LETTER

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Shrub expansion modulates belowground impacts of changing snow conditions in alpine grasslands

Arthur A. D. Broadbent[1] | Michael Bahn[2] | William J. Pritchard[1] | Lindsay K. Newbold[3] | Tim Goodall[3] | Andrew Guinta[2] | Helen S. K. Snell[1] | Irene Cordero[1] | Antonios Michas[4,5] | Helen K. Grant[6] | David X. Soto[6] | Rüdiger Kaufmann[2] | Michael Schloter[4,5] | Robert I. Griffiths[7] | Richard D. Bardgett[1]

[1]Department of Earth and Environmental Sciences, The University of Manchester, Manchester, UK

[2]Institut für Ökologie, Universität Innsbruck, Innsbruck, Austria

[3]UK Centre for Ecology & Hydrology, Wallingford, Oxfordshire, UK

[4]Research Unit for Comparative Microbiome Analysis, Helmholtz Zentrum München, Neuherberg, Germany

[5]Chair for Soil Science, Technical University of Munich, Freising, Germany

[6]National Environmental Isotope Facility, UK Centre for Ecology & Hydrology, Lancaster Environment Centre, Lancaster, UK

[7]UK Centre for Ecology & Hydrology, Environment Centre Wales, Gwynedd, UK

Correspondence
Arthur A. D. Broadbent, Department of Earth and Environmental Sciences, Michael Smith Building, The University of Manchester, Manchester M13 9PT, UK.
Email: arthur.broadbent@manchester.ac.uk

Abstract

Climate change is disproportionately impacting mountain ecosystems, leading to large reductions in winter snow cover, earlier spring snowmelt and widespread shrub expansion into alpine grasslands. Yet, the combined effects of shrub expansion and changing snow conditions on abiotic and biotic soil properties remains poorly understood. We used complementary field experiments to show that reduced snow cover and earlier snowmelt have effects on soil microbial communities and functioning that persist into summer. However, ericaceous shrub expansion modulates a number of these impacts and has stronger belowground effects than changing snow conditions. Ericaceous shrub expansion did not alter snow depth or snowmelt timing but did increase the abundance of ericoid mycorrhizal fungi and oligotrophic bacteria, which was linked to decreased soil respiration and nitrogen availability. Our findings suggest that changing winter snow conditions have cross-seasonal impacts on soil properties, but shifts in vegetation can modulate belowground effects of future alpine climate change.

KEYWORDS
alpine vegetation, belowground ecosystem functioning, biogeochemical cycles, ericaceous, microbial community, snow cover, soil microbiome, winter climate change

INTRODUCTION

Aboveground–belowground linkages shape responses to climate change at the ecosystem scale, but they are still poorly understood (Bardgett et al., 2013; Hagedorn et al., 2019). Disentangling the complexity of belowground responses to climate change is a particular challenge (Fierer, 2017), as is understanding how shifts in soil microbial communities and their functioning influence vegetation change. In turn, plant range expansions affect

soil microbial communities and the ecological processes they regulate, including global biogeochemical cycles (Pugnaire et al., 2019). Here, we disentangle the belowground effects on microbial communities, microbial-mediated functions, and important plant nutrient pools of two widespread climate change impacts in alpine ecosystems: ericaceous shrub expansion and changing snow conditions.

Mountain ecosystems are experiencing disproportionally rapid climate warming (Pepin et al., 2015), leading to large reductions in winter snow cover and earlier spring snowmelt (Beniston, 2012). Snowmelt has been shown to trigger an abrupt transition in soil microbial communities, which is closely linked to marked shifts in soil nutrient dynamics (Broadbent et al., 2021). Furthermore, earlier spring snowmelt, driven by climate change, could disrupt the synchronised seasonal dynamics of plant and soil microbiota nutrient exchange, with potentially negative consequences for plant productivity and diversity (Bardgett et al., 2005). Reduced snow cover also leads to increased freeze-thaw cycles in soil, which cause a release of carbon (C), nitrogen (N) and phosphorus (P) from decaying microbiota that act as a temporal storage pool for soil nutrients during winter, thereby disrupting ecosystem C, N and P retention (Gao et al., 2021; Song et al., 2017).

Climate change is also associated with widespread native shrub expansion (Brigham et al., 2018; Elmendorf et al., 2012; Formica et al., 2014). The ecological impacts of native shrub expansions can resemble those of invasive species (Nackley et al., 2017) and even lead to ecological regime shifts (Brandt et al., 2013). They depend on a variety of factors, including root symbiont type, shrub leaf traits and local edaphic and climatic conditions (Collins et al., 2020). Shrub expansion in alpine ecosystems can also increase winter snow depth, which increases soil temperature, microbial activity, and N availability, although shrub expansion can also decrease snow duration, which could have opposing effects (Sturm, Douglas, et al., 2005; Sturm, Schimel, et al., 2005). However, most studies on shrub expansion have focussed on tall deciduous shrubs in arctic ecosystems, including birch (*Betula* spp.) and willow (*Salix* spp.), which associate with ectomycorrhizal fungi and tend to increase C-cycling rates (Parker et al., 2015). Much less attention has been given to recent widespread expansions of evergreen dwarf shrubs, such as *Calluna vulgaris* and *Vaccinium* spp. (Bragazza et al., 2015; Vowles & Björk, 2019), which are low-growing and associate with ericoid mycorrhizal fungi (ErM). These ericaceous shrubs are associated with different belowground effects than deciduous shrubs, including increased soil C:N ratios and decreased soil nutrient contents (Grau et al., 2019). However, their wider belowground impacts, and the underlying mechanisms involved, remain poorly understood. Changes in snow cover or snowmelt timing are an unlikely mechanism for ericaceous dwarf shrub

impacts on soil properties considering the short stature of these shrubs (Vowles & Björk, 2019). In contrast, shifts in soil microbial communities, such as increased ErM abundance, maybe of more importance for determining their impacts on soil functioning and biogeochemical pools (Orwin et al., 2011).

Here, we examined the combined effects of snow cover change and ericaceous shrub expansion on soil microbial communities and biogeochemical cycles in alpine grasslands. We tested three hypotheses. First, we hypothesised that reduced winter snow cover and earlier spring snowmelt have cross-season legacy effects on soil properties. Specifically, we expected reduced winter snow cover and earlier spring snowmelt to alter microbial community composition and reduce soil respiration, extracellular enzyme activities catalysing key processes in C-, N- and P-cycling, and soil C and N pools and fluxes, including plant N uptake. Second, we hypothesised that expansion of ericaceous shrubs has no influence on snow depth or snowmelt timing due to their short stature, but does cause an increase in ErM abundance and shifts microbial communities towards oligotrophic taxa, which is associated with altered soil functioning and lower soil N availability. Third, we hypothesised that combined effects of shrub expansion and changing snow conditions are additive, rather than synergistic or antagonistic, as recently reported for multiple co-occurring global change impacts (Broadbent et al., 2020; Song et al., 2019).

