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Soil pH and phosphorus availability regulate sulphur cycling in an 82-year-old fertilised grassland

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ABSTRACT

The application of lime and mineral fertiliser is known to mitigate soil acidification and improve soil quality in improved grasslands. However, the long-term effect of simultaneous lime and fertiliser amendments on soil carbon (C) and sulphur (S) cycling is still poorly understood. To examine if soil pH or nutrient availability are the dominant factors regulating C and S cycling, we evaluated the biodegradation of methionine (organic S), gross S transformation, and microbial S utilisation using ^{35}S and ^{14}C dual-labelling. Soil samples (0–10 cm) were collected from one unfertilised control and five annual limed (Ca) treatments with or without nitrogen (N), phosphorus (P), potassium (K) and sulphur (S) fertilisers (Ca, CaN, CaNP, CaNPKCl, $CaNPK_2SO_4$) in an 82-year-old upland grassland experiment in Rengen, Germany. Long-term lime application increased soil pH values but significantly ($p < 0.05$) decreased soil organic C content. Fertilisation had no significant effect on microbial utilisation of ^{35}S-labelled methionine, while microbial immobilisation of $^{35}SO_4^{2-}$ in the limed soils was significantly reduced compared to the control. This is attributed to either the increased soil pH or decreased C availability after liming. Microbial carbon use efficiency (CUE) was significantly higher in soils with applied P fertiliser (i.e., CaNP: 0.66 ± 0.02, CaNPKCl: 0.68 ± 0.02, $CaNPK_2SO_4$: 0.65 ± 0.01) compared to the CaN treatment (0.58 ± 0.01). Moreover, compared to CaN, CaNP and CaNPKCl treatments significantly increased gross S turnover, while no significant effects were observed in the $CaNPK_2SO_4$ treatment. Soil P deficits decreased microbial CUE and S bioavailability. Although P fertiliser addition alleviated microbial P limitation when N fertiliser was added, S fertiliser ($CaNPK_2SO_4$) present constrained S transformation rates. Overall, the importance of P availability for global S cycling in grasslands is shown, especially under N-enrichment conditions. However, the subsequent potential for C loss from long-term liming should be carefully considered in grassland management.

1. Introduction

Sulphur (S) is vital for the synthesis of key proteins and amino acids in plants, controlling forage quality and yield (Eriksen, 2009; Aspel et al., 2022). However, intergovernmental legislation restricted the anthropogenic activities (e.g., emissions from coal-fired power stations) responsible for excessive atmospheric S deposition (Eriksen et al., 2004; Aas et al., 2019). This also has had unintended consequences for global food security, with low anthropogenic S deposition, high crop removal, and S immobilisation increasing the risk of soil S deficiency and S fertilisers demand (Gerson and Hinckley, 2023). In the Northern Hemisphere, atmospheric S deposition rates are projected to decline by 70–90 % by the end of the 21st century (Feinberg et al., 2021). Soil surveys from the UK and Germany have indicated that approximately 23 % and 40 % of soils, respectively are already at high risk of S deficiency (McGrath and Zhao, 1995; Hartmann et al., 2008). However, despite its importance, the mechanisms controlling belowground S cycling, compared to soil carbon (C), nitrogen (N), and phosphorus (P) cycling,

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are poorly understood.

Fertilisation controls biogeochemical processes by affecting soil microbial composition, abundance and activity (Marschner, 2003; Ling et al., 2016; Padhan et al., 2020). Although some studies have explored the effects of organic amendments on soil S transformation (Reddy et al., 2002; Blum et al., 2013; Malik et al., 2021), comparatively few studies have explored the effects of fertilisation, in particular, lime and mineral nutrient additions commonly used in grassland management on soil S cycling (Knights et al., 2001; Wang et al., 2019b). Lime application is known to increase soil pH and mitigate soil acidification (Hejcman et al., 2010a; Li et al., 2018), with greater mineralisation of organic matter and increased nutrient use efficiency found in limed soils (Ignacio Rangel-Castro et al., 2004; Guo et al., 2022). This is attributed to greater microbial activity (Fuentes et al., 2006) and microbial biomass (Kemmitt et al., 2006) under modified soil pH. Organic S turnover in soil is primarily regulated by microbial activity (Ji et al., 2023; Wang et al., 2023). It is therefore reasonable to assume that S mineralisation may increase with lime application.

The effects of mineral fertilisers on soil nutrient cycling and microorganisms are dependent on the fertiliser type, amount and mode of application. For example, additions of C or S can suppress S mineralisation, whereas N additions may enhance mineralisation (Ghani et al., 1992). Typically, the application of balanced mineral fertilisers (e.g., N, P, and potassium (K) can improve soil C, N and S stocks through increased primary productivity (e.g., greater incorporation of crop and forage residues) and subsequent rhizodeposition (Brar et al., 2013; Chaudhary et al., 2017). However, the long-term addition (i.e., >15 years) of imbalanced mineral fertilisers, characterized as sole N/P/K applications or fertilisers with an imbalanced N–P–K ratio, can have negative effects on the microbial community. This is due to the decrease in soil pH or a stoichiometric imbalance between nutrient availability and microbial demands (Geisseler and Scow, 2014; Eo and Park, 2016; Wang et al., 2020; Li et al., 2022). For example, an imbalanced N–P input ratio exceeding 15:1 has been shown to increase the abundance of bacterial taxa with high-N adaptability, leading to reduced organic C mineralisation (Li et al., 2022). In particular, the importance of P availability for microbial elemental turnover and growth strategy has recently been emphasized (Chen et al., 2019; Ji et al., 2023), with a higher N–P input ratio expected to affect soil S cycling.

Predicting the rate of soil S cycling requires a deep understanding of microbial utilisation of organic and inorganic S. Ma et al. (2021a, 2021b) found that microorganisms more easily utilised organic S, as indicated by the decomposition of ^{35}S, ^{15}N or ^{14}C radiolabelled amino acids, than inorganic S, with soil S cycling mainly regulated by the imbalance of elements between substrates and microbes. Additionally, excess glucose-C (2 g C kg^{-1}) or mineral N, P and S addition had little effect on the microbial uptake of S-containing amino acids (Ma et al., 2020; Wang et al., 2023). Yet, much less is known about how long-term fertilisation with lime and mineral nutrients change microbial utilisation of organic and inorganic S, and subsequently, the drivers regulating this S cycling process, remain largely unknown.

