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RESEARCH ARTICLE

Climate change disrupts the seasonal coupling of plant and soil microbial nutrient cycling in an alpine ecosystem

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Abstract

The seasonal coupling of plant and soil microbial nutrient demands is crucial for efficient ecosystem nutrient cycling and plant production, especially in strongly seasonal alpine ecosystems. Yet, how these seasonal nutrient cycling processes are modified by climate change and what the consequences are for nutrient loss and retention in alpine ecosystems remain unclear. Here, we explored how two pervasive climate change factors, reduced snow cover and shrub expansion, interactively modify the seasonal coupling of plant and soil microbial nitrogen (N) cycling in alpine grasslands, which are warming at double the rate of the global average. We found that the combination of reduced snow cover and shrub expansion disrupted the seasonal coupling of plant and soil N-cycling, with pronounced effects in spring (shortly after snow melt) and autumn (at the onset of plant senescence). In combination, both climate change factors decreased plant organic N-uptake by 70% and 82%, soil microbial biomass N by 19% and 38% and increased soil denitrifier abundances by 253% and 136% in spring and autumn, respectively. Shrub expansion also individually modified the seasonality of soil microbial community composition and stoichiometry towards more

N-limited conditions and slower nutrient cycling in spring and autumn. In winter, snow removal markedly reduced the fungal:bacterial biomass ratio, soil N pools and shifted bacterial community composition. Taken together, our findings suggest that interactions between climate change factors can disrupt the temporal coupling of plant and soil microbial N-cycling processes in alpine grasslands. This could diminish the capacity of these globally widespread alpine ecosystems to retain N and support plant productivity under future climate change.

KEYWORDS
alpine ecosystems, climate change, nutrient cycling, plant–soil interactions, seasonality, shrub expansion, snow cover

1 | INTRODUCTION

Ecological processes that regulate ecosystem nutrient cycling and plant production display marked temporal dynamics, especially in strongly seasonal ecosystems, such as alpine and arctic tundra (Bardgett et al., 2005). However, how these ecological processes are modified by climate change and the consequences for ecosystem nutrient cycling and retention remain largely unknown (Bardgett et al., 2005; Makoto et al., 2014; Pugnaire et al., 2019). Addressing this knowledge gap is particularly pressing in alpine ecosystems because they display strong seasonality and are warming twice as fast as the global average (Pepin et al., 2015). Moreover, alpine ecosystems are generally nitrogen (N)-limited and rely on intimate coupling of plant and microbial resource needs across seasons for N retention (Bardgett et al., 2002; Jaeger et al., 1999).

The cycling of labile N pools between plants and soil microbes across seasons is critical for N retention in alpine ecosystems (Bardgett et al., 2005). Plants have a higher demand for resources, including N, during the growing season, whereas their resource needs in the autumn during plant senescence and throughout winter are much lower (Jaeger et al., 1999). Soil microbes therefore experience reduced competition for N from plants during autumn and winter (Bardgett et al., 2002). Further, microbial growth is promoted by large fluxes of labile C from senescing plants in autumn, leading to enhanced N immobilisation, along with decomposing plant litter throughout winter (Schmidt et al., 2007). Yet, how climate change affects the seasonal dynamics of plant and soil N pools and processes remains poorly understood, despite the important implications for N retention in these globally widespread ecosystems.

Alpine ecosystems are experiencing numerous direct and indirect climate change impacts, which include huge reductions in snow cover and widespread shifts in vegetation, respectively (Beniston et al., 2018; He et al., 2023). Reduced snow cover and shifts in vegetation are among the most pronounced and globally widespread signals of climate change in alpine ecosystems (Gobiet et al., 2014; Kellner et al., 2023; Notarnicola, 2020; Pepin et al., 2022; Steinbauer et al., 2018; Zong et al., 2022), yet how they interactively modify key nutrient cycles is poorly understood (Classen et al., 2015), with the vast majority of studies focussing on single global change factors (Rillig et al., 2019). Interactive effects of global change factors can be additive or non-additive. Additive effects, which are the sum of individual effects, are relatively easy to predict and model based on individual effects alone (Song et al., 2019). In contrast, non-additive effects, which can be synergistic or antagonistic, are inherently unpredictable and can profoundly affect C-cycling (Reich et al., 2020). How direct and indirect consequences of climate change interact to modify the seasonal N-cycle in alpine ecosystems is unknown, despite the potential for non-additive interactions to markedly alter annual N fluxes and ecosystem productivity.

Tight temporal coupling between plant and soil N-cycle processes across seasons in alpine grasslands can be disrupted by changes in snow depth due to climate change (Jia et al., 2022). Winter snow cover is predicted to decrease by 80%–90% at 1500 m, and snow melt to occur 5–10 weeks earlier, by the end of the century in the European Alps (Beniston et al., 2018). This will lead to increased freeze–thaw cycles, resulting in damage to plants (Bokhorst et al., 2009), along with a release of N from soil microbiota (Gao et al., 2021; Song et al., 2017). Spring snow melt causes abrupt shifts in soil microbial communities, which are closely linked to rapid fluxes in key plant growth-limiting nutrients (Broadbent et al., 2021). Earlier snow melt due to climate change will advance the timing of these below-ground transitions in biotic and abiotic soil properties, which are crucial for soil N-availability at the start of the growing season (Broadbent et al., 2021). Earlier snow melt could therefore cause a mismatch between soil N-availability and plant N demand (Bilbrough et al., 2000). This temporal mismatch, coupled with damage to plants due to freeze–thaw cycles, could lead to reduced plant N-uptake across the growing season, with negative consequences for plant productivity and ecosystem N retention (Bardgett et al., 2005; Ernakovich et al., 2014).

Climate change is causing widespread ericaceous shrub expansion into alpine grassland, which is co-occurring with reductions in winter snow cover, leading to the potential for interactive effects (Broadbent et al., 2022). It is unclear how ericaceous shrub expansion moderates the effects of reduced snow cover on the temporal coupling of plant and soil N-cycling. However, ericaceous dwarf shrubs are very susceptible to frost damage caused by

a lack of snow cover in winter, or earlier spring snow melt (Bjerke et al., 2017; Parmentier et al., 2018; Wheeler et al., 2014). Given the vulnerability of ericaceous shrubs to frost damage, they may experience large reductions in N-uptake in response to reduced snow cover and earlier snow melt. Substantial reductions in plant N-uptake would disrupt the temporal coupling between plant and soil N-cycling, given that plant N-uptake is a key seasonal transfer of N from soil to plants. This could, in turn, lead to ecosystem N losses via leaching or denitrification.

Here, we used a manipulative field experiment in the European Alps, combined with in situ ^{15}N and ^{13}C labelling, to characterise the temporal coupling of plant and microbial resource needs, and how this relates to soil microbial community composition and functioning. We then tested how this temporal coupling is affected by interactions between two pervasive direct and indirect climate change impacts, namely reduced snow cover and shrub expansion, respectively. Specifically, we tested the hypothesis that reduced snow cover and ericaceous shrub expansion interactively disrupt seasonal N-cycle dynamics via negative additive effects on plant N-uptake, soil N-availability, microbial functions related to N-cycling and by shifting microbial community composition towards oligotrophic taxa, which are associated with reduced ecosystem N retention.

2 | METHODS

2.1 | Experimental design

To test our hypothesis, we established a snow manipulation and shrub expansion experiment in a *Nardus stricta* (L.)-dominated alpine grassland in the Oetztal Alps, Tyrol, Austria, near Vent (mean elevation = 2472 (±1) m; lat., long. = 46.863217, 10.896800), in August 2017. The experiment consisted of 32 plots (2 m × 2 m), which were subjected to a full factorial cross of two snow manipulation treatments (snow removal and snow control) across two vegetation types (shrub invaded and grass control), arranged in a randomised block design across eight blocks ($n = 8$).

