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Potential microbial enzyme activity in seasonal snowpack is high and reveals P limitation

Abigail S. Hoffman[1,2] | **Linda T. A. van Diepen**[2,3] | **Shannon E. Albeke**[4] | **David G. Williams**[1,2]

[1]Department of Botany, University of Wyoming, Laramie, Wyoming, USA

[2]Program in Ecology, University of Wyoming, Laramie, Wyoming, USA

[3]Ecosystem Science and Management, University of Wyoming, Laramie, Wyoming, USA

[4]Wyoming Geographic Information Science Center, University of Wyoming, Laramie, Wyoming, USA

Correspondence
Abigail S. Hoffman
Email: abbyhoffman41@gmail.com

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Abstract

Microbes in snow and ice ecosystems in polar regions contribute substantially to C, N, and P cycling, but few studies have explored microbial activity in seasonal snow. The purpose of this study was to explore the relative importance of snow microbial processing of C, N, and P compounds in atmospheric deposition and litter and detect elemental limitations of snow microbes in Rocky Mountain conifer forests. Enzyme activity in snow was orders of magnitude greater than activity reported for lentic and lotic waters in similar environments. Proportions of C/P- and C/N-acquiring enzymes suggest that snow samples were P limited, or C and P co-limited, while lentic and lotic waters were more N limited. As such, microbes in seasonal snow may change the composition of nutrients and carbon, but these processes are vulnerable to changes in atmospheric deposition and snow extent and duration, which could affect nutrient processing across large areas.

KEYWORDS
aquatic, biogeochemistry, climate change, freshwater, montane, nutrient cycling, subalpine

INTRODUCTION

Seasonal snowpack covers 45.2×10^6 km^2 and 49% of landmass in the northern hemisphere (Lemke et al., 2007). Snowpack is important in mountain ecosystems due to its ability to store water, deliver nutrients to the soil, and insulate soil communities from extreme temperatures. In the western United States, snowpack accounts for 39%–67% of annual precipitation (Serreze et al., 1999), but the region is experiencing increasing precipitation as rain, declining snowpack, and earlier snowmelt due to climate change (Kapnick & Hall, 2012).

Potential microbial activity and limitations across snow ecosystems are not well quantified, although there is evidence of robust microbial communities. Microbial metabolism, including heterotrophic use of diverse carbon substrates (Antony et al., 2012, 2017) and immobilization of N, nitrification, and denitrification (Amoroso et al., 2010; Hodson, 2006; Larose et al., 2013), has been observed in polar snow and ice. In polar ecosystems, photoautotrophic microbes or algae are often the main source of C (Davey et al., 2019; Lutz et al., 2016) and microbial processes are limited by temperature, water availability (Ganey et al., 2017), or P availability (McCutcheon et al., 2021; Mindl et al., 2007). In alpine glaciers and seasonal snow, C inputs come from diverse sources, including vegetation, microbial photoautotrophs, and dust, so microbial limitations are also more diverse

(Bigelow et al., 2019; Hamilton & Havig, 2017; Singer et al., 2012). Similarly, seasonal snow at lower latitudes receives relatively higher inputs of atmospheric reactive nitrogen, dust, and litter than polar snow, which together potentially support significant microbial activity and biogeochemical cycling. While seasonal snow is largely assumed to contribute in a limited capacity to biogeochemical cycling, organic matter processing by microbes in seasonal snow could significantly contribute to fluxes of bioavailable C, N, and P to downstream ecosystems (Arora-Williams et al., 2018; Battin et al., 2016). Measuring microbial activity in seasonal snow is the first step in understanding the relative importance of snow as a biogeochemically active ecosystem compartment.

As global temperatures rise, snowpack has decreased in many areas, and the timing, duration, and extent of snow cover and melt have been altered (Barnett et al., 2005). Such changes are likely altering microbial biogeochemistry of the snowpack and snowmelt. For example, seasonal snowmelt is beginning earlier in winter, but the melt period is lasting longer due to lower radiation at the onset of snowmelt (Musselman et al., 2017). Consequently, the window of time that microbes in the snow can access liquid water under warm conditions is lengthening, which could increase microbial activity (Ganey et al., 2017). But more importantly, microbial biogeochemical processing in seasonal snow will decrease in areas where snow duration and cover are being lost.

The spatial coverage of seasonal snow is large in midlatitudinal regions, albeit shrinking as climate warms, and the importance of microbial nutrient transformations and limitations in seasonal snow relative to other ecosystems is undetermined. Even with low rates of activity in snow, if scaled by aerial coverage, microbes in seasonal snow could contribute significantly to C, N, and P processing, particularly in nutrient-poor mountain ecosystems. It is imperative to understand microbial activity in seasonal snow as climate change may fundamentally alter the importance of annual snowpack as a large-scale biogeochemical reactor.

Extracellular enzymes (EE) produced by microbes to break down organic matter and access energy and nutrients are useful indicators of microbial activity in an ecosystem. Measurements of hydrolytic EE that catalyze terminal reactions of organic matter degradation and yield assimilable carbon (C)-, nitrogen (N)-, or phosphorus (P)-containing molecules are common, which allows for comparisons of potential activity across ecosystems (Allison et al., 2007; Sinsabaugh & Follstad Shah, 2012). Widely measured EE include β-glucosidase (BG), which degrades cellulose and releases C; phosphatase (PHOS), which cleaves phosphates from phospholipids and phosphosaccharides; N-acetyl-β-glucosaminidase (NAG), which degrades chitin and peptidoglycan, releasing N; and leucine aminopeptidase (LAP), which hydrolyzes amino acids such as leucine from polypeptides (Sinsabaugh et al., 2009). Activity of these EE is directly related to nutrient and carbon availability (Allison et al., 2007; Allison & Vitousek, 2005; Olander & Vitousek, 2000). For example, when inorganic P is added, PHOS activity decreases (Olander & Vitousek, 2000), and when readily assimilable C is available, BG activity is suppressed (Chrost & Siuda, 2002). Relationships between inorganic N and peptidases (LAP) or chitinases (NAG) can be more variable because N pathways are more complex, and these N-acquiring enzymes can also be sources of C, but additions of N have been shown to suppress NAG activity (Olander & Vitousek, 2000) and LAP activity (Chrost & Siuda, 2002). Sinsabaugh et al. (2009) found that in nearly 1500 soils and sediment samples, log-transformed ratios of C:N:P-acquiring enzymes averaged 1:1:1. They suggested this empirical ratio represents an equilibrium between microbial biomass and organic matter stoichiometry as it is partitioned by microbial growth and nutrient assimilation efficiencies (Sinsabaugh et al., 2009). Measurements of EE activity in snow can show the potential ability of these unexplored microbial ecosystems to mineralize organic matter and reveal resource limitations in snow and downstream ecosystems.

