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Evidence for phosphorus limitation in high-elevation unvegetated soils, Niwot Ridge, Colorado

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Abstract A key challenge to understanding the effects of climate change and nutrient deposition on ecosystem functioning is our lack of knowledge about nutrient limitations of heterotrophic and phototrophic microbial communities. This is especially true in high elevation ecosystems where it has been shown that earlier melt-out of snow beds and glacial retreat is allowing photosynthetic microbes and plants to move into previously unvegetated areas. We used landscape-level analyses of microbial enzyme stoichiometries combined with soil microcosm fertilization studies to determine which nutrients are limiting to microbes in plant-free or sparsely vegetated, snow bed areas of the Colorado Front Range. Both of these independent approaches indicated that the ultimate limiting nutrient in unvegetated and sparsely vegetated soils is phosphorus (P) for phototrophic microbes, with co-limitation by carbon (C) for the entire microbial community. In contrast, vegetated soils in the same watersheds showed more balanced nitrogen (N), P and C co-limitation similar to patterns seen in other plant-dominated ecosystems. In microcosm experiments, P additions resulted in increased growth rates and percent cover by phototrophs, whereas N additions decreased the relative abundances of phototrophs. Taken together, our findings indicate that the colonization of high elevation ecosystems being impacted by N deposition and climate warming will likely be constrained by P limitation of both heterotrophic and phototrophic microbes and by negative impacts of N on microbial phototrophs. These effects may in turn limit the ability of these fragile ecosystems to immobilize inputs of atmospheric N causing increased runoff of excess N to downstream ecosystems.

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Introduction

High elevation and high latitude ecosystems are under increasing threat from multiple global change drivers. Such systems are especially vulnerable to environmental change because they represent the upward range limits for organisms that are adapted to low nutrient levels and cold temperatures (Pauli et al. 2012). Thus, two of the biggest threats to high elevation communities are nutrient deposition and climate warming (Bowman et al. 2006; Engler et al. 2011; Dullinger et al. 2012; Farrer et al. 2015).

Many high elevation ecosystems are being subjected to nutrient deposition, especially nitrogen (N), as a result of increasing industrial and agricultural activity, exhaust from cars and trucks (Bowman et al. 2006), and dust deposition (Ley et al. 2004; Neff et al. 2008; Mladenov et al. 2012). While the effects of N deposition on microbial communities have been extensively studied (e.g., Fisk and Schmidt 1996; Schmidt et al. 2004; Bowman et al. 2006; Nemergut et al. 2008; Ramirez et al. 2010; Yuan et al. 2016), much of this work has been conducted in areas with continuous plant cover and well-developed soils. In contrast, the effects of increased nutrient loading on microbes in plant-free areas with undeveloped soils have received little attention, even though these areas can be heavily impacted by elevated nutrients (Ley et al. 2004; Knelman et al. 2014; Mladenov et al. 2012; Williams et al. 2015).

Concomitant with increasing nutrient loads, climate warming can cause thinner snowpacks due to increases in rain:snow ratios (Knowles et al. 2006) and earlier melt out of the snowpack in many mountainous areas (Vaughan et al. 2013). A decrease in the depth and duration of snow cover can also have profound effects on the structure and function of the unique microbial communities inhabiting high-elevation ecosystems. During times of deep snowpack, soil temperature and moisture can remain constant (temperatures stay at $\sim$ 0 °C) and favorable for microbial growth for several months, resulting in the bloom of a cold-adapted, heterotrophic microbial community under the snow pack (Ley et al. 2004; Freeman et al. 2009a; Schmidt et al. 2009). In contrast, phototrophic microbes (cyanobacteria and algae) become active in these extreme, high-elevation soils during the short, late-summer growing season (Freeman et al. 2009b). This brief window of photosynthetic activity provides as much carbon (C) to this system as wet and dry deposition combined (Freeman et al. 2009b; Mladenov et al. 2012), but C is not presently accruing due to almost year-round (under-snow and snow-free) heterotrophic activity in these soils (Ley and Schmidt 2002; Ley et al. 2004). Therefore, decreasing snowpack duration could shift the proportion of time during the year that is dominated by phototrophs, with potential consequences for C accumulation in the system.

The present study was undertaken to determine if and to what extent nutrients are limiting to microbial communities, and in particular, phototrophic microbes (cyanobacteria, algae), in unvegetated and sparsely vegetated high-elevation sites along the continental divide of the Colorado Front Range, and to examine potential changes in limitations over time. It is particularly important to understand nutrient limitation in areas with high N input, because the relative degree of limitation by different nutrients could determine to what extent deposited N is either absorbed or passed through to downhill ecosystems, and to what extent atmospheric N is fixed (Seastedt et al. 2004; Bowman et al. 1996, 2006). It is also likely that the well documented high rates of N-deposition in the system studied here (Williams et al. 1996; Baron et al. 2000; Bowman et al. 2006) has driven microbial communities to P limitation, but no efforts have been made to determine what nutrients are most limiting to microbes in this ecosystem. Furthermore, P limitation of microbes is likely because this ecosystem is in an early stage of succession in a cold-dry environment that limits the rate of bedrock weathering and therefore P availability (cf. Darcy et al. 2018). We hypothesize that (1) P and C co-limit heterotrophic microbial activity in unvegetated and sparsely vegetated soils (2) unvegetated and sparsely vegetated soils have greater C limitation than nearby areas with developed soil and vegetation (3) P limits growth of phototrophic microbes in high-elevation unvegetated soils of the Colorado Front Range, and (4) over time, C limitation in unvegetated and sparsely vegetated soils decreases

due to increasing plant cover and carbon inputs. To test these hypotheses, we carried out landscape level surveys of microbial extracellular enzyme activities and conducted microcosm fertilization experiments to determine the limiting nutrients for both the entire microbial community and the phototrophic component of the community at this site.