Here, we investigated the impact of fertilisation on soil S turnover and microbial S (organic S and inorganic S) uptake within an 82-year-old grassland experiment that received varying combinations of lime and mineral fertilisers. We hypothesized that (Fig. S1) soil pH and nutrient availability regulate soil S turnover and microbial S (organic S and inorganic S) uptake in this long-term fertilised grassland. Specifically, we hypothesized that:

(i) Long-term lime and mineral nutrients inputs would increase soil pH, alleviate acid stress on the microbial community and consequently enhance microbial activity, S turnover rates and microbial S uptake.
(ii) The effect of combined lime and mineral nutrients on S cycling depends on whether the mineral nutrient (N–P) is incorporated in a balanced manner. Specifically, (a) the combination of lime with balanced mineral nutrients (N plus P) would increase soil S turnover rates and microbial uptake inorganic S due to the increase in nutrient availability; (b) conversely, the combination of lime with imbalanced mineral nutrients (sole N or P) would decrease or not affect soil S turnover rates and microbial uptake inorganic S since the microbial community is nutrient-limited.

2. Materials and methods

2.1. Site description and field management

The Rengen Grassland Research Station at the University of Bonn was established in 1941 and is located in the Eifel Mountains, Germany (50°14′22″N, 6°49′34″E, 472–475 m a.s.l.) with an average annual precipitation and temperature of 811 and 6.9 °C (1934–1991), respectively (Sochorová et al., 2016). The soil is classified as a Stagnic Cambisol (IUSS Working Group WRB, 2006; Pätzold et al., 2013), comprising approximately 23 % sand, 54 % silt, and 23 % clay in the topsoil (0–10 cm) (Hejcman et al., 2007).

The experiment site was arranged with two blocks (Fig. S2 for field photography and design). Within each block, five fertiliser treatments (Ca; CaN; CaNP; CaNPKCl; $CaNPK_2SO_4$) with five replicates were distributed randomly, creating 25 plots (ca. 15 m^2 each) in each block. Further detail regarding the experimental design is provided by Hejcman et al. (2010c) and Sochorová et al. (2016). In 1988, the unfertilised control treatment was introduced on a strip adjacent to one block. The experimental plots were mown once a year in late summer from 1942 to 1944 and 1950–1961 for hay production, the experiment was not managed (fertilised or cut) during 1945–1950. All plots were mown twice a year in late June/early July and mid-October since 1962. From 1989 until 1995, forage biomass was measured by cutting the sward in each plot to ca. 2 cm (Schellberg et al., 2001). The annual application amount of nutrients of each treatment are as follows (average inputs in kg ha^{-1} yr^{-1}): Ca (715 Ca), CaN (752 Ca, 100 N), CaNP (936 Ca, 100 N, 35 P), CaNPKCl (936 Ca, 100 N, 35 P, 133 K), $CaNPK_2SO_4$ (936 Ca, 100 N, 35 P, 133 K). The nutrients are applied as the following fertilisers: quick lime, limestone ammonium nitrate (KAS 27), potassium chloride, basic slag and potassium sulphate (Smarda et al., 2013). Further experimental information regarding the plant composition and fertilisers is provided in previous studies (Hejcman et al., 2007; Pätzold et al., 2013; Smarda et al., 2013).

2.2. Soil sampling

Soil samples (0–10 cm) were collected from five fertilised treatments and one control ($n = 5$ per treatment) in November 2022. To account for spatial variability, six separate soil samples were obtained from each plot using a soil auger (⌀ 3 cm) prior to pooling into one replicate sample, avoiding both the plot margin and central area. Soil samples were then sieved to < 2 mm to remove debris (e.g., large roots and stones) before air drying. Samples were then ground and sieved to 2 mm for pH determination, and 0.15 mm to determine soil chemical characteristics.

2.3. Soil chemical characteristics

The initial gravimetric moisture content of field-moist soil was determined after drying for 24 h at 105 °C. Air-dried soil was then used in a DI H_2O extraction (1:2.5 w/v) to determine soil pH using a standard electrode. Soil organic carbon (SOC) and total nitrogen (TN) were determined by elemental analysis (vario EL cube; Elementar, Hanau, Germany) after decalcification with hydrochloric acid (5 % HCl) (Pötter et al., 2020). Total sulphur (TS) and total phosphorus (TP) were measured by inductively coupled plasma optical emission spectroscopy (Thermo Scientific ICP-OES 6500, Waltham MA, USA). Soil sulphate was extracted with 0.01 M $CaCl_2$ (1:5 w/v) shaken at 200 rev min^{-1} for 30

min (Eriksen et al., 1995) before determining by ion chromatography (Metrohm IC 850, Germany).

2.4. S-containing amino acids methionine decomposition

Five gram of field moist soil ($n = 5$ per treatment) was placed in a sterile 50 ml polypropylene centrifuge tube before 1 ml of 100 μM ^{14}C-labelled (14.92 mg C kg^{-1}, 1.63 kBq ml^{-1}; PerkinElmer Inc, Waltham, MA) or ^{35}S-labelled methionine (2.98 mg S kg^{-1}, 0.27 kBq ml^{-1}; PerkinElmer Inc, Waltham, MA) was pipetted evenly onto the soil surface. After application, 1 ml of 1 M NaOH was placed above the soil surface to capture respired $^{14}CO_2$. The NaOH traps and soil samples were collected 1 h, 3 h, 6 h, 24 h, 48 h, and 96 h after the application. Soil samples were extracted with 0.5 M K_2SO_4 ($^{14}C_{K2SO4}$) or 0.01 M $CaCl_2$ ($^{35}S_{CaCl2}$) (1:5 w/v, 200 rpm for 30 min). An additional set of samples was extracted with 0.5 M K_2SO_4 after 24 h of $CHCl_3$ fumigation with a k_{EC} correction factor of 0.45 applied (Voroney et al., 2008). All extracts were then centrifuged (18000 *g*, 5 min) and 1 ml of the supernatant was recovered. 4 ml of OptiPhase HiSafe 3 scintillation fluid (Revvity Health Sciences B.V, Groningen, the Netherlands) was added to the NaOH traps and supernatant before determining the ^{14}C and ^{35}S activity by a Hidex 600SLE liquid scintillation counter (Hidex Oy, Turku, Finland). The ^{14}C in microbial biomass was calculated as the difference in ^{14}C activity in K_2SO_4 extracts between fumigated and non-fumigated soil samples.

The ^{35}S recovered by $CaCl_2$ indicated the ^{35}S remaining in the soil (not immobilised by microorganisms). Due to the low sorption of amino acids and sulphate to alkaline soil particles (Curtin and Syers, 1990; Jones, 1999), ^{35}S that cannot be extracted by $CaCl_2$ is recognised as the ^{35}S immobilised in microbial biomass (^{35}S-MB). This was calculated as the difference in ^{35}S activity between the total added ^{35}S-labelled methionine and ^{35}S extracted by $CaCl_2$.^{35}S activity was corrected for its natural decay (half-life ($t_{1/2}$) = 87.51 days).