The objective of this study was to measure potential EE activity in seasonal snow to understand snow microbial ecology and contributions to biogeochemical cycling. We compared these measurements in snow with those in stream and lake water to provide context and to explore how nutrient limitations shift across the landscape, from snowmelt to streams and lakes. We asked the following questions: (1) How does microbial enzyme activity in seasonal snow vary across the landscape and melt season?; (2) How does the extent and magnitude of potential enzyme activity in seasonal snow compare with that of lotic and lentic waters?; and (3) To what degree does C, P, and N limit microbial activity in seasonal snow and how do these limitation patterns compare with downstream lentic and lotic ecosystems?

We expected that microbial activity in subalpine seasonal snow would be a significant contributor to nutrient transformations particularly during the snowmelt period when water and nutrient pulses occur. As melt progressed, we expected that enzyme activity would be higher in forest areas than in meadow areas because forests have more vegetation that likely provides more organic substrates. In addition, we expected EE activity in seasonal snow would be comparable to activity observed in other freshwater ecosystems. Finally, we expected that in

seasonal snow, microbial activity would be P limited due to relatively low P deposition in mountain snow. Understanding microbial activity and limitations in seasonal snow is vital to predicting how changing snow conditions alter biogeochemical cycling.

METHODS

Study sites

We collected samples at three subalpine sites at different elevations in the Snowy Range Mountains in Wyoming, USA, to understand how different snowmelt conditions affected microbial activity (lower elevation, 3000 m [41.34368, −106.21786], mid-elevation, 3180 m [41.36506, −106.23964], and high elevation, 3280 m [41.3763, −106.25461]; Appendix S1: Figure S1). We collected samples for EE analysis from beneath forested vegetation and in adjacent open meadows at each site. The forest vegetation at these sites is dominated by subalpine fir (*Abies lasiocarpa*), Engelmann spruce (*Picea engelmannii*), and Lodgepole Pine (*Pinus contorta*). The meadow vegetation is dominated by short-statured forbs, subshrubs, and graminoids, but plant canopies in the meadows were completely covered by snow for the entire period of our sampling.

Sample collection

We collected samples at each of the three elevation sites once per month from March to June 2019 to capture the snowmelt dynamics. On each sampling date, we used an autoclave sterilized polypropylene scoop to collect 5 surface snow samples in a forested area and an adjacent meadow area for a total of 10 samples per site per date. We assumed that surface snow (∼5 cm in depth) would have the highest microbial activity because particulate organic material accumulates on the surface late during the snow-covered period. We set out to confirm this assumption by sampling also from lower and intermediate depths in the snowpack at each site on the last sampling date. These samples proved to have lower enzyme activity and due to the low sample numbers are not included in our data analysis (Appendix S1: Table S1). We collected separate 300-ml samples for enzyme analyses and 1-L samples for snow chemical analysis into Whirl-Pak bags with autoclaved polypropylene scoops. We kept all samples frozen in a cooler at field sites and then immediately transferred each sample to a −20°C freezer upon return to the laboratory. We stored samples at −20°C for 2–5 months prior to analysis.

Snow chemistry

We melted frozen samples over 6–8 h at 20°C and then filtered and transferred subsamples into polyvials for ion analysis and pre-ashed glass vials for dissolved organic carbon (DOC) analysis. For each sample, we measured pH using a pH meter (YSI Professional Plus, Yellow Springs, OH). We measured concentrations of cations (NH_4^+, Ca^{2+}, and K^+) and anions (NO_2^-, NO_3^-, PO_4^{3-}, and SO_4^{2-}) on a Dionex Dual Integrion RFIC Ion Chromatograph (Dionex, Sunnyvale, CA) in the University of Wyoming Ecology and Biogeochemistry Core Lab. The cation check standards analyzed at concentrations of 10–50 mg/L had analytical precision errors of 4.7%, 0.4%, and 0.4% (NH_4^+, Ca^{2+}, K^+). Check standards for the anions with concentrations ranging from 5 to 7.5 mg/L had analytical precision errors of 0.5%, 2.7%, 1.7%, and 1.1% (NO_2^-, NO_3^-, PO_4^{3-}, and SO_4^{2-}). We measured concentrations of DOC using a Teledyne Tekmar Fusion Total Organic Carbon Analyzer (Teledyne, Thousand Oaks, CA) also in the University of Wyoming Ecology and Biogeochemistry Core Lab. A check standard with a concentration of 0.25 mg/L had an analytical precision error of 10.8%. To correct for any contamination derived from filtering and storage, each measurement was corrected using blank values from nanopure water that was filtered, stored, and analyzed with the samples.

Potential enzyme activity measurements

We assayed enzyme activity using a fluorometric method similar to that outlined in Bell et al. (2013) and Saiya-Cork et al. (2002). We assayed activity of hydrolytic enzymes including α-glucosidase (AG), cellobiohydrolase (CBH), beta-xylosidase (BX), BG, NAG, and PHOS using methylumbelliferyl (MUB)-linked substrates, while LAP was assayed using a 7-amido-4-methylcoumarin (AMC)-linked substrate L-leucine. We measured enzyme activity for all enzymes in three test samples using substrate concentrations of 200, 1000, 2000, and 4000 μM, with incubation times of 1, 2, 4, 6, and 8 h to discern saturating substrate concentrations for enzyme assays. Additionally, we assayed the three test samples at 4 and 20°C to understand the effect of temperature, enabling Q_{10} estimations, and to allow more direct comparisons with results from other studies that conducted assays at 20°C. We report these values in Appendix S1: Table S2 to allow for standardized comparisons.

We melted samples on a bench top at room temperature (∼25°C) for 3–4 h before enzyme analysis. For each sample, we transferred 200 μl of melted snow to a 96-well microplate and then added 50 μl of substrate. BG, CBH,

NAG, and BX were assayed with a substrate concentration of 2000 μM and an incubation time of 1 h. We assayed PHOS and AG using a substrate concentration of 4000 μM and an incubation time of 1 h, while LAP was assayed using a substrate concentration of 4000 μM and an incubation time of 4 h. We incubated well plates at 4°C to closely match temperatures found in snow during melt in the field. We measured fluorescence on a Synergy HTX Multimode Reader (BioTek Instruments, Inc., Winooski, VT) at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. All assays had four replicate wells per sample and substrate and were corrected for background fluorescence of the substrate. We assayed all blanks and standards in buffers with pH of 6.0. We determined the conversion of fluorescence to enzyme activity based on the fluorescence of the standard (10 μM MUB or AMC). We then calculated enzyme activity using the net fluorescence of the sample corrected by the emission coefficient from the standard, sample volume, and incubation time. We report enzyme activity as nanomole of substrate converted per milliliter of water per hour.