MATERIALS AND METHODS

To test our hypotheses, we established two complementary field experiments. The first experiment used a natural gradient in snowmelt timing combined with a shrub manipulation treatment across three alpine valleys in the Austrian Alps to test how earlier snowmelt and shrub expansion affect soil microbial communities, their functioning, and nutrient pools at the landscape scape. For the second experiment, we implemented at one of the sites a multi-year snow manipulation treatment combined with a shrub manipulation treatment. This allowed us to experimentally test how reduced winter snow cover and shrub expansion individually and interactively affect soil microbial communities, their functioning, and nutrient pools and fluxes, including plant N uptake immediately after snowmelt.

Landscape-scale experiment field-sites

The landscape-scale experiment was established across three *Nardus stricta*-dominated alpine grassland sites in three separate valleys in the Oetztal Alps, Tyrol, Austria, near Obergurgl (lat., long. = 46.844833, 11.023783), Soelden (46.978367, 10.972217) and Vent (46.863217, 10.896800). Increases in native ericaceous dwarf-shrub

abundance have been detected across all valleys since 2000 (Kaufmann et al., 2021), with local landowners observing increases for the last 50 years (Markus Pirpamer, *personal communication*). *Calluna vulgaris* is the dominant shrub species at all sites (Figure S1) and accounts for the majority of this increase, along with various *Vaccinium spp.*, albeit to a lesser degree. Shrub encroachment at our sites is most likely a result of climate warming, although changes in grazing regimes may also play a role (Kaufmann et al., 2021; Mayer & Erschbamer, 2017; Mayer et al., 2009). The soil type at all sites is a shallow podzol, the mean annual air temperature, and precipitation, averages for 1981–2010 were 3.2°C and 885 mm, respectively (recorded in Obergurgl, ZAMG, Austria). Mean site elevation ranged from 2279 m (Obergurgl) to 2472 m (Vent), mean soil pH from 4.8 (Obergurgl) to 5.1 (Vent), mean soil C:N from 16.9 (Vent) to 18.5 (Soelden) and plots at all sites were generally south or east facing.

Landscape-scale experimental design

We used in situ shrub manipulation treatments (shrub-invaded, grass-control and shrub removal, Figure S1) on 2×2 m plots arranged in a randomised block design, along a natural gradient of snowmelt timing. We established 81 plots in total, arranged in 9 blocks at each site ($n = 27$ for each vegetation treatment). However, due to instrument failure, 8 plots at the Obergurgl site and 5 plots at the Soelden site had to be excluded from the analysis. This left 68 plots arranged in 7–9 blocks at each site to be used in the analysis ($n = 23, 24,$ and 21 for the shrub-invaded, grass-control and shrub removal treatments). All plots were spaced at least 1 m apart. The shrub removal treatment was randomly allocated to one of two shrub-invaded plots within each block in late August 2017, then all shrubs were removed by carefully extracting whole plants or cutting plants at their bases and where possible removing roots. Roots were only left where removing them would damage the surrounding vegetation. Non-shrub species were left undamaged by shrub removal and no significant regrowth of shrubs was observed throughout the experiment.

Snowmelt timing in the landscape-scale experiment

To measure natural variation in snowmelt timing we installed iButtons (Maximum Integrated) in all plots and Hobo temperature loggers (Onset Computer Corporation) in a subset of plots to measure soil temperature on every plot from August 2017 until sampling in July 2019. We used soil temperature to estimate the date of complete melt-out (snowmelt timing) for every plot in 2018 and 2019. Snowmelt dates were recorded as the first date at which soil temperature rose above 1.5°C,

and began to show diurnal variation due to differences in daytime and night-time temperatures, which indicates the absence of snow cover. Soil temperature sensors failed on a subset of plots ($n = 24$) during 2018/19 winter, but we were able to predict snowmelt timing in 2019 on these plots with over 94% accuracy using snowmelt timing data from the previous year.

Snow manipulation experimental design

At the Vent site, we established the snow manipulation experiment using 48 plots (2×2 m). We removed snow on 24 plots (snow removal treatment) and left snow cover in place on the other 24 plots (snow control treatment). Snow cover treatments were fully crossed with three vegetation treatments (shrub-invaded, grass-control and shrub removal) in 8 blocks, resulting in 8 replicates of each snow cover and vegetation treatment combination. Snow was removed manually a total of five times from the entirety of each of the 24 plots in the snow removal treatment in February and April 2018, and in February, March and April 2019, to a depth of ca. 5 cm above the ground surface. To assess the extent of freeze-thaw cycles in soil following snow removal, soil temperature and moisture were measured throughout 2018 and 2019 using iButtons installed in all plots and Hobo temperature and moisture loggers (Onset Computer Corporation) installed in a subset of 18 plots.

Field sampling

For both experiments, soil was sampled on 22nd–24th July 2019 by taking soil cores (diameter = 2 cm, depth = 10 cm) using a steel corer from five random locations in each plot. Soil cores from the same plot were pooled and homogenised, and any vegetation or litter was separated and discarded from the samples. Sub-samples (approx. 200 mg) for molecular analysis were taken and lysed in the field. The soil corer, and the spatula used to sub-sample for molecular analysis, were both cleaned and sterilised between plots using ethanol (96%). Soil samples were sieved (4 mm), stored at 4°C for up to 2 weeks, and shipped to Manchester for analysis. Vegetation surveys were also conducted, and plot aspect and slope measurements taken, prior to soil sampling.

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). For molecular analyses, DNA was extracted using ZR soil microbe DNA kit (Zymo research) under manufacturer's recommendations

with a few amendments to account for sample preparation. Bacterial and fungal community structure was assessed using 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.

The absolute abundance of selected nitrification [*amoA* from ammonia oxidising bacteria (AOB) and archaea (AOA)] and denitrification (*nirK* and *nirS*) genes was measured using quantitative PCR (qPCR) on an ABI 7300 Real-Time PCR System (Applied Biosystems Inc.).

Biogeochemical cycling and microbial biomass

Plant available NH_4^+-N and NO_3^--N, and dissolved organic nitrogen (DON), were analysed on a Seal AA3 Segmented Flow Multi-chemistry analyser (Seal Analytical, UK). Dissolved (water-extractable) organic carbon (DOC) was analysed using a 5000A TOC analyser (Shimadzu, Japan). Soil pH (1:2.5, soil:water) was determined using a pH metre (Mettler Toledo, UK), and soil water content was determined gravimetrically. Microbial biomass C and N were determined using the chloroform-fumigation technique (Vance et al., 1987). Soil total C and N content (%) was measured on oven-dried soil (105°C) using an Elementar Vario EL elemental analyser (Hanau, Germany) in 2018 and 2019.