2.5. Sulphate immobilisation

To determine microbial utilisation of inorganic sulphate, 1 ml of 50 μM $Na_2^{35}SO_4$ (5.12 kBq ml^{-1}) was added to 5 g of soil ($n = 5$ per treatment). ^{35}S recovery was determined by extracting soil with 0.01 M $CaCl_2$ (1:5 w/v) at 6 h, 24 h, 48 h and 96 h. As described previously, immobilised inorganic sulphate in microbial biomass was calculated as the difference in ^{35}S activity between the total added $Na_2^{35}SO_4$ and ^{35}S extracted by $CaCl_2$ (Ma et al., 2020).

2.6. Gross sulphur transformation rates

Gross SO_4^{2-} immobilisation (GI) and mineralisation (GM) in soils were detected using the isotope pool dilution method (Eriksen, 2005). Briefly, 1 ml of 50 μM $Na_2^{35}SO_4$ (5.12 kBq ml^{-1}) was added to 5 g of soil. After 48 and 96 h, soil was extracted with 0.01 M $CaCl_2$ (1:5 w/v, 200 rev min^{-1} for 30 min) and centrifuged at 18000 *g* for 5 min. The stable ^{32}S–SO_4^{2-} content in the subsequent supernatant was then measured on a PowerWave XS microplate reader (Biotek, Waltham MA, USA). The ^{35}S activity in the $CaCl_2$ extractions ($^{35}S_{CaCl2}$) indicates total extractable S, which was determined on the liquid scintillation counter as described previously.

Afterwards, to determine the ^{35}S–SO_4^{2-} content, 0.75 ml $BaCl_2$ was added to 0.75 ml of the $CaCl_2$ extract to precipitate the $^{35}SO_4^{2-}$, with the suspensions then centrifuged at 18000 *g* for 5 min. The remaining ^{35}S activity in the supernatant of $BaCl_2$ treatments ($^{35}S_{CaCl2+BaCl2}$) was measured using the liquid scintillation counter. Thus, the difference in ^{35}S activity in the two measurements was taken as the amount of ^{35}S–SO_4^{2-} in the solution:

$$^{35}S\text{–}SO_4^{2-} = {}^{35}S_{CaCl2} - {}^{35}S_{CaCl2+BaCl2} \quad (1)$$

Gross S mineralisation and immobilisation rates were calculated as mg S kg^{-1} d^{-1} using the isotope dilution equation provided by Eriksen (2005):

$$m = \frac{(Q1 - Q2)\ln\left(\frac{A1}{A2}\right)}{(t2 - t1)\ln(Q1/Q2)} \quad (2)$$

$$i = m - \frac{(Q2 - Q1)}{(t2 - t1)} \quad (3)$$

Where Q_1 and Q_2 are stable ^{32}S–SO_4^{2-} concentrations and A_1 and A_2 are the amount of ^{35}S–SO_4^{2-} at times t_1 and t_2 after labelling.

2.7. Sulphate and methionine sorption to soil

To measure the sorption of sulphate and methionine to the soil's solid phase, 2 g of field-moist soil was placed in a sterile 20 ml polypropylene vial. To minimise microbial uptake of the added substrates during the sorption assay, the vials were subsequently sealed and heated at 80 °C overnight prior to use (Kuzyakov and Jones, 2006). After cooling, 10 ml of ice-cold 0.01 M KCl containing either ^{14}C-labelled methionine (100 μM; 0.47 kBq ml^{-1}) or ^{35}S-sodium sulphate (100 μM; 2.89 kBq ml^{-1}) was added to the soil and the samples were shaken at 200 rev min^{-1} for 1 h. The samples were then centrifuged (18,000 *g*, 5 min) and the amount of ^{14}C-methionine or ^{35}S-sulphate remaining in the supernatant was determined by liquid scintillation counting as described previously. The experiment was also repeated in the same way, but was performed using a background electrolyte solution of 0.01 M $CaCl_2$. At the end of the experiment, the soil was also shaken with 1 M KCl, as described above, to evaluate the recovery (desorption) of any ^{14}C-methionine of ^{35}S-sulphate held on the solid phase. The net charge of methionine at each soil pH value was calculated using the Henderson-Hasselbalch equation using pKa values of 2.28 and 9.21 for the carboxyl and amino groups, respectively.

2.8. Statistical analysis

Amino acid mineralisation is generally biphasic, describing as a two process double first order kinetic decay model (Glanville et al., 2016):

$$f = \left(a1 \times exp^{-k1t}\right) + \left(a2 \times exp^{-k2t}\right) \quad (4)$$

Where f is methionine remaining in the soil, and a_1 and a_2 are the quantity of ^{14}C partitioned into primary mineralisation (pool 1, catabolic process, microbial release as CO_2) and secondary slower mineralisation (pool 2, biomass production), respectively, k_1 and k_2 are the exponential coefficients for pool 1 and pool 2, respectively, and t represents time.

The half-life ($t_{1/2}$) for pool 1 is calculated using equation (5) below. We did not calculate the half-life for pool 2 because of the unknown connectivity between pool a_1 and pool a_2 (Boddy et al., 2008).

$$t_{1/2} = (\ln 2)/k1 \quad (5)$$

Microbial carbon use efficiency (CUE) was calculated as follows:

$$CUE = a2/(a1 + a2) \quad (6)$$

A two-compartment model was used to describe the S immobilisation after the $Na_2^{35}SO_4$ was added to the soil (Houle et al., 2001). Accordingly, the double first-order model described the depletion of $Na_2^{35}SO_4$ similar to ^{35}S-methionine in equation (4), in this case, f is the ^{35}S remaining in the extractions, a_1 and a_2 indicate the sizes of fast and slow ^{35}S uptake routes respectively, and k_1 is the exponential coefficients describing the rate of ^{35}S transformation of fast uptake route and the half-life of fast uptake route was calculated as Equation (5) (Wang et al., 2023).

One-way ANOVA (Duncan test, significance level of $p < 0.05$) was used to test the differences among the six treatments (^{14}C and ^{35}S

decomposition dataset). A Tukey HSD post-hoc test was used to identify significant pairs in the soil sorption dataset. A paired *t*-test was used to compare the sorption of sulphate and methionine to the solid phase between treatments. Stepwise regression analysis was carried out by SPSS v. 26.0 (SPSS Inc., Chicago, USA) to find the key factors regulating the gross S transformation rates and the $Na_2^{35}SO_4$ immobilisation. Kruskal-Wallis H test ($p < 0.05$) was used when the data was not normally distributed. The curves of kinetic models were fitted to the ^{14}C and ^{35}S data using SigmaPlot (SPSS Inc., Chicago, USA), and the other figures and correlations analysis were carried out in the "ggplot2" R (version 4.2.1) package.

3. Results

3.1. Soil C, N, P and S content and their stoichiometry

After 82 years, soil pH in plots fertilised with lime and N, P or S fertiliser (7.03–7.60) was significantly higher ($p < 0.05$; Table 1) relative to the control (5.36 ± 0.18). However, no significant differences ($p > 0.05$; Table 1) in soil pH were found between the five fertilised treatments when combined with lime.