Compilation of lentic and lotic enzyme activity data

In September of 2020, we searched the literature using Web of Science™ for other studies that measured potential enzyme activity in freshwater systems to provide context for the level of enzyme activity in snow. Search terms focused on extracellular enzyme activity in freshwater lakes and streams and included "extracellular enzyme activity lake water," "extracellular enzyme activity stream," and "extracellular enzymes freshwater." Studies included (1) used fluorometric well plate assays to measure potential activity of C-, N-, and P-acquiring enzymes, with MUB-linked substrates for BG, PHOS, and NAG and AMC-linked substrates for LAP, (2) made measurements in water collected from natural freshwater ecosystems (e.g., studies described as "heavily polluted" or associated with a point pollution source were excluded), and (3) reported measurements in moles of substrate per volume of water per unit of time. Searches returned a total of 1243 studies, of which 12 met the criteria. The vast majority of studies on potential enzyme activity in freshwater ecosystems measure rates in sediments, often in units of nanomole of enzyme activity per gram soil organic matter (or soil organic carbon) per hour, which makes it difficult to compare with potential enzyme activity measured in water. Compared with soils and sediments, potential enzyme activity in all types of water samples is low. We compared our values with freshwater ecosystems to put these measurements in snow into context and because snowmelt (Huss et al., 2017; Stieglitz et al., 2003), streams (Alexander et al., 2007; Lowe & Likens, 2005), and lakes provide important hydrologic connectivity in mountain ecosystems (Viviroli et al., 2003). As such, these comparisons improve understanding of how microbial activity and nutrient limitations change across these connected systems.

We report mean enzyme potentials from 6 studies for 16 different lentic ecosystems (Hoostal et al., 2008; Jackson et al., 2013; Kalwasińska & Brzezinska, 2013; Munster et al., 1992; Song et al., 2018; Velasco Ayuso et al., 2017) and from five studies for 11 different lotic ecosystems (Bullock et al., 2017; Harjung et al., 2019; Hendel & Marxsen, 1997; Sinsabaugh et al., 1997; Sinsabaugh & Foreman, 2001). The only methodological difference between studies was that some studies used a NaOH addition after incubation and before measurement, which increases detection of fluorescent products at low levels, but was not necessary for our measurements. We reported the compartment with the highest total activity in studies where water was sampled from different compartments of a lake or stream. We reported the control treatment measurements if studies described experimental treatments. Data used from these sources are compiled in Appendix S1: Table S3. Enzyme activity in these studies was assayed at in situ temperatures or temperatures ranging from 20 to 25°C (Appendix S1: Table S2).

Enzyme assays are designed to determine potential enzyme activity at optimal, standardized conditions to allow for comparisons between studies. Actual enzyme activity in natural ecosystems is also influenced by substrate concentrations, pH, and temperature (Sinsabaugh & Follstad Shah, 2012). While enzyme activity often increases with temperature, we assayed enzyme activity in snow at 4°C because temperatures in snow are lower than other ecosystems. Based on a few measurements of enzyme activity in snow at 4 and 20°C (Appendix S1: Table S2) and enzyme reaction kinetics (Sinsabaugh & Follstad Shah, 2012) and empirical data suggesting enzymes have temperature optima at 20–25°C (Halemejko & Chróst, 1986; Min et al., 2014), it is likely that potential enzyme activity in snow at 20°C is even higher than reported here. In addition, we compared C/N and C/P proportions for these lakes and streams to understand how microbial activity is limited in different ecosystems. One study was excluded because reported values deviated substantially in orders of magnitude from those of all other studies, and thus, we had low confidence in the validity of the findings.

Analysis of snow, lotic, and lentic ecosystem aerial extent in the Rocky Mountain region

We calculated the spatial coverage of snow, lakes, and streams in the US Rocky Mountain region to quantify the relative extent of microbial activity in snow. We calculated and compared these values for snow and freshwater ecosystems because they have not been reported elsewhere and to emphasize that seasonal snow should be considered as a large-scale biogeochemical reactor, not to imply that biological activity in snow is more important than that in freshwater. We used the US Environmental Protection Agency Level IV Ecoregions of the Conterminous United States (US Environmental Protection Agency, 2013) to classify the extent of coniferous forest in the US Rocky Mountain region. We included Level IV Ecoregions whose classifications contained "forests," "mountains," or "subalpine" in the Northern Rockies (15), Middle Rockies (17), Wasatch and Uinta Mountains (19), and Southern Rockies (21) (Appendix S1: Figure S1). The extent of seasonal snow cover was determined from MODIS/Terra Surface Reflectance 8-Day L3 Global 250 m dataset from 14 March 2019 (Vermote, 2015). The area of overlap between conifer forest and seasonal snow was then calculated in R.

Next, we obtained lake and stream spatial data from the National Hydrography Plus High Res Dataset (US Geological Survey, 2019) for all watersheds that intersected with Rocky Mountain conifer forests as defined above. Lake/pond (FCode 390) and reservoir (FCode 436) data were extracted from the NHDWaterbody dataset, and stream/river (FCode 460), connector (FCode 334), and artificial path (FCode 558) were extracted from the NHDFlowline dataset. We then cropped these features to the extent of the ecoregion conifer forest layer and area, and length of lentic and lotic features was summed. We then calculated area of lotic ecosystems using lengths and stream order classifications from NHD and average stream order widths (Downing et al., 2012). We performed all spatial analyses in R (R Development Core Team, 2008) using the tidyverse, sf, sp, and raster packages.