Soil extracellular enzyme assays

We measured the potential activities of eight soil extracellular enzymes, including 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, i.e., microbial biomass-specific enzyme activity, we divided potential enzyme activities by total amount of microbial biomass C.

Soil respiration

Soil respiration was measured in the snow manipulation experiment at the same time as soil sampling. CO_2 flux measurements were taken using a soil respiration chamber (SRC; PP Systems, Hitchin, UK) attached to an infra-red gas analyser (IRGA; EGM4, PP Systems, Hitchin, UK).

Plant and soil microbial competition for nitrogen

To test how reductions in snow cover and shrub expansion affected plant and soil microbial competition for N, we used in situ ^{15}N labelling on a subset of 20 plots in the snow manipulation experiment (replicate $n = 5$). Shrub removal plots were excluded from this part of the study. On 22nd May 2018, three 20 × 20 cm subplots in each plot were randomly allocated a 50 ml solution containing 2.304 mg ^{15}N as either inorganic-N (126.00 mg L^{-1} of $^{15}NH_4^{15}NO_3$, 98+% enriched; Cambridge Isotope Laboratories) or organic-N (236.70 mg L^{-1} glycine-2-^{13}C-^{15}N, 99% enriched, Cambridge Isotope Laboratories), or an unlabelled control solution. Solutions (50 ml) were applied homogenously via ten evenly spaced and consecutive 5 ml injections. For each injection, a syringe with a 10 cm long side-port needle was inserted to a depth of 7 cm and slowly withdrawn as the solution was injected. This approach allowed us to test whether plant and soil microbial competition for N depended on N form. Subplots were sampled after 24 h (23rd May 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 and transported to the University of Innsbruck (Austria) and separated into soil microbial biomass, root and shoot components for ^{15}N analyses. We calculated total ^{15}N recovered in each component as a percentage of total ^{15}N added, along with the concentration of ^{15}N excess in each component (μmol excess ^{15}N g^{-1}), and the microbial:plant ^{15}N excess ratio (based on total ^{15}N recovered).

Statistical analysis

All statistical analyses were performed in R v. 4.0.0 (R Core Team, 2021) and Rstudio v.1.3.959 (RStudio Team, 2021). Linear mixed effects models (R package "nlme" (Pinheiro et al., 2020)), and Permutational Multivariate Analysis of Variance (PERMANOVA) tests (Anderson, 2001) (R package "Vegan" (Oksanen et al., 2020)) were used to test how soil microbial composition, functioning, biogeochemical cycling, and plant-soil competition for N were affected by changes in snow cover and shrub expansion.

Further method details are available in the Supporting Information.

RESULTS

Landscape-scale experiment

Snowmelt occurred over 15 days in our landscape-scale experiment with no significant differences in snowmelt timing due to vegetation treatments (Figure 1a, $p = 0.42$, $\chi^2 = 1.7$, Df = 2). Soil fungal communities were influenced by both snowmelt timing and shrub expansion (Figure 1b; vegetation treatments: $p < 0.01$, $F = 1.9$, $R^2 = 0.05$; snowmelt timing: $p < 0.01$, $F = 2.2$, $R^2 = 0.03$, $n = 68$; PERMANOVA). Notably, in the shrub-invaded plots the relative abundances of the fungal families
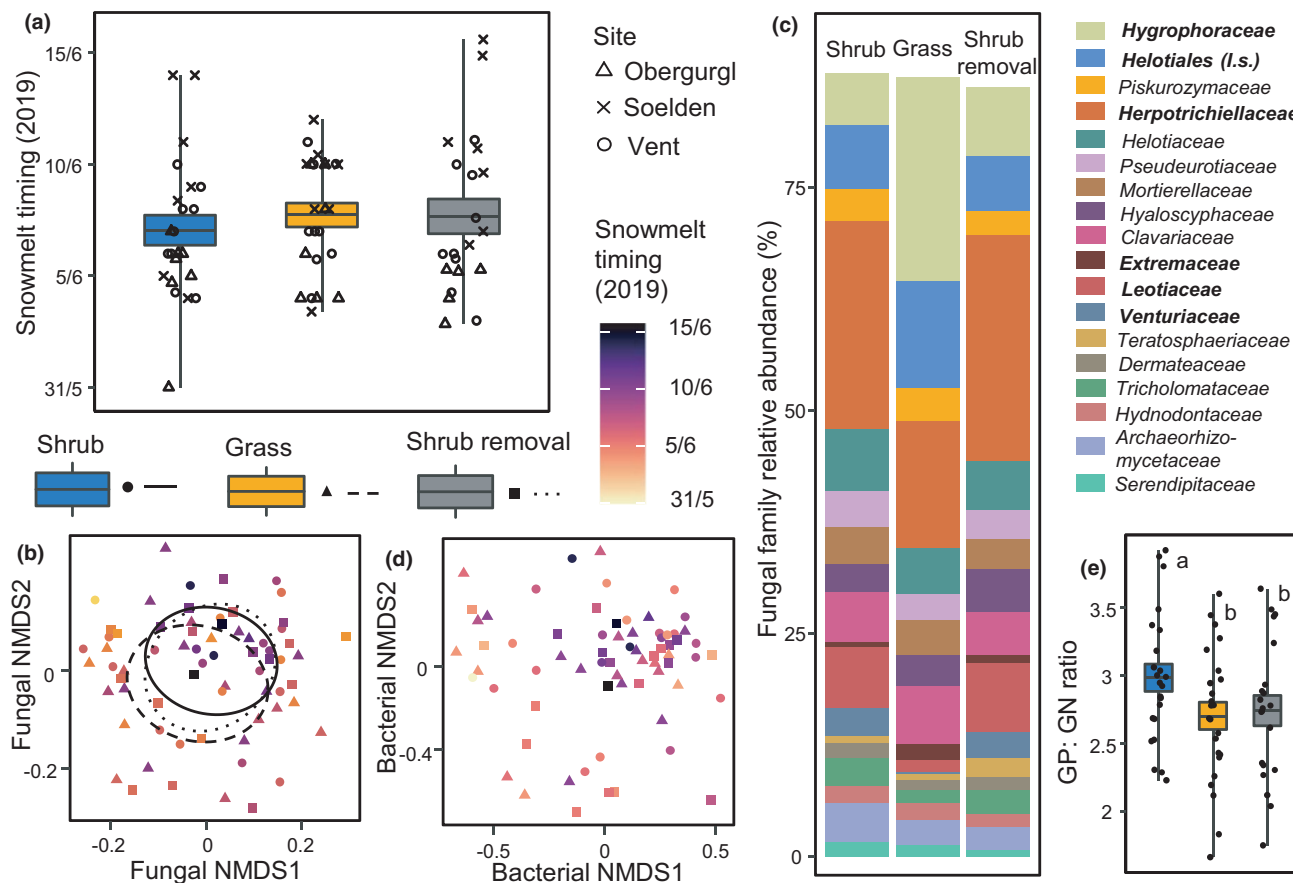
FIGURE 1 Snowmelt timing and shrub expansion effects on summertime soil microbial community composition in the landscape-scale experiment. (a) Mean snowmelt timing across vegetation treatments, (b) fungal community composition (non-metric multidimensional scaling [NMDS] plot, ellipses show the 95% confidence regions for the centroids of vegetation treatments), (c) fungal family mean relative abundances (significant differences [$p < 0.05$] in bold; only families with overall mean abundance >1% are shown), (d) bacterial community composition (NMDS), (e) Gram positive: Gram negative (GP: GN) bacterial ratio. Boxplots show mean, SE and range, dots are individual data points, and significant differences between means are indicated by letters. See Table S1 for details, including exact p and χ^2-values