Snow was manually removed from the entirety of the 16 plots in the snow removal treatment three times (February and April 2018, and February 2019) to a depth of ca. 5 cm above the ground surface. Soil temperature and moisture were measured using iButtons installed in all plots, and Hobo temperature and moisture loggers (Onset Computer Corporation, Bourne, MA, USA) installed in a subset of 18 plots, in order to assess the extent of freeze–thaw cycles in soil following snow removal (Figure S1). Snow removal caused increased freeze–thaw activity in soil, and significantly advanced snow melt timing by 11 days on average in both vegetation types ($\chi^2 = 90.8$, $p < .01$, Figure S1). Mean snow cover was 13% lower in the shrub-invaded plots (102 ± 2 cm [mean ± SE]) than the grass plots (117 ± 2; $\chi^2 = 6.2$, df = 1, $p < .05$), and soil temperature and moisture tended to be lower in shrub plots (Figure S1).

Native ericaceous shrub abundance increased at the site between 2003 and 2015, including upward shifts in elevation most likely in response to climate change (Kaufmann et al., 2021) (Figure S2), with local landowners observing increases in shrub abundance for the last 50 years (Markus Pirpamer, personal communication). *Calluna vulgaris* (L.) is the dominant shrub species at the site and accounts for the majority of this increase in shrub abundance, along with various *Vaccinium* spp. (L.), albeit to a lesser degree. Plant community composition was dominated by *C. vulgaris* in shrub-invaded plots and *N. stricta* in grass control plots (Figure S3). The soil at the site is a shallow (depth ca. 10–30 cm) haplic podzol (European Commission, 2005), with a mean soil pH of 5.1 and a mean C:N ratio of 16.9. The aspect of plots is south or south-east; for further site details, see (Broadbent et al., 2022).

2.2 | Soil sampling

To capture seasonal dynamics across the site, soil was sampled on four key seasonal timepoints in alpine ecosystems. (1) Shortly after snow melt (23 May 2018), which captures the onset of plant growth. This is also a time when alpine plants acquire a substantial proportion of their annual N demand (Bilbrough et al., 2000). (2) Peak plant growth (20 July 2018), which is a period of high microbial turnover and C-sequestration by plants (Bardgett et al., 2005; Schmidt et al., 2007). (3) Onset of plant senescence (14 September 2018), when senescing plants provide a pulse of labile C to the soil, promoting microbial growth and N immobilisation (Bardgett et al., 2005). (4) Mid-snow season (7 February 2019), coinciding with high soil microbial biomass and N immobilisation, attributed to the consumption of dead organic matter under the snow in winter (Schmidt et al., 2007). These timepoints also correspond to the four seasons experienced by alpine ecosystems, that is spring, summer, autumn and winter, respectively, and we refer to these timepoints by their seasonal names throughout the manuscript. The winter timepoint immediately followed snow removal (Figure S1), and was therefore analysed separately from the other timepoints to assess the direct effects of snow removal and associated freeze–thaw cycles in winter.

Soil cores (diameter = 2 cm, depth = 10 cm) were taken using a steel corer from five random locations in each plot. To sample under snow in snow-control plots without disturbing snow cover in winter, we used an extended (2 m) soil corer. Soil cores from the same plot were pooled and homogenised, and any vegetation or litter was separated and discarded from the samples. Five subsamples were immediately taken from each soil sample (approx. 200 mg) for molecular analysis; see SI methods for further details. Sampling equipment was sterilised between plots using ethanol (96%). The remaining soil samples were sieved (4 mm), stored at 4°C for up to 2 weeks and shipped to Manchester for analysis. Vegetation surveys were conducted over 2 weeks in August 2018 by visually estimating plant species relative cover (%)

to the nearest 1% within a 60×60 cm subplot using a modified Daubenmire method (Daubenmire, 1959).

2.3 | Plant N-uptake

To test how reductions in snow cover and shrub expansion affected plant N-uptake, we used in situ ^{15}N and ^{13}C labelling on a subset of 20 plots (replicate $n=5$). Nine 20×20 cm subplots were randomly marked out in each plot. One day before the spring, summer and autumn sampling timepoints (i.e. on 22 May 2018, 19 July 2018 and 13 September 2018), three of the subplots were randomly allocated a 50-mL solution containing 2.304 mg ^{15}N as either inorganic N or organic N, or an unlabelled control solution. The three solutions contained the following compounds dissolved in DI water: solution (1) ^{15}N-labelled inorganic N (126.00 mg L^{-1} $^{15}NH_4{}^{15}NO_3$, 98+% enriched;) with unlabelled organic N (247.09 mg L^{-1} glycine); solution (2) unlabelled inorganic N (131.72 mg L^{-1} ammonium nitrate) with dual ^{13}C-^{15}N labelled organic N (236.70 mg L^{-1} glycine-2-^{13}C-^{15}N, 99% enriched); and solution (3) control solution of unlabelled inorganic and organic N (131.72 mg L^{-1} ammonium nitrate with 247.09 mg L^{-1} glycine), which was used for natural abundance measurements of ^{13}C and ^{15}N.

Subplots were sampled once after 24 h to determine short-term plant N-uptake (i.e. on 23 May 2018, 20 July 2018 and 14 September 2018), and once after 38 days to determine long-term plant N-uptake (on 29 June 2018, 26 August 2018 and 21 October 2018), by taking a core of intact vegetation and soil (diameter = 8 cm, depth = ca. 10 cm) from the centre of each subplot. Immediately after sampling, cores were cooled, transported to the University of Innsbruck (Austria) and separated into root and shoot components. Plant roots were washed in a 0.5 M $CaCl_2$ solution to remove any of the isotopic label attached externally (Wilkinson et al., 2015). The plant material was dried for 48 h at 65°C before being weighed and pulverised. A subsample was subsequently analysed for %C and N and ^{13}C and ^{15}N content. Total N content in shoots and roots was calculated using %N and total biomass values. Stable isotope measurements of C ($\delta^{13}C$) and N ($\delta^{15}N$) were conducted at the NERC National Environmental Isotope Facility (NEIF) at the UK Centre for Ecology and Hydrology in Lancaster, UK.

The concentration of excess ^{15}N and ^{13}C above natural abundance values (μg excess ^{15}N [or ^{13}C] g^{-1}) was calculated separately for each component (shoots and roots) using ^{15}N or ^{13}C atom percent excess and N or C concentrations, respectively (Bardgett et al., 2003; McKane et al., 2002). Atom percent excess was calculated from $\delta^{15}N$ or $\delta^{13}C$ values, as described in (Wilkinson et al., 2015). To account for isotope dilution, concentrations of excess ^{15}N were scaled by the size of the target N pool estimated to be available within each core at the time of injection (Bardgett et al., 2003; McKane et al., 2002); see SI methods for further details. Scaled concentrations of excess ^{15}N in shoots and total shoot mass (g m^{-2}) were used to calculate total shoot N-uptake (mg N m^{-2}) (McKane et al., 2002), whereas concentrations of excess ^{15}N were used to assess root N acquisition. To determine whether organic N was taken up directly by plants, rather than as inorganic N after mineralisation, we compared μg excess ^{13}C g^{-1} with μg excess ^{15}N g^{-1}. ^{13}C enrichment in plant tissues and a positive relationship implied direct organic N-uptake had occurred (Bardgett et al., 2003) (Figure S4).