Statistical analysis

To understand relative activity of C-, N-, and P-acquiring enzymes, we calculated proportions of total activity described by Moorhead et al. (2016). We used proportions instead of ratios because untransformed ratios are not normally distributed, which leads to biases in inferences (Isles, 2020), and log transformations were not practical due to the large number of zeros in our data. Additionally, Moorhead et al. (2016) found that raw proportions were more straightforward to interpret than transformed data. We calculated the proportion of C/P-acquiring enzymes and C/N-acquiring enzymes as:

$$\mathrm{C/P} = \frac{\mathrm{BG}}{(\mathrm{BG} + \mathrm{PHOS})}. \quad (1)$$

$$\mathrm{C/N} = \frac{\mathrm{BG}}{(\mathrm{BG} + \mathrm{NAG} + \mathrm{LAP})}. \quad (2)$$

These proportions indicate the relative activity of C-to-P- and C-to-N-acquiring enzymes, and C/P or C/N proportions equal to 0.5 indicate that activity of C-to-P- or C-to-N-acquiring enzymes is equal. Proportions greater than 0.5 show that C-acquiring enzyme activity is higher than P- or N-acquiring enzyme activity and suggest C may be limiting, while proportions less than 0.5 show P- or N-acquiring enzyme activity is higher than C-acquiring enzyme activity and suggest P or N may be more limiting with respect to C. Ratios have a skewed distribution and must be transformed to avoid biases, but the proportions we calculate are bound between 0 and 1 and transformations do little to change the statistical distribution (Moorhead et al., 2016). Many papers calculate vector angles and lengths from these proportions and use angles greater than 45° to indicate N limitation and angles less than 45° to indicate P limitation and vector length to indicate C limitation (Fanin et al., 2016; Keane et al., 2020; Liu et al., 2020; Moorhead et al., 2013, 2016). We used this same framework and logic in our analysis; however, we found it more intuitive to interpret proportions, which represent relative activity of enzymes, than to interpret angles and vector lengths. Total enzyme activity was calculated as the sum of activity of BG, NAG, LAP, and PHOS, because these have been commonly measured in other studies and thus allow us to compare our data.

Enzyme activity measurements require several assumptions that can influence the interpretations of these data. These interpretations assume that C, N, and P are the primary limitations for microbial communities and that activity of these enzymes integrate microbial resource demands with relative availabilities of C, N, and P (Sinsabaugh & Follstad Shah, 2012). However, substrate availability, pH, and environmental conditions that affect the breakdown of enzymes can also influence measured enzyme activity (Sinsabaugh et al., 2008). For example, NAG and LAP are sensitive to pH (Halemejko & Chróst, 1986; Min et al., 2014), so low activity could be due to poor environmental conditions. Additionally, NAG and LAP catalyze reactions that are sources of both N and C (Sinsabaugh & Follstad Shah, 2012), so it can be difficult to disentangle C and N acquisition if NAG or

LAP activity is very high. While interpretations are made cautiously, many studies support the use of relative activities of EE to understand microbial nutrient limitations (Hill et al., 2012; Sinsabaugh et al., 2008, 2009). Additionally, we limit our comparisons to ecosystems that are relatively similar (e.g., oligotrophic freshwater systems) to limit the influence of differential resource availability. The proportions of different types of enzymes are used to compare the relative activity of different enzymes across samples and systems. The reference value of 0.5 is not meant to be a threshold for interpretation of limitations but represents only where activity of the compared enzymes is equal.

We used analysis of variance and Tukey's honestly significant difference tests to assess significant differences across sampling dates and between cover types (meadow or forest) for all snow chemistry measurements, individual enzyme activity measurements, total enzyme activity, and C/N and C/P proportions. Additionally, because rates of enzyme activity are often related to available C (Sinsabaugh et al., 2008), we calculated enzyme activity per milligram of DOC and used linear regression to explore the relationship between DOC and enzyme activity. We were not able to compare snow enzyme activity per milligram of DOC with other lotic and lentic ecosystems because other studies reported varying measures of C, such as DOC, dissolved organic particulate, dissolved organic matter, or others. We also used linear regression to explore relationships between ion concentrations and enzymes that target those resources (e.g., relationship between PHOS, a P-acquiring enzyme, and PO_4^{3-}). All statistical analyses and figures were completed in R (R Development Core Team, 2008).

RESULTS

Snow chemistry

Dissolved organic carbon, nitrate, phosphate, calcium, and potassium ion concentrations were significantly higher in forest than in meadow sites across all sampling times ($F = 41.76$, 9.74, 23.77, 9.49, and 25.55, respectively, $p < 0.05$; Table 1 and Appendix S1: Table S4). Additionally, DOC, nitrate, ammonium, phosphate, calcium, and potassium ions changed throughout the sampling dates ($F = 26.39$, 20.73, 16.11, 3.95, 13.97, and 4.81, respectively, $p < 0.05$; Table 1 and Appendix S1: Table S4). DOC concentrations increased during the sampling period from March to June in both forest and meadow sites ($p < 0.05$, Table 1). Nitrate and ammonium ions decreased across the sampling period in all sites ($p < 0.05$, Table 1). Phosphate ion concentrations were low throughout the sampling period (Table 1). Potassium ion concentrations also increased across the sampling period ($p < 0.05$, Table 1).

Potential enzyme activity

Total enzyme activity was relatively low across the first three sampling dates for both forest and meadow sites (Table 2). In forest and meadow sites, total enzyme activity increased across the sampling period (Table 3). Total enzyme activity and activity of BG, AG, CBH, BX, NAG, and PHOS were higher in forest sites than in meadow sites ($F = 41.7$, 7.21, 41.72, 35.97, 32.13, and 8.14, respectively, $p < 0.05$; Table 2 and Appendix S1: Table S4). In

TABLE 1 Chemical variables (mean ± SD) measured in seasonal snowfall in the Snowy Range, WY, from March to June 2019 in forested and meadow sites, including dissolved organic carbon (DOC), nitrate, ammonium, phosphate, calcium, and potassium ions, all measured in milligram per liter, and pH