Herpotrichiellaceae, *Leotiaceae* (including the ericoid mycorrhizal species *Pezoloma ericae [= Rhizoscyphus ericae]*), and *Venturiaceae* increased by 64, 389 and 1974%, whereas *Extremaceae*, *Helotiales (Incertae sedis)* and *Hygrophoraceae* decreased by 69, 40 and 74% respectively, compared to the grass control (Figure 1c; Table S1). Soil bacterial composition was significantly related to snowmelt timing ($p < 0.01$, $F = 2.3$, $R^2 = 0.03$, $n = 68$, PERMANOVA, Figure 1d), with shrub expansion having less influence. Nonetheless, the relative abundance of the bacterial families *Acidothermaceae* and *Solirubrobacteraceae* increased by 29 and 49%, respectively, and *Gemmataceae* and *Ktedonobacteraceae* decreased by 29 and 61%, respectively, in the shrub-invaded plots compared to the grass-control (Figure S2; Table S1). The shrub removal treatment did not differ significantly from the shrub-invaded plots in regard to the relative abundances of fungal and bacterial families. We found no significant differences in total fungal or bacterial PLFA marker abundances in relation to snowmelt timing or shrub expansion (Table S1), however, the Gram positive: Gram negative (GP:GN) bacterial ratio was greater

in the shrub-invaded plots compared to both the shrub removal and the grass-control treatments (Figure 1e).

Shrub-associated shifts in microbial community composition were accompanied by significant changes in belowground ecosystem functioning and biogeochemical pools and fluxes (Table S2). Potential phosphatase (PHO) and urease (URE) activity (µmol prod. g^{-1} dry soil h^{-1}) both increased with earlier snowmelt (Figure 2a and b), however, potential PHO activity was consistently lower in shrub-invaded plots compared to grass-control plots across the entire snowmelt gradient. In contrast, earlier snowmelt was associated with a sharp increase in microbial biomass-specific URE activity on shrub-invaded plots, but not in the other vegetation treatments (Figure 2c). Earlier snowmelt on shrub-invaded plots was also associated with lower potential *N*-acetylglucosaminidase (NAG) activity than grass-control plots (Figure S2). Shrub expansion was associated with further changes in soil functioning irrespective of snowmelt timing (Table S2). In particular, the potential activity of cellobiohydrolase (CBH) and β-glucosidase (GLC) was 18 and 23% higher on shrub-invaded plots
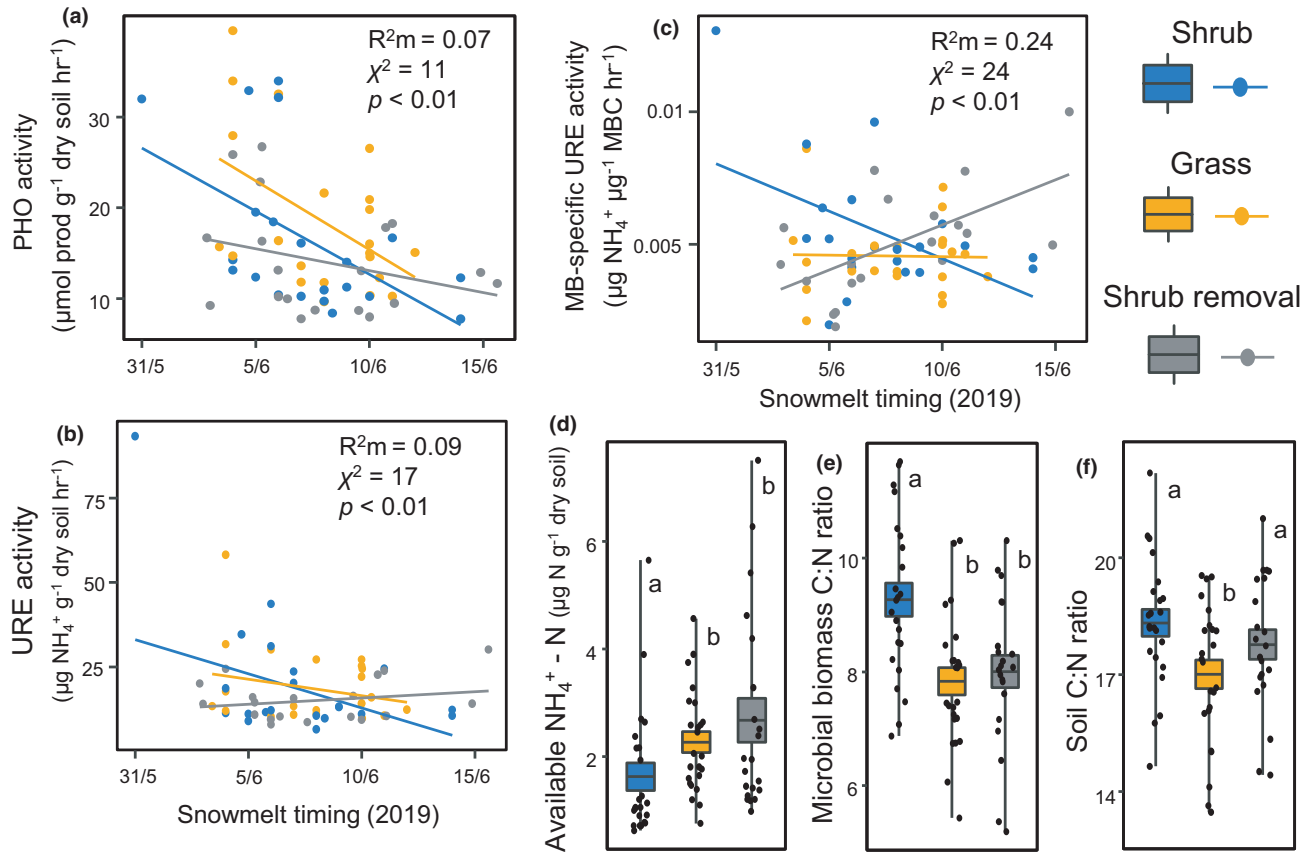
FIGURE 2 Snowmelt timing and shrub expansion effects on summertime soil functioning and biogeochemistry in the landscape-scale experiment. (a) potential phosphatase (PHO) activity, (b) potential urease (URE) activity, (c) microbial biomass (MB)-specific URE activity (lines show model regression fits and points show individual plots, R^2m = marginal R^2 of model, χ^2 and $p = \chi^2$ and p-values of snowmelt timing and vegetation treatment interaction), (d) soil available NH_4^+-N concentration, (e) microbial biomass C:N ratio, and (f) soil C:N ratio. Boxplots show mean, SE and range, dots are individual data points, and significant differences between means are indicated by letters. See Table S2 for details, including exact p and χ^2-values