Soil organic C content significantly decreased on average by 27 % in limed soil compared to the control (Table 1), with SOC in treatments receiving mineral P (CaNP, CaNPKCl, $CaNPK_2SO_4$) significantly lower than the control (42 ± 3.8 g kg^{-1}). The total phosphorus (TP) contents under the treatments with P application (CaNP, CaNPKCl and $CaNPK_2SO_4$) were significantly higher ($p < 0.05$; Table 1) than in treatments without P addition (Control, Ca and CaN). Soil TS was highest in the $CaNPK_2SO_4$ treatment (0.67 ± 0.05 g kg^{-1}), which was significantly greater than the control (0.38 ± 0.02 g kg^{-1}), Ca (0.53 ± 0.02 g kg^{-1}) and CaN (0.52 ± 0.02 g kg^{-1}) treatments.

Accordingly, the stoichiometry of C, N, P and S changed significantly after 82 years of fertilisation (Table S1). The soil C:N:P:S stoichiometry ratios under these treatments were 109:8:1:1 (control), 63:5:0.8:1 (Ca), 70:6:0.7:1 (CaN), 37:4:2:1 (CaNP), 51:5:2:1 (CaNPKCl), 45:4:2:1 ($CaNPK_2SO_4$). Treatments without P addition (Control, Ca, and CaN) had remarkably higher SOC:TP, SOC:TS, TN:TS and TN:TP ratios and lower TP:TS ratios than treatments with P fertiliser (CaNP, CaNPKCl and $CaNPK_2SO_4$).

Table 1
Soil physical and chemical properties under different fertilisation treatments.

Treatments	Soil pH	TP (g kg^{-1})	TS (g kg^{-1})	Sulphate (mg kg^{-1})	SOC (g kg^{-1})	TN (g kg^{-1})
Control	5.36 ± 0.18^{c}	0.43 ± 0.03^{c}	0.38 ± 0.02^{d}	8.43 ± 0.87^{b}	42 ± 3.8^{a}	3.07 ± 0.22^{a}
Ca	7.30 ± 0.15^{a}	0.43 ± 0.03^{c}	0.53 ± 0.02^{c}	8.30 ± 0.24^{b}	34 ± 1.4 ^{bc}	2.92 ± 0.09^{a}
CaN	7.32 ± 0.09^{a}	0.36 ± 0.01^{c}	0.52 ± 0.02^{c}	7.43 ± 0.71^{b}	36 ± 2.7 ^{ab}	2.85 ± 0.08^{a}
CaNP	7.60 ± 0.14^{a}	1.58 ± 0.14^{a}	0.65 ± 0.05 ^{ab}	7.63 ± 0.44^{b}	24 ± 2.3^{d}	2.74 ± 0.27^{a}
CaNPKCl	7.03 ± 0.25^{b}	1.25 ± 0.10^{b}	0.56 ± 0.04 ^{bc}	11.76 ± 1.48^{a}	28 ± 1.5 ^{cd}	2.67 ± 0.14^{a}
$CaNPK_2SO_4$	7.49 ± 0.04^{a}	1.36 ± 0.09 ^{ab}	0.67 ± 0.05^{a}	12.59 ± 1.67^{a}	30 ± 1.8 ^{bcd}	2.95 ± 0.08^{a}

SOC: soil organic carbon, TN: total nitrogen, TP: total phosphorus, TS: total sulphur. Values represent means ± SEM. Different superscripted letters indicate statistical differences among the six treatments ($p < 0.05$).

3.2. Depletion of ^{14}C-labelled methionine

Within 1 h, more than 70 % of the added methionine was immobilised by the microbial biomass (Fig. 1). With increasing incubation time, the $^{14}CO_2$ production increased as the microbial immobilised ^{14}C decreased. Overall, fertilisation had no significant effect on the microbial uptake of ^{14}C-methionine and $^{14}CO_2$ production in soils (Fig. 1). However, after 96 h incubation, the $^{14}CO_2$ production relative to initial ^{14}C addition in treatment CaN (47 ± 1.4 %) was relatively higher than in other treatments (38–45 %). Whereas, ^{14}C immobilised in the microbial biomass in the CaN treatment (24 ± 0.3 %) was relatively lower than the other treatments (29–33 %).

The decomposition of methionine is generally biphasic, described as a two-process double first-order kinetic exponential decay model (Table 2, Fig. S3). Overall, there were no significant differences between treatments with P addition (CaNP, CaNPKCl and $CaNPK_2SO_4$) and the control, regarding the half-life of pool a_1 (catabolic process, microbial respiration) and microbial CUE. However, pool a_2 (anabolic process, biomass production) and CUE under CaN treatment were significantly lower than the unfertilised control and treatments receiving P fertiliser.

3.3. Depletion of ^{35}S-labelled methionine and the immobilisation of $Na_2^{35}SO_4$

After 1 h, about 30 % of the added ^{35}S-methionine could be recovered from the soil in all treatments. In comparison, more than 70 % of the total added $Na_2^{35}SO_4$ was recovered from the fertilised treatments except for the control (36 ± 2.5 %) after 6 h (Table S2). The microbial immobilisation of $^{35}SO_4^{2-}$ was much slower than the immobilisation of ^{35}S contained in methionine (Fig. 2). Fertilisation had no significant effect on the microbial immobilisation of ^{35}S-methionine, with values ranging from 68 to 76 %. However, microbial immobilisation of $Na_2^{35}SO_4$ (after 96 h incubation) in the control treatment was 69 ± 1.3 %, which was significantly higher ($p < 0.05$) than Ca (60 ± 3.7 %), CaN (48 ± 4.3 %), CaNP (37 ± 0.7 %), CaNPKCl (36 ± 0.6 %) and $CaNPK_2SO_4$ (44 ± 2.0 %).

The recovery of ^{35}S-methionine and $Na_2^{35}SO_4$ from soil was best described by a double first-order exponential decay model (Table 3, Fig. S4, $R^2 > 0.98$). Fertilisation had no significant influence on the ^{35}S-methionine depletion in terms of S allocation to pools a_1 and a_2 (fast and slow ^{35}S uptake routes) and the half-life time of S uptake. Control had a higher allocation of ^{35}S to pool a_1 (64 %) for the fast $Na_2^{35}SO_4$ uptake route than other fertilisation treatments (18–30 %), and the half-life time of the fast $Na_2^{35}SO_4$ uptake route was 0.17 h under control, which was lower than other fertilised treatments with half-life ranging from 2.1 to 2.5 h.