Month	DOC* (mg/L)	Nitrate* (mg/L)	Ammonium (mg/L)	Phosphate* (mg/L)	Calcium* (mg/L)	Potassium* (mg/L)	pH
Forest							
March	0.57 ± 0.19^{a}	0.47 ± 0.22^{a}	0.35 ± 0.15^{a}	0.02 ± 0.03	0.11 ± 0.09^{a}	0.04 ± 0.05^{a}	5.78 ± 0.25
April	1.37 ± 1.55^{a}	0.38 ± 0.18^{ab}	0.36 ± 0.21^{a}	0.03 ± 0.03	0.41 ± 0.19^{b}	0.09 ± 0.15^{ab}	5.74 ± 0.34
May	1.33 ± 0.90^{a}	0.27 ± 0.11^{bc}	0.25 ± 0.14^{ab}	0.04 ± 0.03	0.38 ± 0.37^{ab}	0.05 ± 0.05^{ab}	5.71 ± 0.20
June	3.38 ± 1.12^{b}	0.15 ± 0.06^{c}	0.10 ± 0.04^{b}	0.05 ± 0.03	0.18 ± 0.06^{a}	0.13 ± 0.09^{b}	5.73 ± 0.21
Meadow							
March	0.52 ± 0.26^{a}	0.35 ± 0.22^{a}	0.31 ± 0.17^{a}	0.01 ± 0.02	0.05 ± 0.05^{a}	0.01 ± 0.01^{a}	5.79 ± 0.36
April	0.63 ± 0.20^{a}	0.28 ± 0.16^{ab}	0.32 ± 0.21^{a}	0.01 ± 0.01	0.29 ± 0.19^{b}	0.00 ± 0.01^{a}	5.82 ± 0.41
May	0.73 ± 0.34^{a}	0.21 ± 0.10^{b}	0.24 ± 0.16^{ab}	0.02 ± 0.02	0.22 ± 0.26^{bc}	0.01 ± 0.02^{a}	5.78 ± 0.32
June	1.09 ± 0.31^{b}	0.09 ± 0.02^{bc}	0.09 ± 0.02^{b}	0.01 ± 0.01	0.09 ± 0.07^{ac}	0.04 ± 0.02^{b}	5.68 ± 0.32

Note: Asterisks (*) reflect significant differences between forest and meadow sites, and letters reflect significant differences between sampling dates ($p < 0.05$).

TABLE 2 Potential enzyme activity (mean ± SD) measured in seasonal snow in the Snowy Range, WY, from March to June 2019 in forested and meadow sites, including leucine aminopeptidase (LAP), N-acetyl-β-glucosaminidase (NAG), alpha-glucosidase (AG), β-glucosidase (BG), cellobiohydrolase (CBH), β-xylosidase (BX), and phosphatase (PHOS) measured in nanomole per milliliter per hour

Month	BG*	AG*	CBH*	BX*	NAG*	LAP	PHOS*
Forest							
March	1.51 ± 1.17^{a}	0.72 ± 0.47	0.60 ± 0.41^{a}	0.47 ± 0.29^{a}	0.75 ± 0.57^{a}	0.02 ± 0.03^{a}	0.32 ± 0.97^{a}
April	0.90 ± 0.68^{a}	0.84 ± 0.46	0.65 ± 0.34^{a}	0.46 ± 0.46^{a}	0.87 ± 0.46^{a}	0.01 ± 0.01^{a}	0.03 ± 0.10^{a}
May	3.58 ± 4.14^{a}	1.18 ± 0.80	1.92 ± 1.81^{a}	1.05 ± 1.06^{a}	1.10 ± 0.89^{a}	0.02 ± 0.03^{a}	3.44 ± 5.08^{a}
June	15.37 ± 9.46^{b}	1.06 ± 0.54	7.63 ± 4.98^{b}	3.33 ± 2.35^{b}	2.07 ± 1.62^{b}	0.10 ± 0.08^{b}	15.44 ± 10.37^{b}
Meadow							
March	0.37 ± 0.26^{a}	1.08 ± 0.73^{a}	0.28 ± 0.21^{a}	0.26 ± 0.31^{a}	0.70 ± 0.63^{a}	0.02 ± 0.06	0.18 ± 0.74^{a}
April	0.26 ± 0.25^{a}	0.72 ± 0.56^{ab}	0.25 ± 0.28^{a}	0.18 ± 0.20^{a}	0.48 ± 0.40^{ab}	0.02 ± 0.04	0.00 ± 0.0^{a}
May	0.24 ± 0.21^{a}	0.46 ± 0.52^{b}	0.11 ± 0.15^{a}	0.12 ± 0.10^{a}	0.27 ± 0.32^{b}	0.03 ± 0.06	1.68 ± 4.15^{a}
June	2.93 ± 2.13^{b}	0.41 ± 0.25^{b}	1.24 ± 0.83^{b}	0.67 ± 0.42^{b}	0.24 ± 0.17^{b}	0.03 ± 0.03	7.28 ± 5.56^{b}

Note: Asterisks (*) reflect significant differences between activity in forest and meadow sites, and letters reflect significant differences between sampling dates ($p < 0.05$).

TABLE 3 Total potential enzyme activity (total) and proportions of C/N- and C/P-acquiring enzymes measured in seasonal snow in the Snowy Range, WY, from March to June 2019 in forested and meadow sites

Month	Total*	C/N*	C/P
Forest			
March	2.60 ± 2.03^{a}	0.64 ± 0.25^{a}	0.93 ± 0.21^{a}
April	1.81 ± 0.74^{a}	0.47 ± 0.26^{ab}	0.99 ± 0.05^{a}
May	8.15 ± 9.66^{a}	0.69 ± 0.19^{ac}	0.80 ± 0.27^{a}
June	32.99 ± 20.46^{b}	0.88 ± 0.06^{acd}	0.53 ± 0.19^{b}
Meadow			
March	1.28 ± 1.19^{a}	0.39 ± 0.28^{a}	0.94 ± 0.23^{a}
April	0.76 ± 0.47^{a}	0.34 ± 0.33^{a}	1.00 ± 0.0^{a}
May	2.23 ± 4.06^{a}	0.45 ± 0.33^{a}	0.79 ± 0.42^{a}
June	10.49 ± 6.26^{b}	0.89 ± 0.09^{b}	0.44 ± 0.35^{b}

Note: Asterisks (*) reflect significant differences between activity in forest and meadow sites, and letters reflect significant differences between sampling dates ($p < 0.05$).

both meadow and forest sites, activity of BG, CBH, BX, and PHOS increased between the first three sampling dates in March, April, and May and the final sampling date in June ($F = 37.5$, 32.68, 23.05, and 40.01, respectively, $p < 0.05$; Table 2 and Appendix S1: Table S4). In the forest sites, NAG activity also increased from the first three sampling dates to the final sampling, but in meadow sites, NAG activity decreased ($F = 8.22$, $p < 0.05$; Table 2 and Appendix S1: Table S4). Although there were significant differences in activity of LAP between different cover types and sampling dates ($F = 5.6$, $p < 0.05$; Appendix S1: Table S4), LAP activity was low throughout all sites and sampling dates (Table 2). In snow, NAG activity was significantly higher than LAP activity (Table 2). Generally, EE activity in snow from March to May was on the same order of magnitude as lentic and lotic ecosystems, while in June, EE activity in snow was significantly higher than in lentic and lotic ecosystems (Figure 1 and Appendix S1: Table S3). The exception was LAP activity, which was higher in lotic ecosystems than snow and was similar between lentic ecosystems and snow (Appendix S1: Table S3).