than grass-control plots. Similarly, microbial biomass-specific activity of CBH, GLC and β-xylosidase (XYL) was 50, 53 and 23% higher in the shrub-invaded treatment than the grass-control treatment. These enzyme activities remained high in the shrub removal treatment and did not differ significantly from the shrub-invaded treatment ($p > 0.05$). Plant-available ammonium concentrations were 28 and 39% lower in shrub-invaded plots compared to grass-control and shrub removal plots, respectively (Figure 2d). Microbial biomass C and N were 19 and 30% lower in the shrub-invaded treatment compared the grass-control treatment (Table S2). Moreover, both the microbial biomass and soil C:N ratios were higher on shrub-invaded plots than grass-control plots, by 18% and 8% respectively (Figure 2e and f).

Snow manipulation experiment

Snow removal led to extensive freeze-thaw cycles in soil across all vegetation treatments (Figure S3). It also significantly advanced snowmelt timing in 2018 ($\chi^2 = 90.8$, $p < 0.01$), but not in 2019 (2019; $\chi^2 = 0.7$, $p = 0.41$, $n = 16$,

Df = 1; Figure S3). Vegetation treatments did not significantly affect mean snow depth (106 ± 1 cm, $p = 0.1$, $\chi^2 = 4.6$, Df =2). As in the landscape-scale experiment, soil fungal communities were markedly different in the shrub-invaded treatment compared to the grass-control treatment (Figure 3a, $p < 0.01$, $F = 2.2$, $R^2 = 0.09$, $n = 16$, PERMANOVA; Table S3). The relative abundance of the dominant fungal family in the grass-control treatment, *Hygrophoraceae*, decreased by 73% in the shrub-invaded treatment. In contrast, *Herpotrichiellaceae* increased by 174% to become the dominant family, and *Leotiaceae*, which includes the ericoid mycorrhizal species *Pezoloma ericae [= Rhizoscyphus ericae]*), increased by 947%, Figure 3b). Bacterial community composition also showed significant differences across the vegetation treatments, albeit to a lesser extent than fungal communities (Figure 3c, $p = 0.04$, $F = 1.4$; and $R^2 = 0.06$, $n = 16$, PERMANOVA; Table S3). Specifically, the bacterial family *Gemmataceae* decreased by 31% in shrub-invaded compared to grass-control plots, whereas *Solirubrobacteraceae* increased by 54% (Figure S4). The snow removal treatment led to a 102% increase in the relative abundance of the bacterial family *Bacillaceae*,
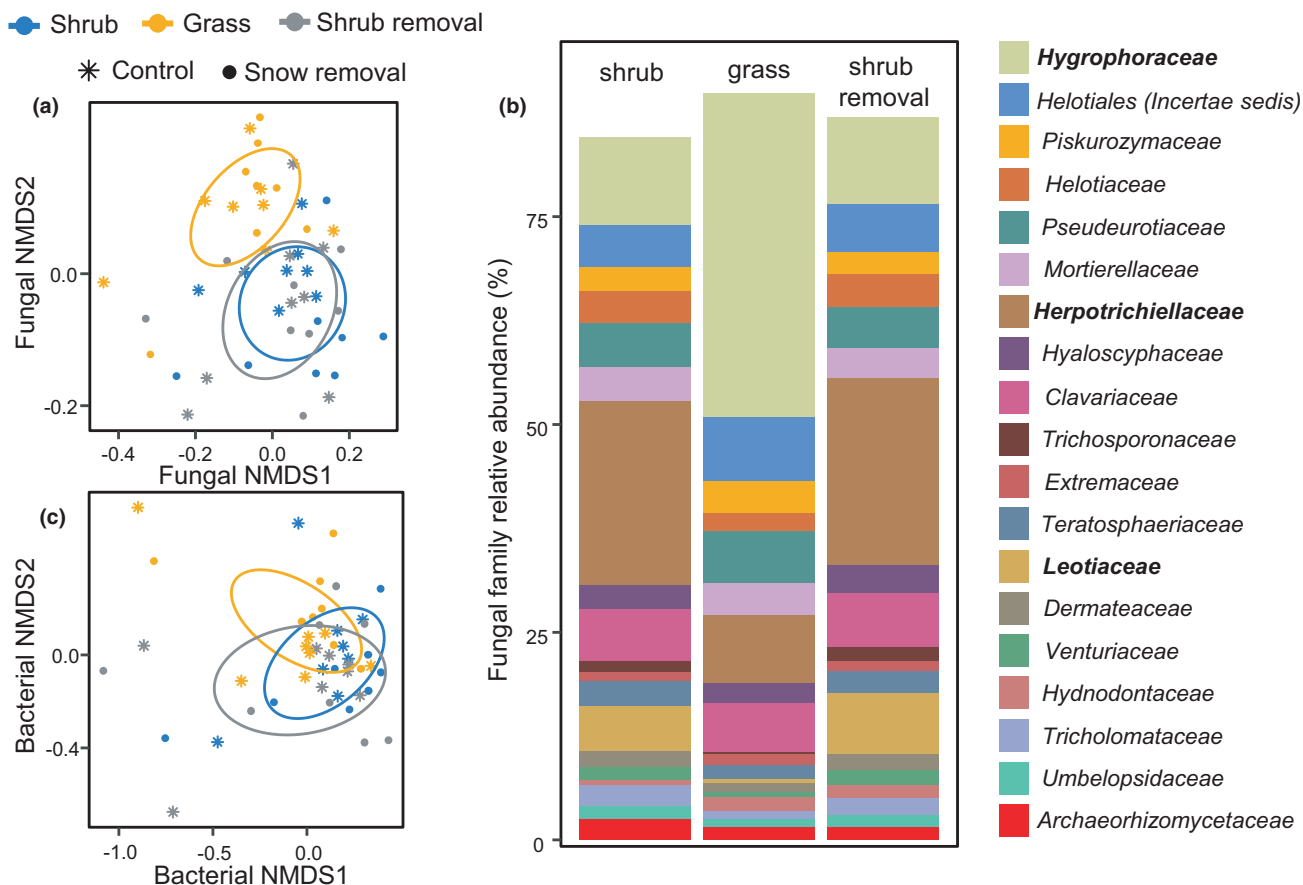
FIGURE 3 Shrub expansion effects on summertime soil microbial community composition in the snow manipulation experiment. (a) Fungal community composition (NMDS), (b) fungal family mean relative abundances (significant differences [$p < 0.05$] in bold; only families with overall mean abundance >1% are shown), (c) bacterial community composition (NMDS). Ellipses in NMDS plots show the 95% confidence regions for the centroids of vegetation treatments. See Table S3 for details including exact p and χ^2-values

and a 7% decrease in the GP:GN bacterial ratio (Figure S4, Table S3). Again, we found no significant effects of snow removal on total fungal or bacterial PLFA marker abundances ($p > 0.05$, Table S3).