3.4. Gross S transformation rates

In general, the gross S immobilisation rate (GI) was higher than the gross S mineralisation (GM) rate (Fig. 3, S5). A significant difference in GM and GI was observed between treatments CaN and CaNP, indicating a strong effect of P addition. Additionally, GM in treatments with P addition ordered as $CaNPK_2SO_4$ < CaNP < CaNPKCl, GM increased with additional P nutrients, but declined under the treatment $CaNPK_2SO_4$, which indicated the negative effect of S fertiliser on GM. Treatment CaNP had the highest GI (2.08 mg S kg^{-1} soil per day), followed by treatment CaNPKCl, and GI had a significantly positive relationship with TP ($R = 0.71$, $p < 0.001$) and TS ($R = 0.59$, $p < 0.001$) content.

3.5. Sulphate and methionine sorption to soil

Overall, the sorption of sulphate and methionine to the solid phase was low, although the sorption of methionine was greater than for sulphate ($p < 0.01$; Table S3). The sorption of sulphate was highest in the control soil ($p < 0.001$), while in contrast, it had the lowest methionine

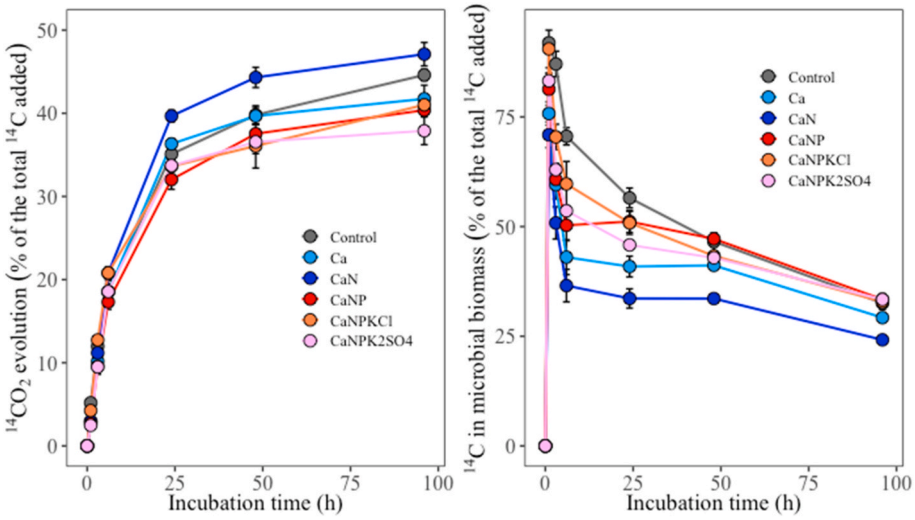

Fig. 1. Time course of the proportion of ^{14}C-labelled methionine immobilised in soil microbial biomass (^{14}C-MB) and released as $^{14}CO_2$ under different fertilisation treatments. Data are means ± SEM.

Table 2
Effects of fertilisation on the kinetic parameters describing the decomposition of ^{14}C-labelled methionine.

Treatments	a_1	a_2	CUE	k_1	a_1 $t_{1/2}$ (h)
Control	33.6 ± 0.96^{c}	65.9 ± 2.14^{ab}	0.66 ± 0.01^{ab}	0.13 ± 0.01^{b}	5.63 ± 0.38^{ab}
Ca	38.3 ± 1.23^{b}	62.3 ± 2.76^{b}	0.62 ± 0.01^{bc}	0.10 ± 0.01^{b}	6.26 ± 0.44^{a}
CaN	43.0 ± 1.25^{a}	57.7 ± 2.04^{c}	0.58 ± 0.01^{c}	0.11 ± 0.01^{b}	6.21 ± 0.25^{a}
CaNP	35.2 ± 1.70^{bc}	65.2 ± 1.52^{ab}	0.66 ± 0.02^{a}	0.12 ± 0.02^{b}	6.12 ± 0.72^{a}
CaNPKCl	32.1 ± 2.06^{c}	68.2 ± 4.11^{a}	0.68 ± 0.02^{a}	0.17 ± 0.02^{a}	4.16 ± 0.45^{b}
$CaNPK_2SO_4$	35.1 ± 1.33^{bc}	65.8 ± 2.98^{ab}	0.65 ± 0.01^{ab}	0.13 ± 0.01^{b}	5.59 ± 0.43^{ab}

a_1 and a_2 are the quantity of ^{14}C partitioned into primary mineralisation (pool 1, catabolic process, microbial release CO_2) and secondary slower mineralisation (pool 2, anabolic process, biomass production), respectively. k_1 is the exponential coefficient for pool 1. a_1 $t_{1/2}$ indicates the half-life for pool 1. CUE: microbial carbon use efficiency. Different superscripted letters indicate statistical differences among the six treatments ($p < 0.05$). Values represent mean ± SEM.

sorption ($p < 0.01$). The sorption of sulphate and methionine were similar in a background electrolyte of 0.01 M KCl and 0.01 M $CaCl_2$ ($p = 0.41$ and $p = 0.22$, respectively). The 1 M KCl extract recovered 97.5 ± 1.6 % of the added sulphate and 95.9 ± 0.8 % of the added methionine (sulphate *vs.* methionine, $p = 0.40$). Chemical equilibria calculations indicated that the net charge of methionine was neutral at all the soil pH values.

3.6. Relationship between soil properties and S cycling and microbial carbon use efficiency

The gross S transformation rates were negatively correlated with SOC and SOC to nutrients ratios (SOC:TN, SOC:TS and SOC:TP) (Fig. 4). Microbial CUE was positively correlated with TP and TP:TS ratio (Fig. 4 and S6, TP: R = 0.52, $p = 0.005$; TP:TS: R = 0.56, $p = 0.002$). The microbial immobilisation of $Na_2^{35}SO_4$ at different time points was highly correlated with the soil pH, SOC, TP and TS and their stoichiometric ratios (Fig. 4). Soil TP:TS ratio and pH jointly explained 40 % of the variance of CUE (Table 4). Soil TP or TP:TS ratio was the main factor regulating gross S transformation rates and microbial CUE. Within 48 h incubation, more than 80 % of the variation in $Na_2^{35}SO_4$ immobilisation was mainly explained by soil pH and TP:TS ratio jointly. Soil pH was the dominant factor affecting $Na_2^{35}SO_4$ immobilisation.

4. Discussion

4.1. Effect of long-term fertilisation on soil chemical properties

Our results were in line with previous findings that lime addition has positive effects on plant yield and soil pH (Pagani and Mallarino, 2012; Sochorová et al., 2016). Most importantly, previous studies utilizing this field experiment have reported that plants in treatments without P fertiliser display a negative P balance, indicating that the experiment is limited by P, rather than N (Chytrý et al., 2009; Hejcman et al., 2010b; Smarda et al., 2013). This finding was evident by the soil stoichiometric ratios in our study (Table S1). The low P content under the Ca and CaN treatments, may potentially derived from enhanced forage production (Sochorová et al., 2016) and high forage P removal, stimulated from lime and N addition (Liao et al., 2020). Additionally, although grass yield increases with P addition, long-term P fertiliser applications can decrease species richness in this grassland (Hejcman et al., 2010a).