2.4 | Soil biogeochemical pools

To measure plant-available inorganic N, 5 g (fresh weight) of soil was extracted with 25 mL of 1 M KCl and then analysed on a Seal AA3 Segmented Flow Multi-chemistry analyser (Mequon, WI, USA). Dissolved (water-extractable) organic carbon (DOC) and nitrogen (DON) were determined by extracting 5 g (fresh weight) of soil in 35 mL ultrapure (Milli-Q®) H_2O and analysed using a 5000A TOC analyser (Shimadzu, Japan) or Seal AA3 Segmented Flow Multi-chemistry analyser (Mequon, WI, USA), respectively. To calculate DON, we measured total dissolved N and total dissolved inorganic N (NH_4^+-N and NO_3^--N) simultaneously in each extract, and then subtracted total dissolved inorganic N from total dissolved N (Jones & Willett, 2006). Soil pH (1:2.5, soil:water) was determined using a pH meter (Mettler Toledo, UK), and soil water content was determined gravimetrically. Microbial biomass C and N were measured using the chloroform-fumigation technique (Vance et al., 1987); see SI methods for details. All extracts were filtered through ashless Whatman No. 42 filter papers. Soil total C and N content (%) were measured on oven-dried soil (105°C) using an Elementar Vario EL elemental analyser (Hanau, Germany).

2.5 | Soil extracellular enzyme assays

We measured a suite of eight extracellular soil enzymes that catalyse key C-, N- and P-cycle processes. These included β-glucosidase (GLC), cellobiohydrolase (CBH), β-xylosidase (XYL), phenol oxidase (POX), peroxidase (PER), *N*-acetylglucosaminidase (NAG), phosphatase (PHO) and urease (URE). To calculate potential enzyme activity relative to microbial biomass, that is microbial biomass-specific enzyme activity, we divided potential enzyme activities by the total amount of microbial biomass C. For detailed methods, see (Broadbent et al., 2022).

2.6 | Quantitative PCR

The absolute abundances of selected nitrification [*amoA* from ammonia-oxidising bacteria (AOB) and archaea (AOA)] and denitrification (*nirK* and *nirS*) genes were measured using quantitative PCR (qPCR) on an ABI 7300 Real-Time PCR System (Applied Biosystems Inc, USA). All reactions contained 1×Power SYBR Green PCR Master Mix (Life Technologies LTD, UK), bovine serum albumin (BSA; 0.06%; Sigma-Aldrich Chemie GmbH, Germany), PCR primers and 2 μL of DNA template to a final volume of 25 μL.

Each primer (5 pmol) were used for genes *amoA* of AOA, *nirK* and *nirS*, but 7.5 pmol for *amoA* of AOB. PCR reactions for denitrification genes additionally contained dimethyl sulfoxide (DMSO, 2.5%; Sigma-Aldrich Chemie GmbH). The detailed PCR thermal profiles are described in (Broadbent et al., 2022). Positive standards containing cloned plasmids with the genes of interest were prepared using the ZeroBlunt TOPO kit (Invitrogen AG, USA), following the manufacturer's protocol, and were included in the analysis. The sources of the genes were *Nitrosomonas* sp., fosmid clone 54d9, *Azospirillum irakense* DMS 11586 and *Pseudomonas stutzeri* for *amoA* of AOB, *amoA* of AOA, *nirK* and *nirS*, respectively. The efficiency of qPCRs was calculated based on the linear curve of the positive standards according to the formula $E = 10^{(-1/\text{slope})}$ and was in the range of 75%–95%. DNA samples were diluted 1:100 before quantification in order to minimise PCR inhibition based on a pre-experiment test. Zero values indicate that no amplification was observed.

2.7 | Soil microbial community composition

Phospholipid fatty acid (PLFA) analyses were used to characterise soil microbial community composition across different kingdoms, based on the methods of Bligh and Dyer (Bligh & Dyer, 1959). PLFAs were extracted from freeze-dried soil (Frostegård et al., 1991), as modified by Buyer and Sasser (Buyer & Sasser, 2012), and analysed on a gas chromatograph (Agilent 7890A Gas chromatograph, Santa Clara, CA, USA). The abundance of PLFAs was expressed in nmol PLFA g^{-1} dry soil. PLFAs were assigned as indicators of fungal and bacterial abundance; see SI methods for details. Total PLFA abundance and the ratios of fungal to bacterial markers and Gram positive (GP) to Gram negative (GN) bacterial markers were also calculated and analysed.

For molecular analyses, DNA was extracted using the ZR soil microbe DNA kit (Zymo research) under the manufacturer's recommendations with a few amendments to account for sample preparation; see SI methods for details. Bacterial and fungal community structure was assessed using the rarefied sequence abundance of the genetic regions encoding for 16S small subunit ribosomal RNA (16S rRNA) and the internal transcribed spacer region 2 (ITS2)—targeting bacteria and fungi, respectively.

Extracted DNA was quantified using the nanodrop 8000 UV–Vis spectrophotometer (ThermoFisher scientific, MA, USA), and amplicons were generated using the protocols fully outlined in (Seaton et al., 2022) and SI methods. Sequences were processed using the DADA2 (Callahan et al., 2016) pipeline in R V.3.0.17 (R Core Team, 2021) to quality filter, merge, denoise and assign taxonomies, with the addition of a cutadapt step for ITS (Martin, 2011). Full bioinformatics settings are outlined in Seaton et al. (2022) and SI methods. After quality filtering, a total of 2,224,136 bacterial (16S rRNA) and 1,229,923 fungal (ITS2) sequences were used in the analysis for the spring, summer and autumn timepoints. To account for the effect of sequencing depth bias, the resultant ASV tables were rarefied to an even depth of 8035 (16S rRNA) and 8262 (ITS2). For the winter timepoint, 618,266 bacterial (16S rRNA) and 317,044 fungal (ITS2) sequences were used in the analysis, and ASV tables were rarefied to an even depth of 6384 (16S rRNA) and 5274 (ITS2).

2.8 | Statistical analysis

To test how plant N-uptake, soil biogeochemical pools, microbial functioning and microbial community composition varied across seasonal timepoints (spring, summer and autumn), and were affected by changes in snow cover (snow removal and snow control) and shrub expansion (shrub invaded and grass control), we used repeated measures linear mixed effects models (LMMs, R package 'nlme' (Pinheiro et al., 2023)) and permutational multivariate analysis of variance (PERMANOVA) tests (Anderson, 2001) (R package 'Vegan'); see SI methods for details. To determine whether any significant interactions were antagonistic, synergistic or additive, we tested for significant differences between predicted mean additive effects and actual interactive effects (Fong et al., 2018); see SI methods for details.

All statistical analyses were performed in R v 4.0.5 (R Core Team, 2021) and Rstudio 1.4.1106 (RStudio Team, 2021).

3 | RESULTS

3.1 | Seasonal coupling of plant and soil N-cycle processes

Many ecosystem properties showed pronounced and significant seasonal dynamics indicative of temporal coupling between key plant and soil N-cycle processes ($p < .05$; Figures 1 and 2; Tables S1–S3). In spring, we detected high root N concentrations and content that were associated with high inorganic N-uptake and soil NH_4^+ concentrations (Figures 1 and 2a). High soil ammonium concentrations in spring were linked to a high potential for ammonia oxidation by archaea, based on the quantitative assessment of the archaeal *amoA* genes. In contrast, potential denitrification (based on quantitative assessment of the *nirK and nirS* genes), soil NO_3^- concentrations and microbial biomass N were all low in spring compared to other seasons. Fungal biomass was high in spring, which was associated with elevated potential activity and microbial investment in peroxidase, an enzyme produced primarily by fungi to degrade complex C-compounds, including lignin. The relative abundances of key bacterial taxa, for example *Rhizobiales* and *Frankiales*, and fungal taxa, that is *Chaetothyriales*, were also highest in spring (Figure 2a).