Changes in microbial community composition associated with reduced snow cover and shrub expansion were accompanied by significant shifts in summertime belowground functioning and biogeochemical pools and fluxes. As at the landscape-scale, the shrub-invaded treatment exhibited higher potential and microbial biomass-specific CBH and GLC activity, along with lower potential PHO activity, compared to the grass-control treatment, regardless of snow manipulation treatments (Table S4). The snow removal treatment did not influence enzyme activities as strongly as the shrub-invaded treatment, but it did cause an 8% decrease in potential NAG activity, and a 38% increase in microbial biomass-specific peroxidase (PER) activity, regardless of vegetation treatments (Table S4). In situ soil respiration, a general measure of biological activity in the soil, was lower in the snow removal and shrub-invaded treatments (Figure 4a, Table S4). Soil temperature and moisture during respiration measurements did not vary significantly across snow or vegetation manipulation

treatments ($p > 0.05$). Following snow removal, the abundance of denitrifying genes decreased in the grass-control treatment (especially *nirS*; Figure 4b), but increased in the shrub and shrub removal treatments (especially *nirK*; Figure 4c). There were no significant differences in the archaeal and bacterial ammonia oxidising genes (*amoA*). As in the landscape-scale experiment, the shrub-invaded treatment had lower soil microbial biomass N than the grass-control treatment, but only in the absence of snow removal (Figure 4d). Following snow removal, microbial biomass N decreased by 34% in the grass-control treatment but was unaffected in the other vegetation treatments. The shrub-invaded treatment was generally associated with lower soil N availability, as indicated by higher microbial biomass and soil C:N ratios, and lower plant-available NH_4^+-N concentrations (Figure 4e–g; Table S4). Soil %C was lower on snow removal plots, whereas the shrub-invaded treatment was associated with higher soil %C than the grass-control treatment (Figure 4h, Table S4).