Microbial enzyme activity limitations

At the final sampling date in June, forest snow tended to be C and P co-limited (Table 3, Figure 2). Similarly, meadow snow samples tended to be C and P co-limited, with some samples more strongly P-limited (Figure 2). In contrast, lentic waters have a wide range of nutrient limitation, with proportions of C/N-acquiring enzymes ranging from 0.01 to 0.92 and proportions of C/P-acquiring enzymes ranging from 0.003 to 0.94 (Figure 2 and Appendix S1: Table S3). Lotic waters were more strongly N limited in comparison with snow with proportions of C/N-acquiring enzymes averaging 0.11 ± 0.11 and proportions of C/P-acquiring enzymes averaging 0.19 ± 0.13.

BG ($p < 0.001$, $R^2 = 0.40$), PHOS ($p < 0.001$, $R^2 = 0.28$), NAG ($p < 0.001$, $R^2 = 0.23$), and LAP ($p < 0.001$, $R^2 = 0.11$) activities were all positively correlated with DOC concentrations (Figure 3). Additionally, total enzyme activity per milligram of DOC did not differ between landscape types ($p > 0.05$) but was higher at

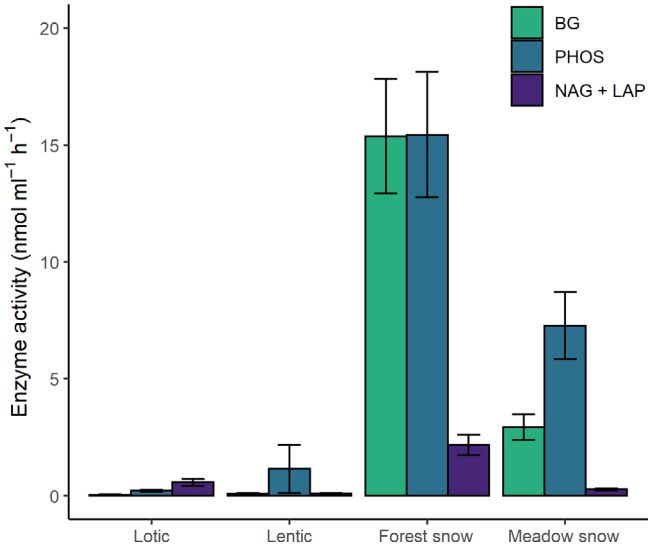

FIGURE 1 Mean (±SE) potential activity of C-, P-, and N-acquiring enzymes including β-glucosidase (BG), phosphatase (PHOS), and N-acetyl-β-glucosaminidase (NAG) + leucine aminopeptidase (LAP) assayed in seasonal forest and meadow snow in the Snowy Range, WY, in June 2019 (Table 2), and mean (±SE) potential activity of enzymes in lentic and lotic waters from a literature search

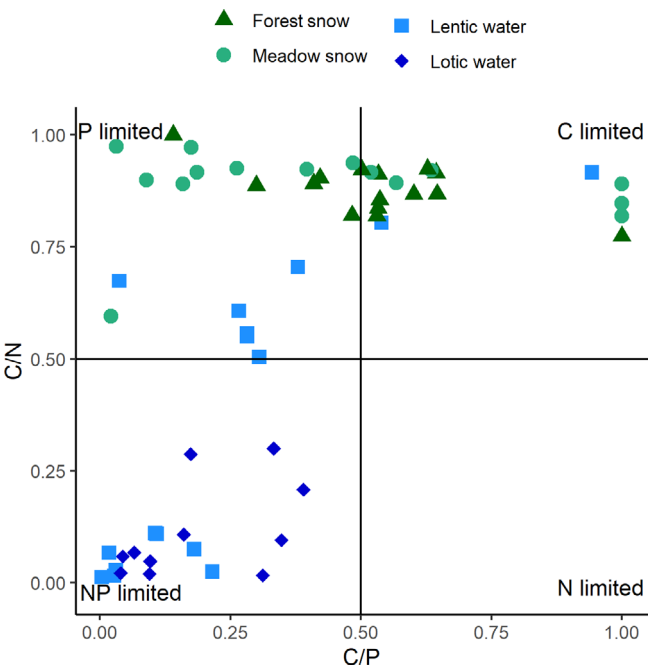

FIGURE 2 Proportions of C/N-acquiring enzymes and C/P-acquiring enzymes in seasonal snow in forest (dark green triangles) and meadow (light green circles) ecosystems in the Snowy Range, WY, in June 2019 and from freshwater studies pulled from the literature including lentic (light blue squares) and lotic (dark blue diamonds) ecosystems. The horizontal and vertical lines drawn at C/P = 0.50 and C/N = 0.50 show where C-to-P- and C-to-N-acquiring enzyme activity is equal

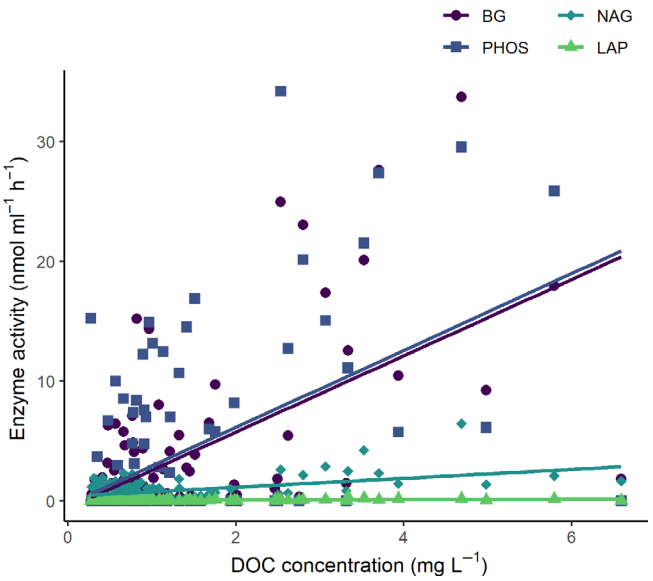

FIGURE 3 Relationship between dissolved organic carbon (DOC) concentrations and β-glucosidase (BG, $R^2 = 0.40$, $p < 0.001$), phosphatase (PHOS, $R^2 = 0.28$, $p < 0.001$), *N*-acetyl-β-glucosaminidase (NAG $R^2 = 0.23$, $p < 0.001$), and leucine aminopeptidase (LAP, $R^2 = 0.11$, $p < 0.001$) in seasonal snow collected in June 2019 in the Snowy Range, WY