In summer, we observed a shift in plant N allocation from roots to shoots, and more acidic, nutrient-poor and drier soil conditions (Figures 1 and 2b). Specifically, shoot total N increased, whereas root total N, soil NH_4^+ and DON concentrations, pH and moisture

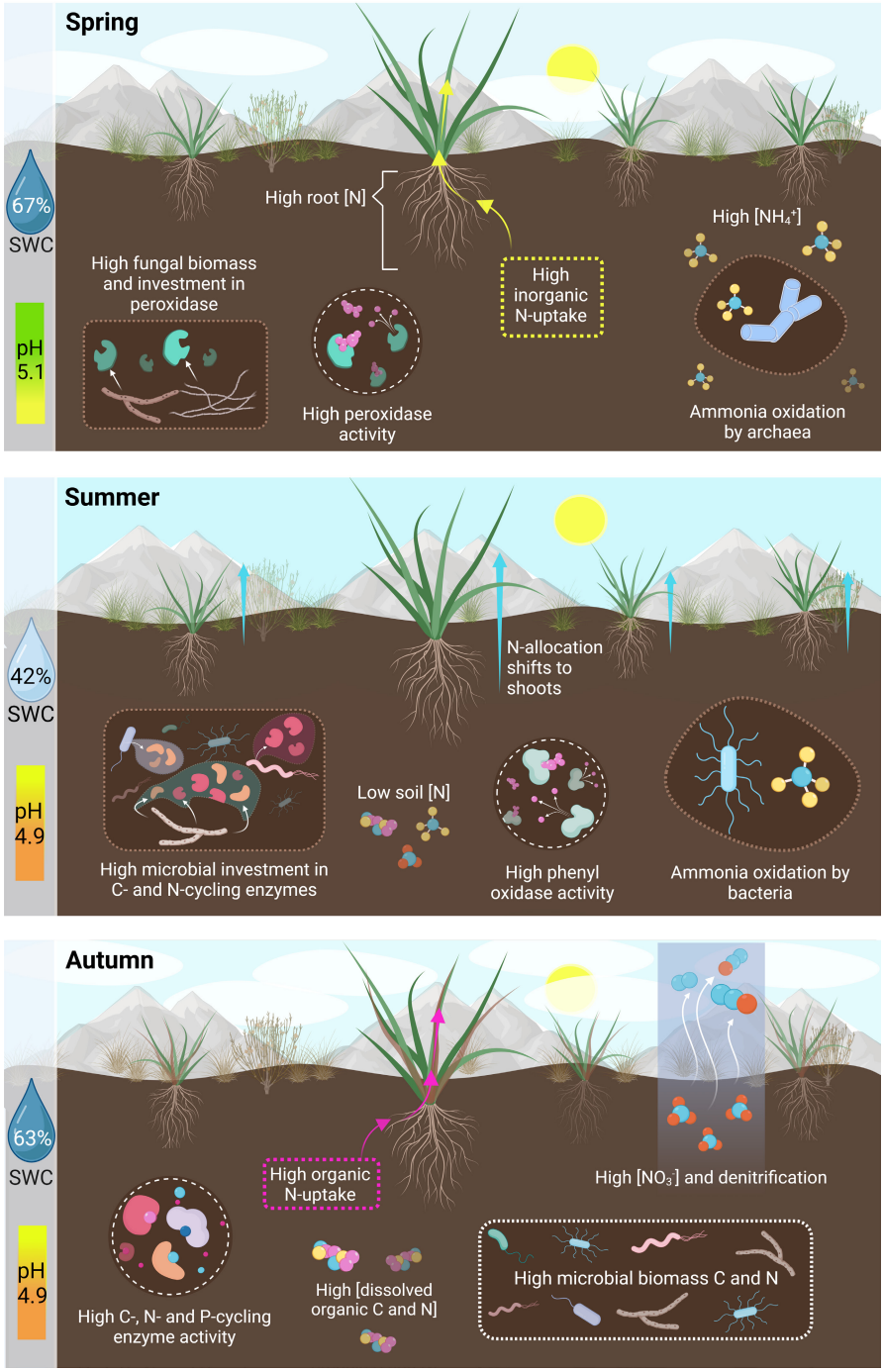

FIGURE 1 Seasonal dynamics of key plant and soil properties related to N-cycling in alpine grassland. Molecules are coloured by constituent elements (N, blue; C, pink; H, yellow; O, red). SWC, soil water content. For further details, including actual means and SE, exact *p* and χ^2-values see, Tables S1–S3 and Figure 2. Created with BioRender.com.

all decreased compared to spring. Ammonia-oxidising bacteria increased in abundance, based on the quantitative assessment of the bacterial *amoA* gene. Microbial investment in a range of C- and N-cycling enzymes was highest in summer, based on microbial biomass-specific enzyme activities, indicating high microbial turnover of nutrients. Potential enzyme activities, however, were generally low in summer, apart from high phenol oxidase activity, which degrades complex C-compounds.

In autumn, we documented marked changes in plant–soil interactions, with increased importance of organic N-uptake by plants linked to high DOC and DON concentrations in soil (Figures 1 and 2c). Microbial C-limitation was reduced, as demonstrated by high DOC concentrations and high C:N ratios of microbial biomass and soil, which were likely related to pulses of labile C from senescing plants. Soil NO_3^- concentrations were highest in autumn, as were denitrifier abundances, based on quantitative assessment of the *nirK and nirS* genes. Potential activities of C-, N- and P-cycling enzymes were also highest in autumn, which is likely related to high soil microbial biomass. A range of functionally important microbial taxa, including the bacterial orders *Acidobacteriales*, *Solibacterales* and *Myxococcales*, and ericoid mycorrhizal fungi (i.e. *Helotiales*), were also at their maximum relative abundances in autumn (Figure 2c).