However, it is interesting to note that in our study, long-term liming (especially CaNP) significantly decreased SOC by 27 %. Our finding supports similar research on this grassland from Sochorová et al. (2016), who found that while long-term fertiliser applications can improve forage production, they can also significantly (−20 %) reduce SOC. This can be attributed to the following mechanisms: (1) greater C allocation to aboveground shoots instead of roots when soil is enriched with N and P (Sochorová et al., 2016), (2) there was approximately double forage P content within the CaNP than other treatments (Hejcman et al., 2010c), with this P-rich organic substrates, greater C tends to be removed due to faster degradation of high-quality inputs (Freschet et al., 2013) or (3) when the soil microbial P limitation is removed, soil C pool decreases (Li et al., 2022). However, we are unable to draw a full C balance of this meadow grassland experiment owing to the limited historical records. Further research is needed to better understand the mechanisms that control the accrual of soil C stocks in these limed grassland systems. Nevertheless, based on our findings, careful consideration of the application of lime and other management practices is warranted to enhance below-ground C sequestration. Summarizing, microbial activity is likely to be limited by both C and P in limed soil without P fertiliser, and by C in limed soil with P fertiliser.

The soil S pool increased significantly following lime application, with a greater positive effect observed when applied with P fertiliser

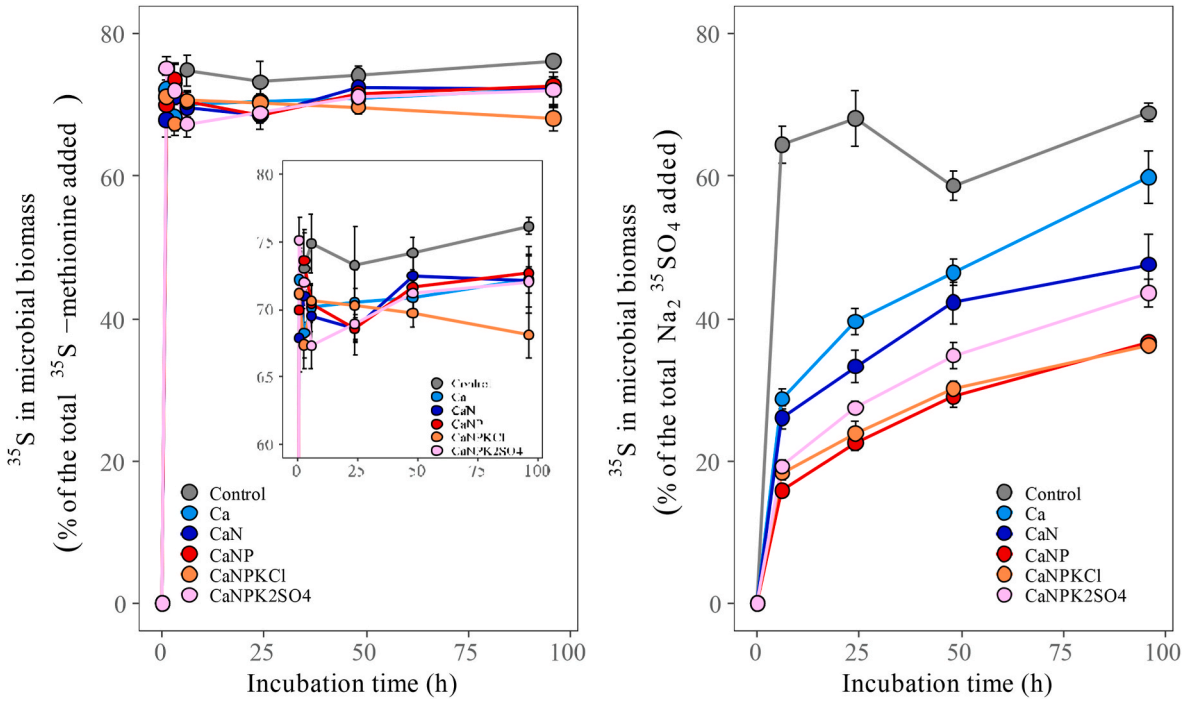

Fig. 2. Time course of the microbial immobilisation of ^{35}S-labelled methionine and inorganic $Na_2^{35}SO_4$ under different fertilisation treatments. Data are means ± SEM.

Table 3
Effects of fertilisation on the kinetic parameters describing the decomposition of^{35}S-labelled methionine and the recovery of $Na_2^{35}SO_4$.

	Treatments	a_1	k_1	a_2	k_1 $t_{1/2}$ (h)
^{35}S-methionine	Control	72.86	3.74	27.15	0.185
	Ca	69.54	27.08	30.46	0.026
	CaN	69.78	3.58	30.22	0.194
	CaNP	70.97	4.23	29.03	0.165
	CaNPKCl	70.00	23.62	30.00	0.029
	$CaNPK_2SO_4$	71.57	23.06	28.43	0.030
$Na_2^{35}SO_4$	Control	63.73	4.11	36.27	0.169
	Ca	30.47	0.335	69.53	2.1
	CaN	29.56	0.301	70.44	2.3
	CaNP	17.94	0.272	82.06	2.5
	CaNPKCl	20.25	0.312	79.74	2.2
	$CaNPK_2SO_4$	21.80	0.273	78.20	2.5

a_1 and a_2 are the estimated pool sizes for the fast and slow substrate uptake routes. k_1 and k_2 are the exponential coefficients for the fast and slow substrate uptake routes, respectively. k_1 $t_{1/2}$ indicates the half-life for the fast substrate uptake route.

Table 4
Effects of fertilisation on the stepwise regression analysis describing microbial carbon use efficiency (CUE), gross S transformation and $Na_2^{35}SO_4$ immobilisation.

Variables	Model	R^2	F	*P* value
CUE	0.661*TP:TS-0.372*pH	0.400	10.005	<0.0001
GM	0.652*TP:TS	0.403	19.260	<0.0001
GI	0.707*TP	0.480	25.915	<0.0001
6h	0.464*SOC:TS-0.527*pH	0.888	99.238	<0.0001
24h	−0.353*TP:TS-0.746*pH	0.839	71.163	<0.0001
48h	−0.559*TP:TS-0.596*pH	0.845	68.025	<0.0001
96h	0.583*SOC:TS-0.342*TP:TS	0.696	28.670	<0.0001

GM: gross S mineralisation rate, GI: gross S immobilisation rate, SOC: soil organic carbon, TP: total phosphorus, TS: total sulphur, SOC:TS: the SOC to TS ratio, TP:TS: TP to TS ratio. 6 h, 24 h, 48 h, and 96 h indicate the immobilised ^{35}S at different time points after the addition of $Na_2^{35}SO_4$.

relative to N fertiliser without P addition. It was shown that the interaction of lime and P fertiliser is more efficient in increasing the S pool. This was consistent with previous studies which found that long-term N fertiliser and lime addition did increase the mineralisation of organic S (Knights et al., 2001; Šiaudinis et al., 2017). The greater S accumulations in our study under fertilised treatments may be due to two reasons: (1) the field experiment had an initially low soil S content, as indicated by the higher SOC:TS ratio (110, Table S1) in the unfertilised control, suggesting a greater potential for S immobilisation, and (2) the P fertiliser addition in P-limited soil increased soil microbial activity and thus favoured S mineralisation (Nguyen and Goh, 2012).