later sampling dates ($F = 8.34$, $p < 0.001$), increasing from 4.0 ± 3.8 nmol ml^{-1} h^{-1} mg DOC^{-1} in March to 11.0 ± 7.6 nmol ml^{-1} h^{-1} mg DOC^{-1} in June. PHOS activity was positively correlated with PO_4^{3-} concentrations ($p < 0.001$, $R^2 = 0.15$) and was more strongly correlated at the final sampling date when enzyme activity was highest ($p < 0.001$, $R^2 = 0.34$). Neither NAG nor LAP activity was correlated to NO_3^- or NH_4^+ concentrations ($p > 0.05$). C-acquiring enzymes were all correlated, with BG correlated with BX ($p < 0.001$, $R^2 = 0.93$), CBH ($p < 0.001$, $R^2 = 0.95$), and AG ($p < 0.001$, $R^2 = 0.10$). LAP and NAG were weakly correlated ($p < 0.001$, $R^2 = 0.09$).

Spatial extent of seasonal snow, lentic, and lotic ecosystems

Spatial analyses of US Rocky Mountain forests show that snow covers 209,616 km^2 in these ecosystems, while lentic water bodies cover 1086 km^2 and lotic water bodies run for 406,429 km and cover 1561 km^2 (Appendix S1: Table S5).

DISCUSSION

There was high enzyme activity of C-, N-, and P-acquiring enzymes in meadow and forest snow samples,

particularly during the late snowmelt period. Measurements of EE activity during late snowmelt were on average an order of magnitude higher than in lentic and lotic waters. While it is difficult to compare directly with soils because units of enzyme activity and environmental conditions are different, soils and sediments typically have much higher enzyme activity per gram of soil than that observed in a milliliter of snowmelt or freshwater (Sinsabaugh et al., 2008, 2009). However, seasonal snow covers a large percentage of land, particularly in mountain ecosystems, so nutrient processing within the snowpack may contribute to nutrient and C availability in downstream ecosystems.

Due to the large spatial extent of seasonal snow, particularly in comparison with other freshwater ecosystems, it is essential to consider snow microbial processing of atmospheric deposition and its influence on nutrient exports in snowmelt. In Rocky Mountain conifer forests, we found that snow covers nearly 80 times as much area as lentic and lotic waters. The area covered by streams and lakes is a fraction of the land covered by snow, although both extensive stream networks and snow coverage facilitate important hydrologic and biogeochemical connectivity across broad scales. Additionally, freshwater sediments are important in biogeochemical cycling, and sediments can have higher potential enzyme activity than either water or soils (Sinsabaugh et al., 2009).

Relative proportions of enzyme activity targeting C, N, or P suggest that during snowmelt, when snow microbial processing is highest, microbial activity was limited by C, P, or C and P. Microbes use EE to access nutrients and C, so proportions of C/P enzymes near 0.5, such as those observed in the forest snow samples, indicate C and P are co-limiting to microbial activity, while proportions of C/P enzymes less than 0.5, such as those observed in the meadow snow samples, indicate P is limiting microbial activity later in the melt season (Figure 3). Similarly, in polar snow environments, P has widely been found to be limiting to microbial activity due to excess N from atmospheric deposition and C fixed by autotrophic organisms across large open areas (Darcy & Schmidt, 2016; McCutcheon et al., 2021; Mindl et al., 2007). The eastern Snowy Range, WY, where we collected our samples, has low atmospheric N deposition (National Atmospheric Deposition Program—National Trends Network, 1992–2020) compared with other mountain ranges, so microbial biogeochemical processing of N and P in snow may be altered by changing deposition patterns. Spatial and temporal patterns of dust deposition are not well quantified in many mountain environments but could drive snow microbial productivity where P is limiting.

In comparison with snow, lentic enzyme potentials exhibit a broad range of nutrient and C limitations (Figure 3). The range of limitations observed in lakes is unsurprising given the range of lentic ecosystems EE potentials were drawn from and highlights the need for future research to understand how nutrient limitations are connected across freshwater ecosystems from headwater streams and alpine lakes to major rivers and lakes in low-elevation basins. In alpine watersheds, studies suggest that C is limiting (Bigelow et al., 2019; Velasco Ayuso et al., 2017), so microbial activity in snow may provide essential bioavailable C. Additionally, long-term atmospheric N deposition has led to P limitation in freshwater ecosystems, but recent declines in N deposition have shifted some areas back to N and P co-limitation (Bergström et al., 2020). However, P deposition in dust and from forest fires is also increasing, and P inputs may have a greater impact on ecosystems because of the nutrient requirements by organisms (Brahney et al., 2015). Furthermore, lower elevation ecosystems receive more N and P from runoff and terrestrial weathering that could alter nutrient limitations and explain the large variation in nutrient limitations across lentic ecosystems.

Lotic waters are more N-limited in comparison with the snow samples. This could be because many of these measurements are from larger river systems that received nutrients from runoff, and composition of runoff contributions alters nutrient limitations from natural systems that lack those inputs. However, even an intermittent headwater stream in Spain (Harjung et al., 2019) and a first-order stream in Germany (Hendel & Marxsen, 1997) show N limitation. Microbial activity could shift from P-limited in snow to more N-limited in headwater streams due to rapid N immobilization in soils or due to temporal variation in both these ecosystems. Recent studies show that forest soils and stream sediments efficiently retain NO_3^- leading to low NO_3^- concentrations in the stream water (Addy et al., 2019; Fuss et al., 2019). Additionally, in mountain streams with high snowmelt discharge, streams experience a pulse of nutrients at the onset of snowmelt, followed by very low nutrient concentrations as discharge dilutes and flushes streams (Williams et al., 2015). Ecosystems with snowmelt-driven hydrology experience temporally variable fluxes of nutrients (Costa et al., 2017), which may contribute to the apparent discontinuity in nutrient limitations between connected ecosystems. More research on the contributions of microbes in snow, lakes, and streams to biogeochemical cycling and nutrient availability in freshwater ecosystems is needed to understand spatial and temporal patterns in microbial activity and the extent of microbial processing across ecosystems with differing nutrient inputs. Very few of the enzyme measurements pulled from the

literature are from mountain ecosystems, so further research should focus on nutrient cycling in mountain ecosystems and how snow microbial activity and snowmelt impact nutrient cycling from alpine ecosystems to downstream rivers and lakes.