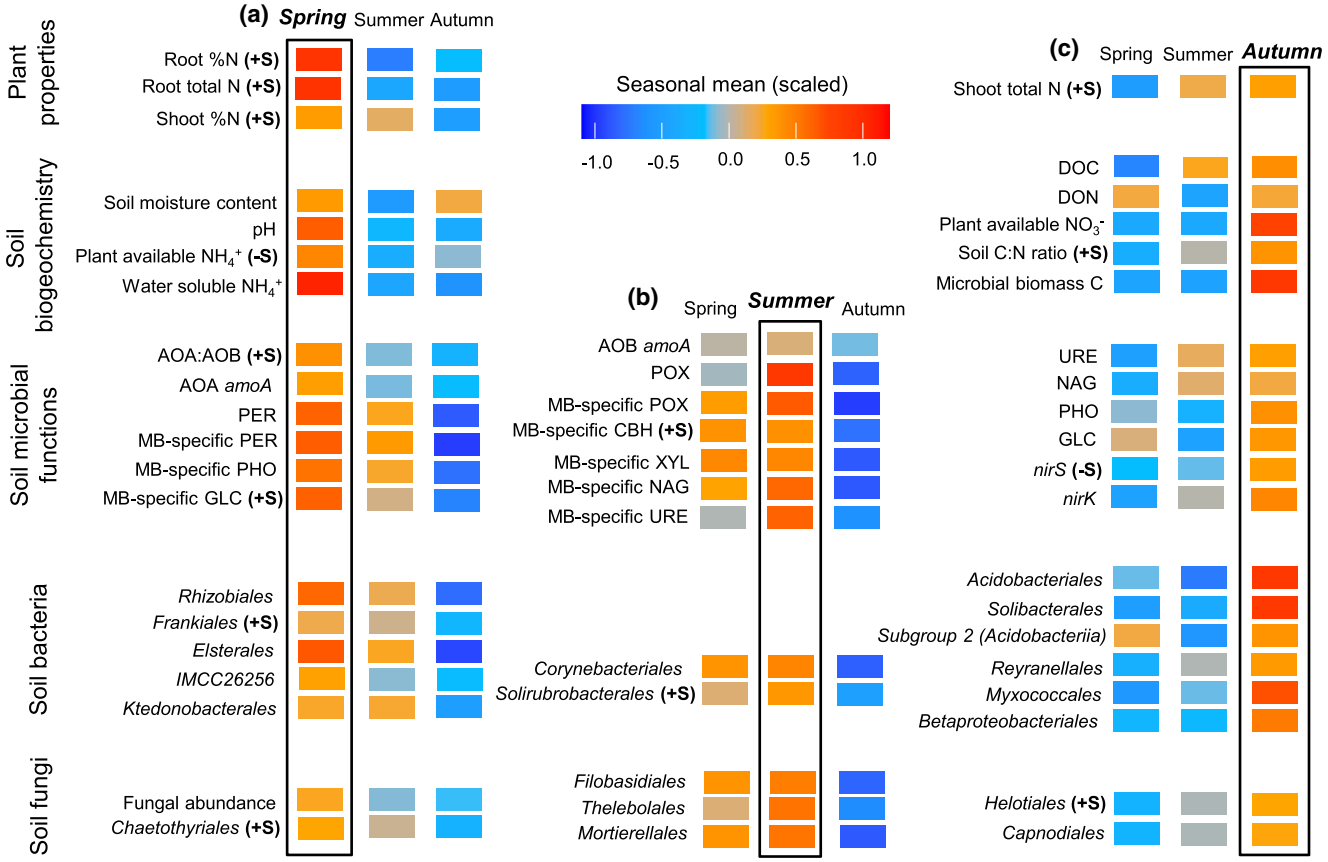

FIGURE 2 Seasonal dynamics of full range of plant and soil properties assessed in an alpine grassland. Properties are grouped by the season at which they were at their maximum: (a) spring; (b) summer; and (c) autumn. Some properties were significantly higher (+S) or lower (−S) under shrub expansion. AOA, ammonia-oxidising archaea; AOB, ammonia-oxidising bacteria; CBH, cellobiohydrolase; GLC, β-glucosidase; NAG, *N*-acetylglucosaminidase; PER, peroxidase; PHO, phosphatase; POX, phenol oxidase; URE, urease; XYL, β-xylosidase. For purposes of visualisation, all variables were scaled to have a mean of 0 and a standard deviation of 1. Statistical analyses were performed on unscaled data $n = 8$. For further details, including actual means and SE, exact p and χ^2-values, see Tables S1–S3.

3.2 | Direct and indirect climate change impacts disrupt seasonal N-cycle

Reduced snow cover and shrub expansion interactively disrupted the seasonal dynamics of key N-cycle processes, leading to large reductions in plant N-uptake and microbial biomass N, along with marked increases in denitrifier abundances (Figure 3; Table S4). On their own, snow removal and shrub expansion both increased organic N-uptake into shoots in autumn compared to control plots, thereby amplifying the strong seasonal dynamics of plant organic N-uptake (Figure 3a). However, together, they had an antagonistic effect, whereby organic N-uptake was reduced by 82% in autumn, 70% in spring and 58% in summer, compared to control plots (Figure 3a). Organic N was taken up directly by plants, at least in part, as demonstrated by enrichment of plant tissue with ^{13}C, which would not occur if organic N had been mineralised and then taken by plants as inorganic N. Moreover, there was a significant relationship between ^{13}C and ^{15}N excess g^{-1} in roots when dual-labelled ^{15}N and ^{13}C organic N was added ($\chi^2 = 19.6$, $p < .01$, Figure S4), providing further evidence of direct organic N-uptake. Together, snow removal and shrub expansion also reduced inorganic N-uptake by 83% and 80% for shoots (Figure 3b) and roots (Figure 3d), respectively, with particularly strong effects on uptake into roots in spring and summer, and uptake into shoots in summer and autumn. This reduction was driven by strong individual effects, as there was no significant interaction (Table S4).

Snow removal and shrub expansion had pronounced interactive effects on the seasonal dynamics of soil N-cycling. Specifically, they had a negative additive effect that reduced soil microbial biomass N by 19% in spring, 26% in summer and 38% in autumn (Figure 3e). Their interaction also led to a strong synergistic effect that increased denitrifier abundances by 253% in spring, 217% in summer and 136% in autumn, based on the quantitative assessment of the *nirK* gene (Figure 3f). Snow removal decreased the abundance of ammonium-oxidising bacteria by 67%, from 248 ± 92 to 81 ± 19 copies (bacterial *amoA* gene) ng^{-1} DNA, irrespective of vegetation types or seasonal timepoints ($\chi^2 = 5.7$, $p < .05$). Climate change effects on nitrifier and denitrifier abundances were mostly driven by changes in specific functional groups, rather than overall differences in the biomass of the microbial community. Total PLFA abundances were not significantly affected by snow removal ($\chi^2 = 0.48$, $p = .49$), but were reduced by 16% with shrub expansion ($\chi^2 = 6.3$, $p = .01$).