4.2. *Effect of long-term fertilisation on ^{14}C-methionine decomposition*

Microbial carbon use efficiency describes the relative partitioning of carbon between anabolic and catabolic metabolism processes (Sinsabaugh et al., 2013; Jones et al., 2018). The acidic soil environments with low soil pH were found to be the most constraint factor shaping soil microbial communities and decreasing microbial activities (Liu et al., 2018; Wang et al., 2019a). Jones et al. (2019) also found that soil pH was the major driver of microbial CUE across 970 agricultural soils with CUE progressively declining as the soil pH fell below 5.5, but no significant changes of CUE were observed if the soil pH was higher than 5.5 (5.5–7.5). These studies all suggested that soil microorganisms tend to be suppressed by high Al^{3+} and H^+ (and possibly Mn^{2+}) at low soil pH, but not at higher soil pHs when Al^{3+} becomes largely insoluble. Thus, microbial CUE was not expected to be greatly affected by soil pH in our study due to the neutral soil conditions prevailing after lime addition. Although Jones et al. (2019) reported no effect of nutrient stoichiometry on microbial CUE, our data suggests that CUE was more affected by soil P content and TP:TS stoichiometry. Our findings, however, are supported by the results of Creamer et al. (2014) who showed a strong response in CUE to nutrient addition.

Microbial CUE in this study significantly decreased in the CaN treatment relative to control and treatments receiving P. This conflicts with the findings from Barcelos et al. (2021), who found that lime and N fertiliser had minimal effect on CUE in a no-till field experiment. The

most limiting nutrient (e.g., N or P) may control the soil organic matter decomposition process since microbes require a careful nutrient balance (Sinsabaugh et al., 2013). Relative to P and K availability, N availability is believed to control C cycling by increasing microbial CUE (Spohn et al., 2016). However, in our study, greater microbial respiration was found in soils not receiving P fertiliser, especially within the N addition treatment (CaN plots). Increased respiration corresponds to a lower CUE in nutrient-limited conditions (Craine et al., 2007; Manzoni et al., 2012). Therefore, our findings suggested that the microbial community tends to be more P-limited when decomposing high-quality C (with a low C to N ratio), resulting from the high P demands of fast-growing microorganisms under treatments without P fertiliser (Ca and CaN). A previous study also found that soil P limitation of microbial decomposition may have profound implications for C cycling (Cleveland et al., 2002). We thus concluded that microbial P limitation in limed soil with N decreased the microbial C allocated to growth, resulting in greater microbial respiration. Lime with balanced nutrient input alleviated microbial P limitation and further increased CUE.

4.3. *Effect of long-term fertilisation on S cycling*

Gross S immobilisation rates were higher than the gross mineralisation rates (Fig. 3, S5), which indicated a net immobilisation of S in the microbial biomass. Our results suggested that soil P content controls gross S transformation rates, subsequently confirming our second (ii) hypothesis: higher P availability corresponds to higher S turnover rates under lime with balanced (N + P) nutrient addition (ii a), while low P availability leads to lower S turnover rates following the application of lime without P fertiliser (ii b).

A previous study indicated that the succession of microbial S function during saline-alkali soil restoration was mainly driven by the availability of P (Ji et al., 2023). The deficiency of soil P limited soil microbial activity under N-sufficient conditions, subsequently constrained S flux rates. The addition of P fertiliser with N and lime mitigated this negative effect on S transformation. This is in accordance with the former finding that P addition increased the decomposition rate of organic residues with a strong interaction with N (Cheshire and Chapman, 1996). Moreover, as biological mineralisation is driven by the microbial requirement for organic C for energy, the lower SOC content under treatments CaNP and CaNPKCl may be driving the higher GI. Additionally, our results indicate that higher gross S transformation rates correspond to higher S content. However, relative to the other treatments with higher S content (CaNP and CaNPKCl), soils treated with S ($CaNPK_2SO_4$) fertiliser had lower gross S transformation rates. The stimulation of sulfatase activity leads to higher mineralisation due to the decline of SO_4^{2-} level (McLaren et al., 1985). Therefore, we assume

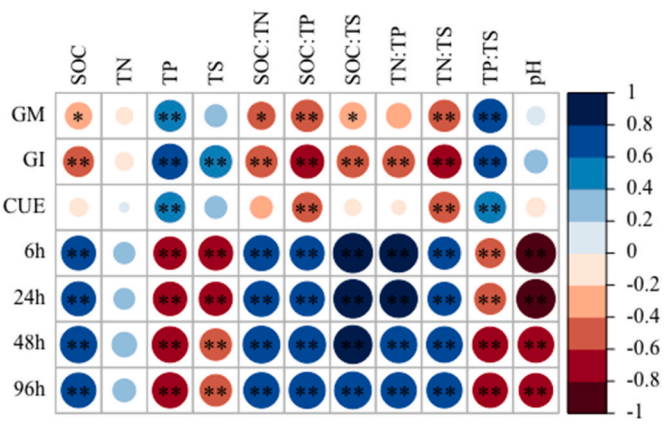

Fig. 4. The Pearson's correlation relationship between carbon use efficiency (CUE), gross S transformation rates (GM and GI), the immobilisation of $Na_2^{35}SO_4$ and soil properties.
GM: gross sulphur mineralisation rate, GI: gross sulphur immobilisation rate, 6h, 24h, 48h, and 96h indicated the $^{35}SO_4^{2-}$ was immobilised as biomass at different time points after the addition of $Na_2^{35}SO_4$, SOC: soil organic carbon, TN: soil total nitrogen, TP: soil total phosphorus, TS: soil total sulphur, SOC:TN: the SOC to TN ratio, SOC:TP: the SOC to TP ratio, SOC:TS: the SOC to TS ratio, TN:TP: the TN to TP ratio, TN:TS: the TN to TS ratio, TP:TS: the TP to TS ratio. The colour gradient in the heatmap indicates Pearson's correlation coefficients. The Asterisks in the square represent different significance levels at $p < 0.05$ (*) and $p < 0.01$ (**).