Total EE activity was higher in forest snow than meadow snow, likely because forests have more organic matter inputs and higher DOC concentrations, which increase the activity of hydrolytic enzymes (Sinsabaugh et al., 2008). Additionally, oxidative stress due to high solar radiation is important in shaping microbial communities in the polar cryosphere (Maccario et al., 2019), so enzyme activity in subalpine meadow environments may also be lower than forest environments because microbes are more stressed by radiation.

Activity of all measured enzymes, except LAP, was significantly higher at the last sampling date in June than at the earlier sampling dates from March to May in both forest and meadow samples. The relatively low activity of all enzymes from March to May indicates that environmental factors other than C, N, or P are limiting activity. Other studies have found that microbial activity in cold environments is limited by the lack of liquid water (Ganey et al., 2017). As snow duration and melt patterns change and freeze–thaw events increase, microbial activity could increase earlier in the year as melt begins earlier and lasts longer (Musselman et al., 2017).

Forest snow had higher concentrations of nutrients and DOC than meadow snow at the last sampling date when microbial activity was significant. DOC concentrations increased during the sampling period, with the highest DOC availability measured in the forest sites in June. This increase in DOC could be explained by microbial EE activity breaking down larger organic molecules or by leaching of C from organic matter. An increase in K^+ ions in the forest sites indicates that leaching from organic matter occurred (Hafner et al., 2005) in the forest sites, but is of a small magnitude and does not explain all of the increase in DOC. In contrast, ion concentrations generally decreased during the sampling period because ions are preferentially exported in snowmelt (Williams et al., 1996), leading to decreasing concentrations of NH_4^+ and NO_3^- and consistently low concentrations of PO_4^{3-}.

Enzyme activity and DOC concentrations could be positively correlated because microbes require C as an energy source, so enzyme activity tends to increase with organic matter (Sinsabaugh et al., 2008). However, DOC is also a product of enzyme activity, so the positive relationship between DOC and enzyme activity could be reinforced because C enzyme activity produces assimilable C-containing compounds. BG, a C-acquiring enzyme, is more tightly correlated to DOC than PHOS, NAG, or LAP, which suggests there is positive feedback between DOC and BG activity. Additionally, enzyme activity per milligram of DOC was higher at later sampling dates, suggesting that increasing DOC concentration was not the only driver of increased enzyme activity and that other nutrient or temperature or water limitations are important. Due to the importance of available carbon for microbial energy, future studies in lakes and streams should explore the importance of C as a driver of microbial enzyme activity. In comparison with soils, where soil C positively correlated with enzyme activity, freshwater ecosystems and snow could have more complex relationships between C and enzyme activity because they have a greater range of proportions of heterotrophic and autotrophic microbes that affect C sources and C storage mechanisms are different.

Many studies report negative correlations between enzyme activity and the resources that they make available. For example, PHOS tends to be negatively correlated with P availability because microbes produce enzymes to target resources that are least available (Hill et al., 2012; Sinsabaugh & Follstad Shah, 2012). Contrary to findings in other studies, in snow there was a positive relationship between PHOS and phosphate. In snow, relationships between enzyme activity and limiting nutrients may be different from other ecosystems because resources are rapidly lost during melt when activity is highest, so nutrients do not accumulate, and enzyme activity is not inhibited as observed in other ecosystems. These results suggest that microbial EE activity in snow contributes bioavailable substrates to downstream ecosystems in snowmelt and microbes contribute to nutrient retention and availability in seasonal snow.

There was no relationship between N-acquiring enzymes and N ion concentrations. This could be because N-acquiring enzyme activity is low in this study, which is likely because LAP activity and NAG activity are depressed by the presence of nitrate and ammonium (Sinsabaugh & Follstad Shah, 2012). Additionally, NAG degrades chitin, while LAP hydrolyses peptides and amino acids, and both reactions are sources of C and N, so the relationship between their activity and inorganic N is not straightforward (Sinsabaugh & Follstad Shah, 2012). NAG and LAP also perform differently depending on environmental conditions. NAG has a pH optimum around 5.5 (Min et al., 2014), while the pH optimum for LAP is around 7.5 (Halemejko & Chróst, 1986). LAP may also be more sensitive to low temperatures (Halemejko & Chróst, 1986). In most studies, LAP activity is higher than NAG activity, and LAP is more commonly measured in freshwater enzyme studies, but relatively low temperatures and pH likely depress LAP activity in snow. More research is needed to understand

the mechanisms that drive microbial activity in different snow environments, particularly because correlations between environmental variables and EE activity in the snow can be misleading due to the export of resources in snowmelt.

LAP and NAG degrade compounds such as chitin, peptidoglycan, and polypeptides (Sinsabaugh & Follstad Shah, 2012). The low N enzyme activity measured in this study suggests these compounds are not significant sources of C. However, the negative relationship between C/N and C/P proportions in snow samples in Figure 2 shows N-acquiring enzyme activity, while still low in comparison with C-acquiring enzyme activity, may be higher when C is more limiting. Similar to studies in alpine ecosystems (Bigelow et al., 2019), this suggests that in subalpine snow ecosystems, N-acquiring enzymes can be sources of C for microbial communities.

Microbial activity in seasonal snow contributes to bioavailable C, N, and P exported to aquatic and terrestrial ecosystems and may fuel microbial activity in early spring in these receptor ecosystems. It is well known that nutrient and C subsidies from arctic glaciers can alter downstream ecosystems (Fegel et al., 2019; Saros et al., 2010), but the importance of nutrient inputs and forms (reduced vs. oxidized N, high vs. low molecular weight compounds) from seasonal snow is unknown. Several studies have shown that microbes in the atmosphere are important ice nucleators, are ubiquitous in snow and are uniquely adapted to the cold, dry, oligotrophic environments found in snow and the atmosphere (Harding et al., 2011), so microbial activity in snow is likely widespread. It is important to understand microbial activity in seasonal snow because these ecosystems cover large areas of land surface and are rapidly being altered by climate change, which could impact biogeochemical inputs to many ecosystems. Additionally, poorly quantified dust inputs could contribute important C and P resources, which alter microbial metabolism. Microbial activity in seasonal snow likely impacts the composition and input of inorganic nutrients and organic compounds to terrestrial and aquatic mountain ecosystems at the start of the growing season. Future research is needed to understand how these processes scale across the landscape and alter total inputs and chemical composition of those inputs to terrestrial and aquatic ecosystems.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data (Hoffman, 2021) are available from Figshare: https://doi.org/10.6084/m9.figshare.14442284.v1.

ORCID

Abigail S. Hoffman https://orcid.org/0000-0002-4053-6278

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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