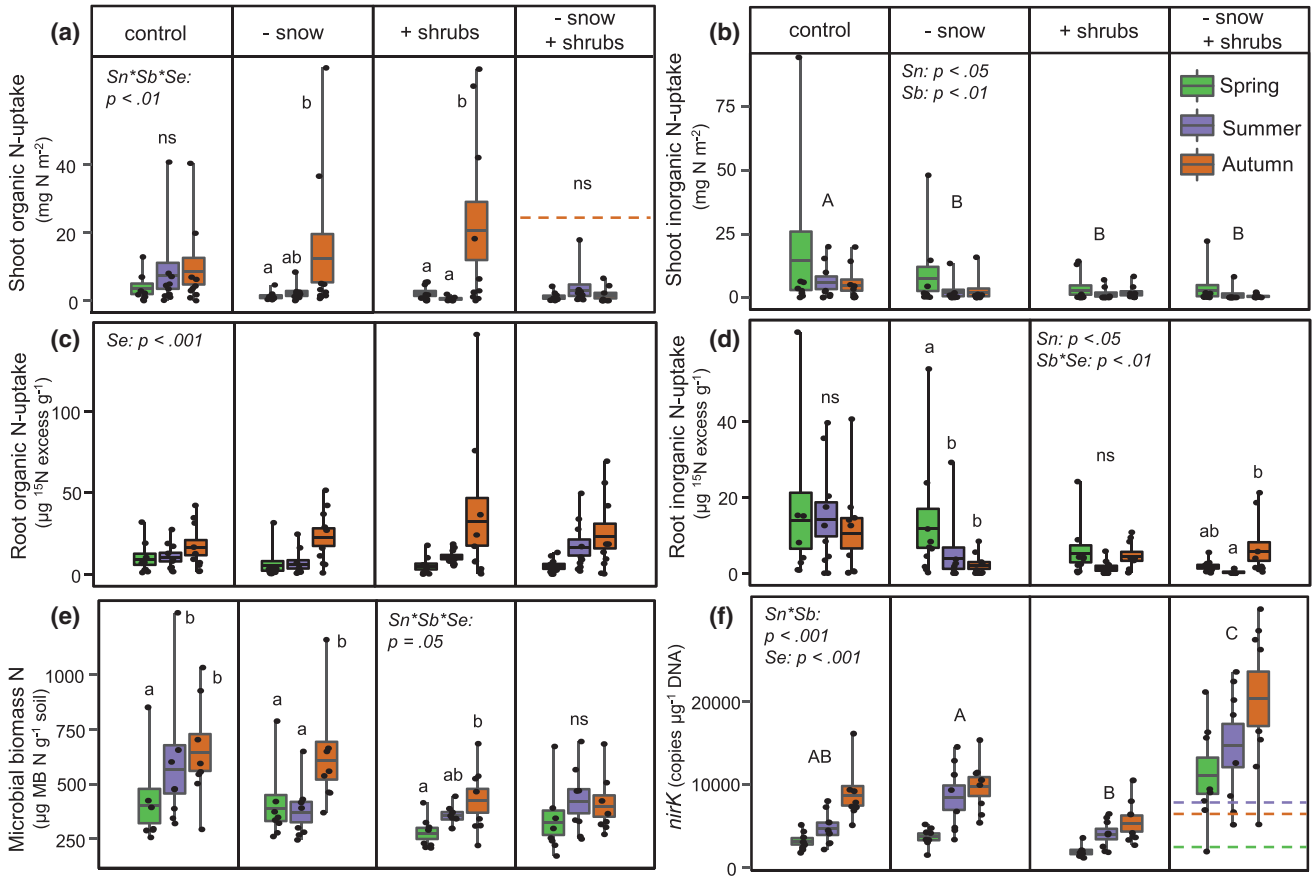

FIGURE 3 Snow removal (Sn) and shrub expansion (Sb) effects on seasonal dynamics (Se) of plant N-uptake and soil N-cycling in alpine grassland. (a) Shoot organic N-uptake, (b) shoot inorganic N-uptake, (c) root organic N-uptake, (d) root inorganic N-uptake, (e) microbial biomass N and (f) *nirK* gene abundances. Snow removal and shrub expansion effects on seasonal dynamics are shown by lower case letters, which indicate post-hoc significant differences ($p < .05$) between seasonal means within panels (a, d, e). Seasonally consistent effects of snow removal and shrub expansion are shown by upper-case letters (b, f). For interactions between snow removal and shrub expansion that were antagonistic (a, autumn only) or synergistic (f, all seasons), the predicted additive effects are shown by dashed lines. Boxplots show mean, SE and range, dots are individual data points, $n = 8$; ns, not significant. See Table S4 for details, including exact p, df and χ^2 values.

In winter, marked individual and interactive effects of snow removal and shrub expansion were observed on soil N-cycling and microbial communities (Figure 4; Table S5). Specifically, snow removal decreased dissolved organic N (DON) by 34% (Figure 4a) and the fungal:bacterial (F:B) ratio by 11%, regardless of vegetation type (Figure 4b). Snow removal and shrub expansion had a strong synergistic effect that increased denitrifier abundances by 262% (Figure 4c). They also interactively modified soil bacterial communities (Figure 4d, snow and vegetation interaction; $p < .05$, $F = 1.4$, $r^2 = .04$, PERMANOVA), including decreases in the relative abundances of bacterial orders *Rhizobiales*, *Elsterales* and *Subgroup 2* (*Acidobacteriia*), and increases in *Acetobacterales* (Figure S5). Snow removal had no significant effects on soil fungal community composition in winter ($p = .59$, $F = 0.9$, $r^2 = .03$, PERMANOVA).

Shrub expansion shifted microbial communities towards oligotrophic taxa, irrespective of snow manipulations, and altered the seasonal dynamics of soil microbial community structure and stoichiometry (Figure 5; Table S4). Specifically, shrub expansion was associated with a marked increase in the Gram positive (GP):Gram negative (GN) bacterial ratio in spring and autumn (Figure 5A), and the F:B ratio in spring (Figure 5B). The microbial biomass C:N ratio also increased with shrub expansion in spring and autumn (Figure 5C). Soil fungal community composition shifted in response to shrub expansion, showing the greatest divergence in autumn (Figure 5D, vegetation: $p < .01$, $F = 4.8$, $r^2 = .05$, seasonal timepoints: $p < .01$, $F = 1.5$, $r^2 = .03$, PERMANOVA). This was likely driven by higher abundances of the order *Helotiales*, especially in autumn (Figure 2c), which included ericoid mycorrhizal fungi (ErM) associated with ericaceous dwarf shrubs, such as *Pezoloma ericae* [= *Rhizoscyphus ericae*] and *Oidiodendron maius*. Bacterial community composition was also affected by shrub expansion, although to a lesser extent than fungi (Figure 5E, $p < .01$, $F = 1.5$, $r^2 = .03$).

4 | DISCUSSION

Reduced snow cover and shrub expansion interactively disrupted seasonal N-cycle dynamics, likely leading to cross-seasonal ecosystem N losses. When combined, both climate change factors reduced plant N-uptake and soil microbial biomass N, and markedly increased

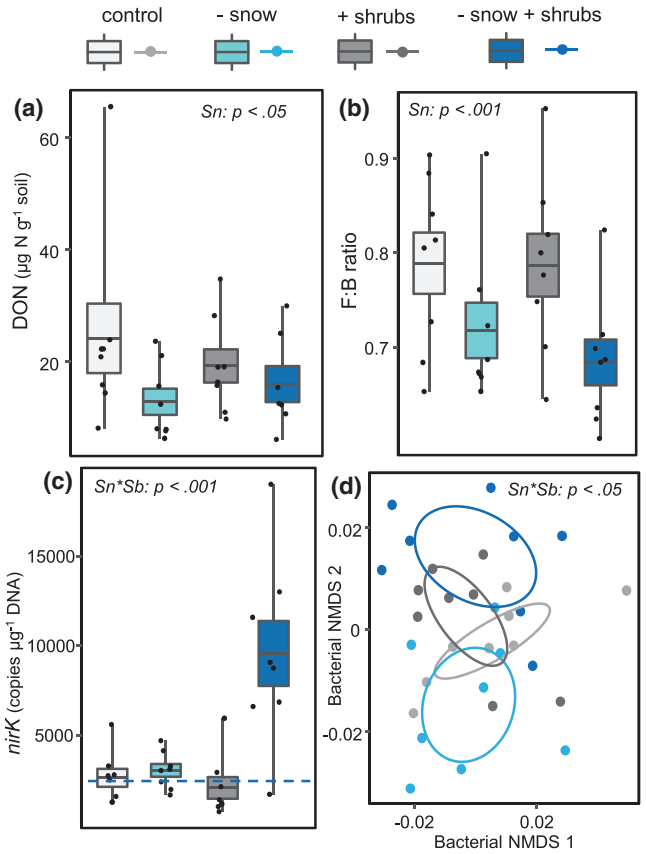

FIGURE 4 Snow removal (Sn) and shrub expansion (Sb) effects on soil properties in winter, shortly following snow removal. (a) Dissolved organic nitrogen concentrations (DON), (b) Fungal:Bacterial (F:B) ratio, (c) *nirK* gene abundances and (d) soil bacterial community composition (non-metric multidimensional scaling [NMDS] plot, ellipses show the 95% confidence regions for the centroids of snow treatments and vegetation types). For the synergistic interaction between snow removal (Sn) and shrub expansion (Sb) (b), the predicted additive effect is shown by the dashed blue line. Boxplots show mean, SE and range, dots are individual data points, $n = 8$. See Table S5 for details, including exact p, df and χ^2 values.

denitrifier abundances. Effects were most pronounced in spring (i.e. shortly after snow melt) and autumn (i.e. at the onset of plant senescence), which are critical timepoints in ecosystem N-cycling due to transfers of N between plant and soil microbial pools. Reduced snow cover and shrub expansion also had strong individual effects on soil N-cycling and microbial community composition. Specifically, reduced snow cover led to a decrease in the F:B ratio and soil DON pools in winter, along with cross-seasonal reductions in nitrifier abundances. Shrub expansion caused shifts in microbial community composition and stoichiometry towards more N-limited conditions and oligotrophic taxa associated with slower nutrient cycling, particularly in spring and autumn. Together, these findings largely confirm our hypothesis that reduced snow cover and ericaceous shrub expansion interactively disrupt seasonal N-cycle dynamics. This suggests the capacity of alpine ecosystems to retain N and support plant productivity may be diminished under future climate change.