Nitrogen demand by the soil microbial community was significantly higher in shrub-invaded plots immediately following snowmelt, as demonstrated by in situ ^{15}N stable isotope labelling (Table S5). Specifically,
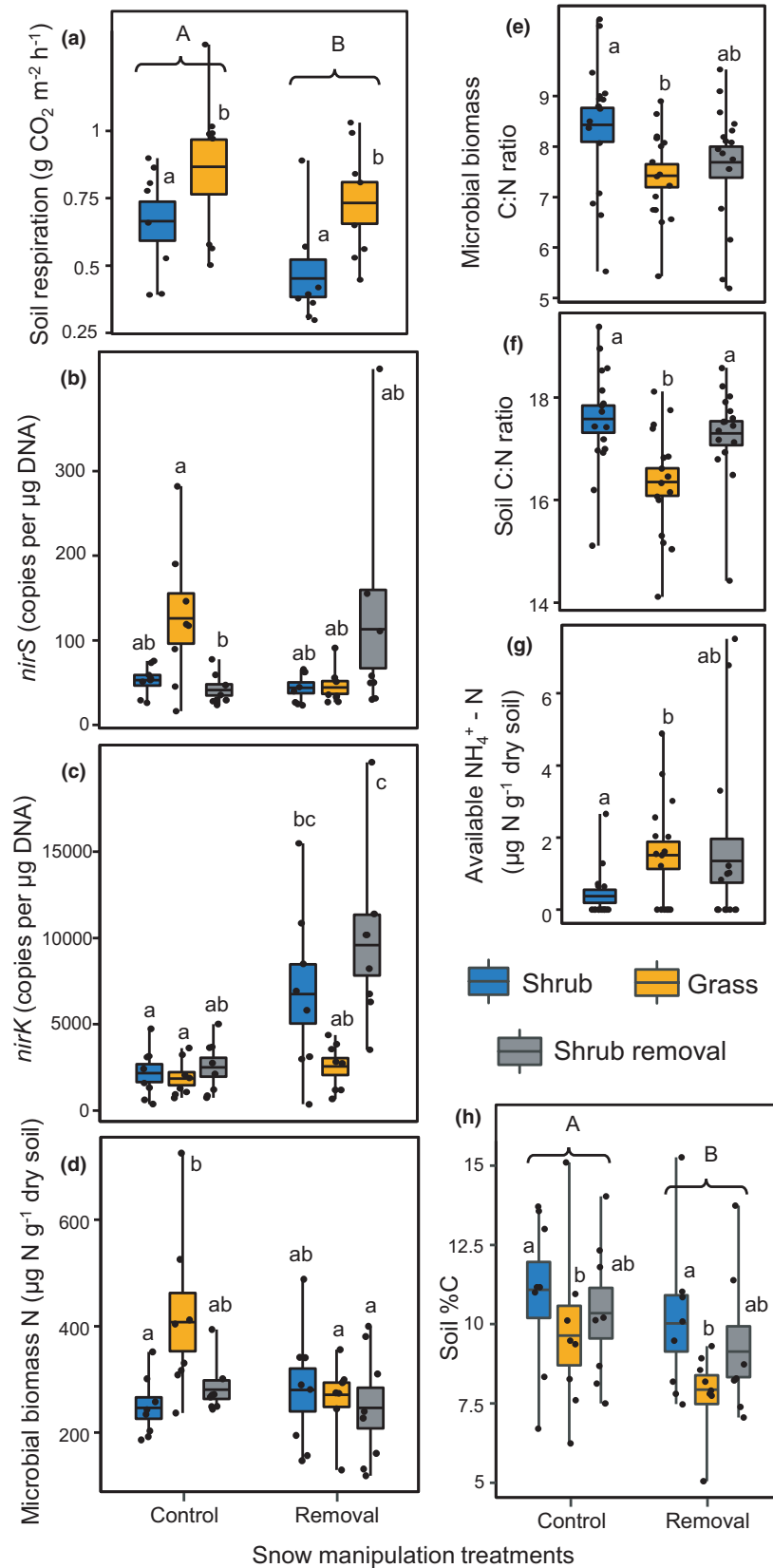
FIGURE 4 Snow removal and shrub expansion effects on summertime soil functioning and biogeochemistry in the snow manipulation experiment. (a) soil respiration, (b) *nirS* abundance, (c) *nirK* abundance, (d) microbial biomass N, (e) microbial biomass C:N ratio, (f) soil C:N ratio, (g) available NH_4^+-N concentration, (h) soil %C. Boxplots show mean, SE and range, dots are individual data points, and significant differences between means are indicated by letters (for a and h lower- and upper-case letters indicate significant differences between vegetation and snow manipulation treatments, respectively). See Table S4 for details including exact *p* and χ^2-values
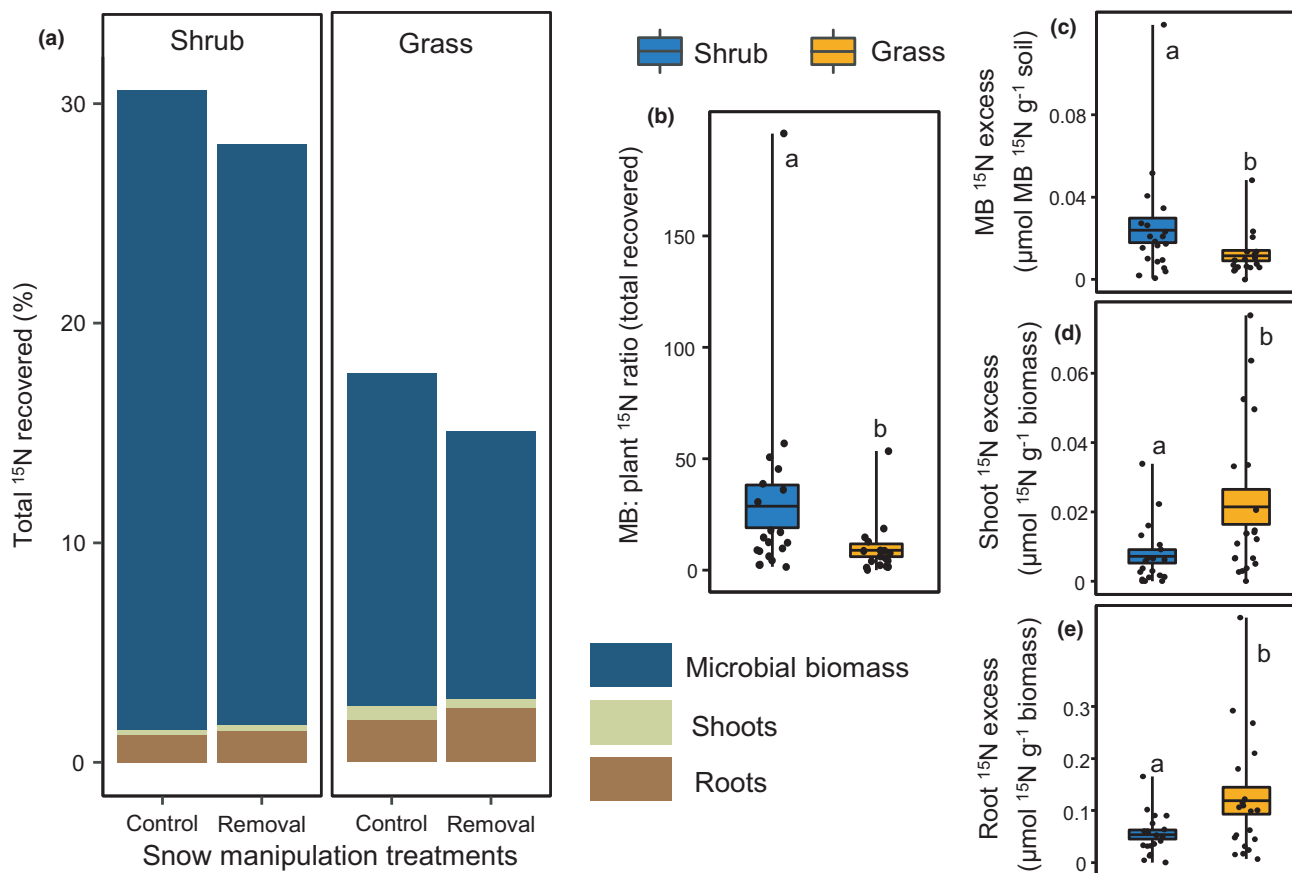
FIGURE 5 Snow removal and shrub expansion effects on springtime ^{15}N content in soil microbial and plant biomass following ^{15}N labelling in the snow manipulation experiment. (a) Total ^{15}N recovered (as % of ^{15}N added), (b) microbial biomass (MB): plant ^{15}N ratio (based on total ^{15}N recovered), (c) ^{15}N excess in microbial biomass, (d) ^{15}N excess in shoot biomass, and (e) ^{15}N excess in root biomass. Boxplots show mean, SE and range, dots are individual data points, and significant differences between means are indicated by letters. See Table S5 for details including exact p and χ^2-values

total ^{15}N recovered in the soil microbial community was 106% higher in the shrub-invaded treatment compared to the grass-control treatment following ^{15}N labelling (Figure 5a). In contrast, total ^{15}N recovered in plant shoot and root biomass was 59 and 39% lower, respectively, in the shrub-invaded treatment (Figure 5a). Moreover, the microbial: plant ratio of total ^{15}N recovered was 221% higher in the shrub-invaded treatment than the grass-control treatment (Figure 5b). Concentrations of ^{15}N excess in microbial and plant biomass followed the same significant patterns as total ^{15}N recovered (Figure 5c–e; Table S5). These results were broadly consistent, although not statistically significant ($p = 0.23$; Table S5) after accounting for target N pool dilution, with microbial biomass ^{15}N concentrations being 126% higher in the shrub-invaded treatment than the grass-control treatment.

DISCUSSION

Our findings partially supported our first hypothesis that changing snow conditions have summertime legacy effects on soil microbial communities, soil functioning, and biogeochemical pools and fluxes. In our landscape-scale experiment, earlier snowmelt was related to shifts in summertime bacterial and fungal community composition. Whilst these shifts were relatively small ($r^2 = 0.03$), they are important because our snowmelt gradient only spanned 15 days. This is narrow compared with the 50–130 day earlier snowmelt predicted for 2100 in the European Alps (Beniston et al., 2003), which will likely lead to strong legacy effects on summertime microbial communities. Indeed, stronger effects of snowmelt timing have been found on microbial community composition when the extreme ends of snowmelt timing gradients are tested (Zinger et al., 2009).