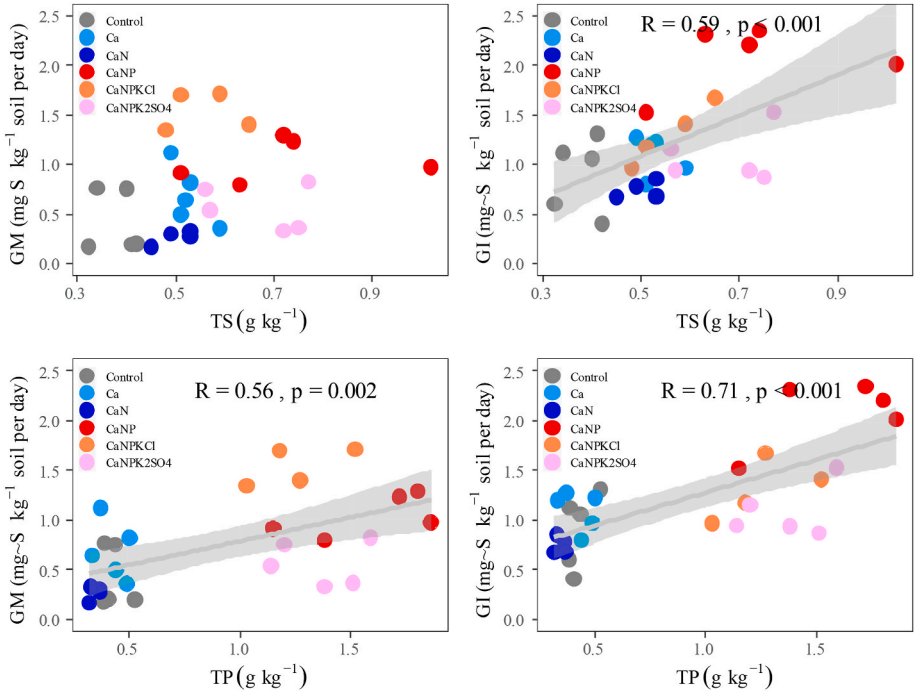

Fig. 3. Gross S mineralisation (GM) and immobilisation (GI) rates versus soil total sulphur (TS) and total phosphorus (TP) content under different treatments. The R value indicates Pearson's correlation coefficients.

that the lower microbial sulfatase enzyme production for organic S degradation with extra inorganic S inputs leads to lower S transformation rates.

However, contrary to our expectations, our first hypothesis (i) was rejected as long-term fertilisation had no significant effect on microbial utilisation of organic S, with microorganisms rapidly immobilising the amino acids regardless of soil nutrient conditions and soil pH. In our study, over 70 % of the S was immobilised in microbial biomass. Microorganisms immobilised most of the S as biomass, with the immobilisation process dominating methionine decomposition. The short-term (<3 h) organic S utilisation by microorganisms has been found to be determined by the amino acid structure, not by the added substrate, while long-term microbial metabolism of organic S as sulphate is driven by microbial controlled stoichiometry (Ma et al., 2021a).

Soil microorganisms had a faster and higher uptake of S-containing amino acids compared with the inorganic S. It indicated that soil microbes prefer to use organic S rather than inorganic S as an S source. This result was consistent with Ma et al. (2021b), who found that the microorganisms had a greater capacity to utilise low-concentration amino acids, with the decomposition of amino acids relying more on microbial biomass (biological process) as the most active organic S fraction, even though it only accounts for less than 3 % of the organic S (Banerjee and Chapman, 1996). Our results from the gross S transformation rates based on the isotope dilution method were linear with the finding from the immobilisation of inorganic S. Most importantly, in our study, the immobilisation of inorganic S significantly decreased with long-term lime application, and not only tightly linked with soil pH, but also positively correlated with soil C availability. With adequate available C and N conditions, the immobilisation of SO_4^{2-} to organic S fractions happens with high microbial activity (Fitzgerald et al., 1982). In our study, relative to limed soil, higher C availability in unfertilised plots may lead to the higher microbial immobilisation of sulphate. Therefore, contrary to our first hypothesis (i), long-term application of lime and mineral nutrients decreased microbial utilisation of inorganic S, and it was mainly attributed to either increased soil pH or the low C availability resulting from long-term lime application. However, two different effects, i.e., the soil pH increase and organic carbon content decrease, resulted from the long-term lime application simultaneously, the individual contribution of soil pH and C availability to the decrease in microbial utilisation inorganic S therefore needs further study.

4.4. Sulphate and methionine sorption to soil

The soil was sterilised prior to performing the sorption experiments and an ice-cold electrolyte was used to minimise microbial uptake (Rousk and Jones, 2010). The high recovery of ^{14}C and ^{35}S labels at the end of the experiment indicated minimal microbial immobilisation of the added label. Overall, the amount of methionine and sulphate sorption were very low in agreement with previous studies on the sorption of neutral amino acid in soil (Rothstein, 2010; Ma et al., 2022). We ascribe the higher sulphate sorption in the control treatment to the lower pH values in this soil relative to the others (Marsh et al., 2011). In the case of the zwitterion methionine, the lower sorption in the control soil relative to other treatments can also potentially be ascribed to alterations in soil surface pH (Yeasmin et al., 2014).

5. Conclusion

Soil pH and nutrient availability regulate soil C and S cycling in this grassland system. However, in contrast to our first hypothesis (i), soil pH and nutrient availability had no significant effect on microbial uptake of organic S as the low molecular weight amino acid (methionine) is preferred by microorganisms relative to inorganic S and rapidly immobilised as microbial biomass. Microbial inorganic S immobilisation in particular significantly decreased in long-term limed soils, despite liming increasing soil pH values. This might be attributed to the decreased soil organic carbon content under liming, especially when combined with N fertiliser, which enhanced microbial C limitation. Therefore, the application of lime in grassland management should be considered carefully, as exogenous carbon is commonly recommended with lime to compensate for the potential C loss. Our second hypothesis (ii) was confirmed, after long-term liming with N, low P availability became the primary factor limiting soil microorganisms, further decreasing CUE and constraining gross S transformation. The application of lime plus N and P fertilisers was found to increase P availability, resulting in higher gross S transformation rates. Additionally, our results indicate lower microbial gross S transformation rates with S fertiliser addition. Our findings highlight the importance of P bioavailability on soil S cycling in grassland soils, further promoting our understanding if the microbial-regulated S cycles in these managed ecosystems.

CRediT authorship contribution statement

Qiqi Wang: Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Sara L. Bauke:** Writing – review & editing, Visualization, Data curation. **Thomas F. Döring:** Writing – review & editing, Resources. **Jinhua Yin:** Writing – review & editing, Data curation. **Emily C. Cooledge:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Davey L. Jones:** Supervision, Resources, Methodology. **David R. Chadwick:** Supervision, Resources, Methodology. **Albert Tietema:** Writing – review & editing, Supervision. **Roland Bol:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2024.109436.

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