Plant N-uptake is a crucial seasonal N-cycle process that underpins plant productivity, but it was strongly disrupted by interactions between reduced snow cover and shrub expansion. Plant inorganic N-uptake was higher in spring, which coincided with high plant-available NH_4^+ concentrations, a key source of inorganic N for alpine plants (Miller et al., 2007). However, reduced snow cover and shrub expansion together substantially reduced inorganic N-uptake across all seasons. Meanwhile, organic N-uptake was higher in autumn, coinciding with seasonal peaks in DOC and DON. This suggests that plant preferences for different N forms change seasonally to match the most abundant soil N forms, as observed in other alpine ecosystems (Hong et al., 2019). However, reduced snow cover and shrub expansion interacted to severely reduce organic N-uptake in autumn. This likely occurred because reduced snow cover caused frost damage to vegetation, as occurs with arctic browning due to extreme winter warming events (Bjerke et al., 2014; Bokhorst et al., 2008; Myers-Smith et al., 2020).

Ericaceous shrubs are particularly susceptible to the negative effects of reduced snow cover and associated frost damage (Bjerke et al., 2017; Krab et al., 2018; Parmentier et al., 2018; Treharne et al., 2019; Zong et al., 2022). In contrast, alpine grasses may be more resilient to frost damage and earlier snow melt (Bjerke et al., 2018; Möhl et al., 2023), given they survive at higher altitudes with more extreme temperatures. Moreover, some alpine grass species have a flexible phenology, which allows them to start their growth earlier in the year, and thereby track earlier snow melt (Möhl et al., 2022). Ericaceous shrub expansion was associated with lower snow depths, soil temperatures and soil moisture (Figure S1). This contrasts with the observed effects of larger deciduous shrubs, which trap snow in their perennial woody biomass, leading to higher soil temperatures due to the insulating properties of snow (Sturm et al., 2005; Vowles & Björk, 2019). The microclimatic effects of ericaceous shrub expansion were not a focus of our study, but they could exacerbate the effects of reduced snow cover on plant and soil processes and would warrant investigation in future studies. Our findings highlight that direct climate change effects on key ecosystem functions, such as plant N-uptake, will be strongly modified by indirect climate change effects, such as shifts in vegetation.

Disruption of the seasonal dynamics of plant N-uptake due to reduced snow cover and shrub expansion was closely linked to decreased soil microbial biomass N, and a marked increase in the abundance of denitrifiers. Specifically, decreased inorganic N-uptake into roots in spring shows that a key seasonal transfer of N from soil to plants was disrupted. This was linked to a large increase in denitrifier abundances (+253%). Similarly, in autumn, denitrifier abundances, which were already very high, increased by 136% due to the combination of reductions in snow cover and shrub expansion. This coincided with large reductions in plant organic N-uptake (−82%) and soil microbial biomass N (−38%). Together, these findings demonstrate that temporal coupling between key plant and soil N-cycle pools and processes was disrupted by reduced snow cover and shrub expansion. Specifically, seasonally important transfers of N from soil to plants following spring snow melt, along with

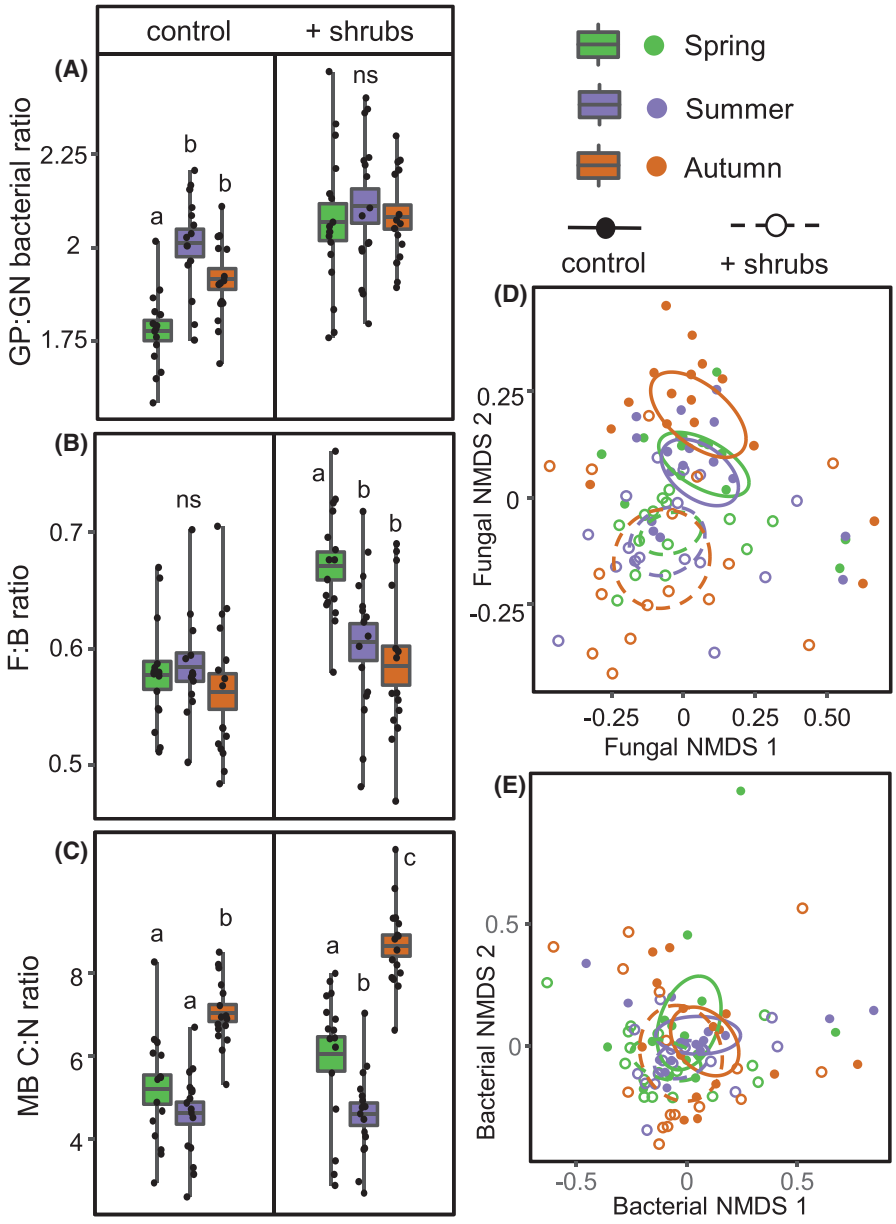

FIGURE 5 Shrub expansion effects on the seasonal dynamics of soil microbial community composition and stoichiometry. (A) Gram positive:Gram negative (GP:GN) bacterial ratio, (B) Fungal:bacterial (F:B) ratio, (C) Microbial biomass (MB) C:N ratio, (D) fungal community composition and (E) bacterial community composition (non-metric multidimensional scaling [NMDS] plots, ellipses show the 95% confidence regions for the centroids of vegetation types in each seasonal timepoint). Shrub expansion effects on seasonal dynamics are shown by lower case letters, which indicate post-hoc significant differences ($p < .05$) between seasonal means within panels (A–C). Boxplots show mean, SE and range, dots are individual data points, $n = 8$, ns, non-significant. See Table S4 for further details, including exact p, df and χ^2 values.

immobilisation of N in microbial biomass following plant senescence in autumn, were strongly disrupted, and this was accompanied by marked increases in denitrifier abundances. Nitrogen that was not immobilised in plant and soil microbial biomass in spring and autumn, respectively, was thus likely lost via denitrification, leading to reduced ecosystem N retention.