Contrary to our first hypothesis, earlier snowmelt was also related to greater summertime potential phosphatase and urease activity, suggesting that winter climate change could lead to increased summer nutrient cycling rates. In our snow manipulation experiment, snow removal led to harsher conditions for soil microbial communities by increasing the frequency of freeze-thaw cycles in winter. Soil functioning did not fully recover from this perturbation by the summer, as evidenced by

increased relative abundances of resilient bacterial families (i.e., *Bacillaceae* (Mandic-Mulec et al., 2015)), higher fungal investment in enzymes that degrade recalcitrant compounds (i.e., PER, (Sinsabaugh et al., 2008)), lower biological activity in soil (measured via soil respiration), and a decline in soil %C, which all occurred due to snow removal, regardless of shrub presence. The decline in soil %C was surprising given the short timeframe of our experiment. The effect was not present in the previous year (2018; see Figure S4) and may not necessarily be a treatment effect, so it should be interpreted with caution. Reduced snow cover also led to a decreased potential for denitrification and lower microbial biomass N retention, but only in the absence of shrubs (i.e. in the grass-control treatment). Our snow removal treatment did not have as strong effects on soil properties as might be expected from recent meta-analyses of studies testing the impacts of increased freeze-thaw cycles (Gao et al., 2021; Song et al., 2017). However, many of these studies investigated the immediate impact of freeze-thaw in controlled laboratory settings, which may not reflect real-world impacts, as tested here (Henry, 2007). Nevertheless, our findings demonstrate that even short-term reductions in winter snow cover, and relatively small advances in snowmelt timing, can have significant cross-season legacy effects on alpine soil microbial communities and functioning in the field.

Our results supported our second hypothesis that ericaceous shrub expansion does not alter snow depth or snowmelt timing, but it does shift soil microbial communities towards ErM and oligotrophic bacteria. As hypothesised, these microbial shifts were also accompanied by altered soil functioning and decreased soil N availability. That shrub expansion did not affect snow depth or snowmelt timing in our study is most likely due to the short stature of ericaceous shrubs (Vowles & Björk, 2019), which is similar to the dominant grass species (*N. stricta*) at our sites. The mechanisms governing ericaceous shrub impacts on soil functioning and biogeochemistry are thus more likely related to changes in soil biota. Specifically, shrub expansion shifted soil fungal communities towards ErM (i.e. *Pezoloma ericae [= Rhizoscyphus ericae]* in the *Leotiaceae*) (Bruzone et al., 2017) and saprotrophs associated with nutrient poor substrates (*Herpotrichiellaceae*) (Untereiner & Malloch, 1999). ErM are resistant to decay due to their melanised hyphae (Clemmensen et al., 2015) and may therefore immobilise soil nutrients for longer time periods than other fungi, potentially accounting for lower soil N availability. However, reduced input of more recalcitrant litter associated with evergreen ericaceous shrubs could also contribute to decreased soil N availability (Adamczyk et al., 2016). Shrub expansion shifted bacterial communities towards taxa associated with cellulose and xylan degradation (the genus *Acidothermus* in the *Acidothermaceae* (Barabote et al., 2009; Berry et al., 2014) and *Solirubrobacteraceae* in the

class *Thermoleophilia* (Bhatnagar et al., 2018)), which could partially explain the observed increases in cellulolytic enzyme activities associated with shrub expansion. Bacterial communities also shifted towards *K*-selected growth strategies, specifically an increased GP: GN bacterial ratio, which has been related to lower soil nutrient availability and more recalcitrant C compounds in soil (Fanin et al., 2019; Fierer et al., 2007).

The shrub-induced shift toward oligotrophic soil conditions and associated microbial taxa, likely explains the overall reduction in soil respiration associated with shrub expansion. We also found increased soil %C under shrubs in our snow manipulation experiment. However, this was not detected in the previous year 2018 (Figrue S4), or at the landscape scale, so it should be treated with caution. Our findings generally contrast with the impacts of deciduous shrub expansion, such as increased soil C-turnover (Parker et al., 2015), which highlights the importance of shrub species identity and root symbiont types in controlling belowground impacts (Collins et al., 2020). Soil properties in the shrub removal treatment were generally more similar to the grass treatment than the shrub treatment, which indicates that shrub treatment effects were directly related to shrub presence. However, these differences were not always significant, likely due to the relatively short time (2-years) since removal of shrubs. Decomposition of fine roots also likely occurred following shrub removal, which may have led to nutrient release and impacts on soil microbial communities and processes. Nevertheless, the wide-ranging belowground effects associated with shrubs in our study reveal the interlinked responses of soil microbial communities and processes to shifting vegetation in alpine grasslands, and the consequences for plant nutrient pools and fluxes.

We found that shrub expansion modulated a number of the impacts of changing snow conditions. This included a negative additive effect on soil respiration (Figure 4a), supporting our third hypothesis. Reduced soil CO_2 emissions following combined ericaceous shrub expansion and reductions in snow cover could potentially lead to a negative climate change feedback. However, some shrub impacts opposed or mitigated the effects of changing snow conditions, contradicting our third hypothesis. In our landscape-scale experiment, the increase in potential phosphatase activity associated with earlier snowmelt was mitigated by shrub expansion, which was associated with consistently lower activity in this important P-cycling enzyme across the entire gradient of snowmelt timing. The combination of shrub expansion and earlier snowmelt also led to increased microbial investment in urease, a key N-cycling enzyme. These findings suggest a potential switch toward microbial investment in N-cycling rather than P-cycling enzymes following earlier snowmelt and shrub expansion in alpine grassland. In the snow manipulation experiment, the combination

of reduced snow cover and shrub expansion led to a switch in denitrifying gene abundances from *nirS* to *nirK*. The higher potential for denitrification in the grass-control treatment may account for the lower total recovery of ^{15}N following ^{15}N labelling, although a similar decline was not seen in the combined shrub and snow removal treatment, despite the increase in *nirK* gene abundances. Shrub expansion protected microbial biomass N from the negative effects of reduced snow cover, likely due to higher abundances of stress-tolerant ericoid fungi associated with shrub expansion (Tibbett & Cairney, 2007). Shrub-associated changes in soil fungal composition may therefore allow microbial communities to more effectively retain N during increasingly frequent freeze-thaw events under future climate change (Campbell et al., 2010).

Mountain ecosystems are experiencing rapid environmental change on multiple fronts. Our findings highlight the important role of interactions between plants and soil microbial communities in shaping the response of these globally widespread ecosystems to environmental change. Specifically, our results suggest that future reductions in alpine snow cover and duration due to climate change may have legacy effects on soil microbial communities and their functioning that persist across seasons with consequences for biogeochemical cycles. However, we also show that these legacy effects may be modulated by ericaceous shrub expansion, which triggers marked shifts in soil microbial community composition and functioning, that are linked to altered soil nutrient pools and fluxes. Combined, our results suggest that belowground impacts of changing winter snow conditions persist across seasons with consequences for biogeochemical cycles, but that shifts in vegetation may be an important modulator of belowground effects of future alpine climate change.

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CONFLICT OF INTEREST

The authors declare there are no conflicts of interest.

AUTHORSHIP

PEER REVIEW

DATA AVAILABILITY STATEMENT

ORCID

Arthur A. D. Broadbent https://orcid.org/0000-0002-8438-7163
David X. Soto https://orcid.org/0000-0003-4210-293X
Robert I. Griffiths https://orcid.org/0000-0002-3341-4547

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