteobacteria among P-solubilizing bacteria (Widdig et al. 2019). Meanwhile, those finds demonstrate the taxonomic composition of functional genes are similar. Among Proteobacteria, the Gammaproteobacteria were the most frequently reported to harbour phoX (Ragot et al. 2016), and this class’s abundance significantly decreased under N input, which was supported by findings of Dai et al. (2020).

Because the most-abundant genes were related to the P uptake and transport systems, we determined the taxonomic assignments of genes involved in phosphate-specific transport systems in our study. These genes enable microorganisms to effectively compete with plants for available phosphorus from soil solution (Richardson and Simpson 2011). Most of genes coding for phosphate-specific transport systems were harboured by Actinobacteria, which indicated that the highly abundant phylum Actinobacteria influenced soil microbial P turnover (Bergkemper et al. 2016).

Increases in P availability caused by N input

The concentration of soil available P was evaluated by the relative abundance of the phnG and ugpC gene via the random forest analysis (Fig. 5). The phnG gene belongs to the C-P lyase multienzyme complex performing the cleavage of C-P in organic phosphonates resulting the release of available P from multiple organic P pool (Bergkemper et al. 2016). Despite the total relative abundance of C-P lyase genes (phn NLKMFCHIJO) was significantly decreased under LN input, the individual relative abundance of phnG gene has increasing trend under HN input (P > 0.05, Table S7). The ugpC gene is part of the Glycerol-3-phosphate transporter transforming the
sn-glycerol-3-phosphate (G3P) under the condition of phosphate starvation (Wuttge et al. 2012). The relative abundance of ugpC is decreased under LN input ($P > 0.05$, Table S7), which resulted in the decrease of P absorbed by microorganisms from soil and the increase of available P for plant.

The changing patterns of soil available P and microbial biomass P concentration affected by N addition in this study could be attributed to the change in soil factors, because the soil P dynamics were strongly related to soil properties (i.e. pH, inorganic N, NO$_3^-$-N) and the alkaline phosphatase activity (Fig. 4). This result further indicated that the changes in edaphic environments regulated the changing patterns of soil available P and microbial biomass P.

Numerous studies have reported that N fertilization stimulates plant growth, increases P uptake by plants, and finally decreases soil inorganic P availability (Vitousek et al. 2010; Yang et al. 2015). However, in our study, the soil available P significantly increased in the LN and HN treatments compared to the CK treatment. This might be because a large amount of N stimulated the secretion of phosphatase and further promoted the accumulation of available P in soil (Tian et al. 2016). The alkaline phosphatase activity in our study further proved this finding. Among the soil P cycle processes, the soil P transformation mediated by biotic factors, such as the mineralization regulated by phosphatase, played especially vital role in making P taken up by plants (Nannipieri et al. 2011). A meta-analysis of 34 fields with N addition found that plants and microbes under nitrogen addition can allocate additional N to produce phosphatase enzymes to accelerate the mineralization of soil organic P to mitigate P limitation (Marklein and Houlton 2012). In general, P availability is considered regulated by soil pH (Adhikari et al. 2017). Plant root and mycorrhizal microorganisms could secrete organic acids resulting in the decrease of soil pH, thus accelerating the solubilization of inorganic P (Rosling et al. 2016; Liu et al. 2018a, b). Nitrogen input can decrease soil pH by inducing significant accumulation of nitrate (Guo et al. 2010; Wang et al. 2017; Yang et al. 2015), which increases dissolution of phosphate and enhances soil P availability.

Phosphorus availability is also regulated by microorganisms (Alori et al. 2017). On the one hand, microorganisms directly mineralize and solubilize soil phosphorus through the release of hydrolase enzymes, thereby increasing plant available phosphorus (Richardson and Simpson 2011); on the other hand, microorganisms can efficiently utilize P and then immobilize P into their biomass (Achat et al. 2010). Fixed P in microbial tissues could be returned to the soil and become an important source of available P to plants after the death of microbial cells (Turner et al. 2013). Our study showed that the soil microbial biomass P significantly decreased under HN addition. N deposition increased soil total N and available N contents, limiting microorganism growth as a result of C restriction and then inhibiting the ability of microbes to fix P (Demoling et al. 2008; Deng et al. 2017). Phosphorus fixation by microorganisms mainly occurs in soil (Wei et al. 2018), and decreases in fixed P by microorganisms could theoretically increase the soil available P.

The significant positive correlations between the relative abundance of the pstS gene and soil pH under N input (Table S3) indicate that soil acidification had a greater influence than other factors on the potential capacity of soil microbial P uptake and transport (Hsieh and Wanner 2010). The random forest analysis revealed that the concentration of microbial biomass P was determined by the relative abundance of the pstS gene (Fig. 5). It is likely that the reduction in pH caused by N input directly decreased the microbial capacity for P uptake and transport (Dai et al. 2020) and thus decreased microbial biomass P. However, a direct positive correlation between soil available P and the abundances of functional genes related to P cycling was not observed. Similar findings regarding the rates of soil C and N mineralization also revealed that it was difficult to find a clear relationship between functional genes and correlated nutrient cycling processes via only investigation of functional genes (Zhang et al. 2019). One possible reason was probably because the metagenomics approach could obtain only the functional potentials of the microbial community and not their real activities (Grafe et al. 2018). Future studies should combine transcriptomic and proteomic characterization methods to demonstrate the mechanisms affecting functional genes.

Conclusions

The highlight of our study was considering the role of functional genes in exploring the effects of N inputs.
on P availability in alpine grassland soil. In our study, positive relationships existed between the soil pH, \( \text{pstS} \) gene and microbial biomass P. The conceptual figure depicting the N input regulate soil P dynamic via soil biotic and abiotic factors were showed in Fig. 7. Compared to the abundance of microbial functional genes, soil properties played important roles in regulating soil P dynamics. Shifts in soil pH, alkaline phosphatase activity, and soil total N and \( \text{NO}_3^-\) contents were determinants of soil available P and microbial biomass P. Our findings indicated that soil properties had greater effect on soil phosphorous dynamics than functional gene abundances under short term nitrogen input.

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**Fig. 7** The conceptual figure of the effects of N addition on soil microbial biomass P and available P via alteration of soil properties and functional gene. \( \text{NO}_3^-\)-N nitrate nitrogen, Aalp Alkaline phosphatase activity. The soiled one-way arrows indicate that N input significantly influenced the factors \( (P < 0.05) \). The yellow dashed one-way arrows indicate that there was significant relationship \( (P < 0.05) \) between two factors in our study and causal connections were found by previous studies. The blue dotted line indicate that the causal connection was found by previous studies without significant relationship \( (P > 0.05) \).
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