Increased denitrification in alpine ecosystems under future changes in snow cover could lead to N losses via higher emissions of N_2O, a powerful greenhouse gas, or N_2, as suggested by recent studies in seasonally snow-covered ecosystems (Blankinship & Hart, 2012; Jia et al., 2021, 2022). We detected a high potential for nitrification throughout spring and summer, as shown by high abundances of ammonia-oxidising functional groups. Archaea dominated ammonia oxidation at our site, particularly in spring, as in a previous study (Broadbent et al., 2021). Ammonia-oxidising bacteria become important in summer and autumn, indicating temporal niche partitioning of ammonia oxidation across the growing season, as found in other grassland ecosystems (Regan et al., 2017). Under control conditions, this high potential for nitrification was associated with a build-up of N in microbial biomass. However, snow removal markedly reduced the abundances of nitrifiers, and disrupted the seasonal increase in microbial biomass N. Our study site was not as strongly N-limited (mean soil C:N ratio between 16 and 17, Table S3) as other alpine ecosystems (Hagedorn et al., 2019; Mooshammer et al., 2014). This could explain why N was not immobilised by soil microbes, but rather lost via denitrification, following snow removal and shrub expansion. Reductions in ecosystem N retention due to reduced snow cover and shrub expansion could ultimately constrain plant growth. This could, in turn, counteract any future warming-induced increases in plant productivity, such as those recently observed in the European Alps (Rumpf et al., 2022).

Another potential route for ecosystem N losses due to reduced snow cover is the leaching of DON during freeze–thaw cycles, as evidenced by the immediate decreases in soil DON concentrations following snow removal in winter. This was accompanied by a reduction in fungal biomass relative to bacteria (i.e. decreased F:B ratio), and shifts in bacterial community composition. These findings suggest that bacterial biomass is less susceptible to the negative impacts of snow removal in winter than fungi. This was likely due to rapid shifts in bacterial community composition, with increases in some taxa (e.g. *Rhizobiales*, *Elsterales* and *Acetobacterales*) compensating for decreases in other taxa (e.g. *Acetobacterales*). However, the responses of bacterial taxa depended on vegetation type, which highlights the importance of plant–microbial interactions for microbial community responses to climate change. As is the case with most snow manipulation experiments, reductions in snow cover and advances in snow melt timing applied in our study are relatively small compared to the huge reductions in snow cover (80%–90% at 1500 m) and earlier snow melt (5–10 weeks at 1500 m) predicted for the end of the century in the Alps (Beniston et al., 2018; Rixen et al., 2022). This suggests the effects we detected will likely become more pronounced under future climate change. While reductions in snow cover due to climate change are relatively widespread globally (Thackeray et al., 2019), some seasonally snow-covered ecosystems are experiencing increases in snow depth, such as parts of northeast China and the Mongolian plateau (Tan et al., 2019). Here, deepened snow cover has also been shown to loosen temporal coupling between microbial and plant N-utilisation, largely due to increased N-leaching and N_2O emissions during spring thaw (Jia et al., 2021, 2022). Changes in snow conditions, whether increases or reductions, clearly have important implications for ecosystem nutrient cycling. However, our study demonstrates that the type of vegetation present and future shifts in vegetation due to climate change are essential for understanding and predicting how changes in snow cover will affect ecosystem nutrient cycling and retention.

Shrub expansion had an especially strong influence over soil microbial community structure and stoichiometry during spring and autumn in our study. These seasons are typically neglected in ecological research, but we found that they are crucial for below-ground processes in alpine ecosystems, including resource transfers between plants and soil microbes. The presence of ericaceous shrubs was associated with an increase in the Gram positive: gram negative bacterial and the microbial biomass C:N ratios in spring and autumn, which are both associated with microbial N-limitation, and an increase in the use of recalcitrant C-substrates by microbial communities (Fanin et al., 2019). The presence of shrubs was also associated with a much higher F:B ratio in spring. This is pertinent for alpine ecosystem functioning because spring was a key timepoint for soil fungal biomass and functioning (e.g. peroxidase activity). Together, our findings demonstrate that the effects of ericaceous shrub expansion on soil microbial communities show pronounced seasonal variation. Future studies, especially those conducted within one season (usually summer), should take this into account. Moreover, our results provide clear evidence that ericaceous shrub expansion is associated with a cross-seasonal shift towards microbial functional groups associated with nutrient-poor soil and slower fungal (and GP bacterial) energy channels (Wardle et al., 2004). Ericaceous shrub expansion will therefore likely lead to increased accumulation of soil organic carbon in alpine ecosystems, as found recently in boreal forests (Fanin et al., 2022).

In conclusion, by characterising the seasonal dynamics of a wide range of plant and soil N-cycling processes in alpine grassland (Figures 1 and 2), we show that spring (i.e. shortly after snow melt) and autumn (i.e. the onset of plant senescence) are key timepoints for the seasonal N-cycle. Moreover, using experimental field manipulations, we demonstrate that two pervasive climate change effects, reduced snow cover and shrub expansion, interactively disrupt the temporal coupling of key plant and soil microbial N-cycle processes during these seasonal timepoints. This included antagonistic effects that markedly reduced plant N-uptake, and synergistic effects that markedly increased soil denitrifier abundances, which together indicate ecosystem N losses. Disruption of temporal coupling between plant and soil N-cycle processes in alpine grasslands could therefore diminish the capacity of these globally widespread ecosystems to retain N, with potentially far-reaching implications for ecosystem productivity. More generally, our study shows that direct and indirect climate change factors, which are co-occurring in many ecosystems worldwide, can have non-additive interactive effects that precipitate sudden shifts in biogeochemical cycling. These effects are impossible to predict based on the individual effects of either climate change factor on its own, which highlights the importance of studying the interactive effects of direct and indirect climate change factors.

AUTHOR CONTRIBUTIONS

Arthur A. D. Broadbent: Data curation; formal analysis; investigation; methodology; project administration; visualization; writing – original draft; writing – review and editing. **Lindsay K. Newbold:** Data curation; formal analysis; investigation; methodology; resources; writing – review and editing. **William J. Pritchard:** Formal analysis; investigation; methodology; writing – review and editing. **Antonios Michas:** Data curation; formal analysis; investigation; methodology; writing – review and editing. **Tim Goodall:** Data curation; formal analysis; investigation; methodology; writing – review and editing. **Irene Cordero:** Formal analysis; investigation; methodology; writing – review and editing. **Andrew Giunta:** Investigation; methodology; project administration; writing – review and editing. **Helen S. K. Snell:** Investigation; methodology; writing – review and editing. **Violette V. L. H. Pepper:** Visualization; writing – review and editing. **Helen K. Grant:** Investigation; methodology; writing – review and editing. **David X. Soto:** Investigation; methodology; writing – review and editing. **Ruediger Kaufmann:** Methodology; resources; writing – review and editing. **Michael Schloter:** Conceptualization; methodology; resources; writing – review and editing. **Robert I. Griffiths:** Conceptualization; data curation; funding acquisition; methodology; resources; writing – review and editing. **Michael Bahn:** Conceptualization; investigation; methodology; project

administration; resources; writing – review and editing. **Richard D. Bardgett:** Conceptualization; funding acquisition; investigation; methodology; project administration; resources; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the European Nucleotide Archive (raw sequences reads) under project accession PRJEB44750 at https://www.ebi.ac.uk/ena/browser/view/PRJEB44750. Other data that support the findings of this study are openly available in Figshare at https://doi.org/10.6084/m9.figshare.22760909.v1. Environmental metadata and ENA sequence submission can be cross-referenced using the 'Sample_ID' in the environmental metadata file and the ENA 'sample_alias' field.

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