Materials and methods

Field sites and soil sampling

We used a field survey of extracellular enzyme activities at multiple spatial (5 to 2000 m) scales over multiple years (2002, 2007, 2015) to estimate nutrient limitations of the microbial community across a high-elevation landscape. The initial sampling in 2002 used a nested sampling scheme to compare unvegetated soils (n = 90) in the headwater areas of Green Lakes Valley and Lake Isabelle Valley to dry meadow tundra sites on Niwot Ridge (n = 30). More details of the landscape and sampling scheme are described elsewhere (Caine 2010; King et al. 2008). In 2007 and 2015 sampling was narrowed down to the headwater areas of Green Lakes Valley as described by King et al. (2010, 2012) and included sites that were unvegetated and some that were then becoming sparsely vegetated (< 32 individual plant stems per square meter, n = 65). We also sampled plots with > 32 stems m^{-2} (n = 9), and plots that increased in vegetation from 2007 to 2015 from < 32 to > 32 stems m^{-2} (n = 21). Soils at this site typically freeze during the fall and spring (before and after the insulating snowpack develops) and remain around 0 °C while covered in snow in the winter. N deposition at Niwot Ridge has declined in recent years to 9 kg ha^{-1} $year^{-1}$ (Litaor et al. 2018), but is still elevated compared to other nearby areas (e.g. Rocky Mountain National Park, < 5 kg ha^{-1} $year^{-1}$) and above the estimated critical loads for plant responses (4 kg ha^{-1} $year^{-1}$, Bowman et al. 2006). Nitrate export from the aquatic system has declined as well (Caine 2018). Plant colonization of high-elevation unvegetated soils is limited mostly by the long-lasting snowpack that covers many parts of the landscape for 10 months of the year (Ley et al. 2004; Erickson et al. 2005; Freeman et al. 2009a), but plant cover has been increasing in recent years, concurrent with warming summer temperatures and earlier snowmelt (Bueno de Mesquita et al. 2017, 2018). The mean increase in stems in plots that we considered to increase in density was 124 per plot (SE = 16.78). Previous work has described the variation in biogeochemical parameters across this landscape including low pH (mean = 5.1, SE = 0.04) and low availability of nutrients (Ley et al. 2004; King et al. 2008, 2010, Bueno de Mesquita et al. 2017; Porazinska et al. 2018). Across the 65 unvegetated to sparsely vegetated soil samples (2007), inorganic phosphorus levels averaged 0.22 (SE 0.014) µmol g dry $soil^{-1}$ (King et al. 2010) while inorganic nitrogen levels averaged 0.14 (SE 0.022) µmol g dry $soil^{-1}$ (Porazinska et al. 2018). In the densely vegetated plots, inorganic phosphorus levels averaged 0.19 (SE 0.052) µmol g dry $soil^{-1}$ and inorganic nitrogen levels averaged 0.40 (SE 0.133) µmol g dry $soil^{-1}$. Note that inorganic P is from the Olsen extraction method and should be interpreted with caution in our acidic soils. Olsen P has also been shown to overestimate P in low-P samples (Recena et al. 2015).

Sampling was done during the first 2 weeks of September in 2002, 2007 and 2015, after all sites across the landscape had become snow free. Samples were taken at multiple spatial scales (ranging from 5 to 2 km apart) in order to gain an understanding of spatial variation and autocorrelation among microbial, plant and biogeochemical variables as described elsewhere (King et al. 2008, 2010, 2012; Porazinska et al. 2018). For the present study, we used these samples to obtain ecosystem-wide estimates of enzyme stoichiometries (described below) across the 3 years—2002, 2007, and 2015. Soil samples were collected by homogenizing a 5 cm^2 patch of soil in situ to a depth of 4 cm and then filling a 50 ml sterile conical tube or plastic bag with soil. In plots with sparse vegetation, soils were collected from areas not directly adjacent to plants. Samples were immediately placed on ice and transported to the lab where they were frozen at − 20 °C until enzyme assays and DNA extractions could be done. We have previously tested the effects of frozen storage of these soils and found no effect on enzyme activities (King et al. 2008; Weintraub et al. 2007). We also used unvegetated soils from this same landscape to conduct the microcosm experiments described below.

Enzyme activities

Soils used for enzyme stoichiometry comparisons were analyzed in the years they were collected (i.e. 2002, 2007 and 2015) after being frozen at − 20 °C for less than 6 months as others have done for seasonally cold soils (cf. King et al. 2008; Weintraub et al. 2007; Schmidt et al. 2016; Zeglin et al. 2009). Activities of four enzymes were measured in soil slurries consisting of 2 g of each soil sample in 125 ml of 50 mM sodium acetate buffer, pH 5.0 (a typical pH for these soils), homogenized at 3000 rpm for 1 min using an Ultra-Turrax homogenizer (IKA Works Inc., USA) and incubated in the dark for 20 h at 14 °C using controls, fluorescent substrates, and volumes as described by Weintraub et al. (2007) for cold soils. Fluorescence was measured using a Synergy HT Multi-Detection Microplate Reader (Biotek, USA). Activities (nmol h^{-1} g dry $soil^{-1}$) of the following enzymes were measured: N-acetylglucosamidase (NAG) and leucine aminopeptidase (LAP, only measured in 2002) for N acquisition, β-glucosidase (BG) for C acquisition, and acid phosphatase (Phos) for P acquisition.

The ratios of measured enzyme potentials (enzyme stoichiometry) were used to indicate which nutrients are limiting to soil microbial processes because such ratios reflect the relative allocation of microbial resources to the acquisition of P, N and C. In other words, the ratio of enzyme activities in a given sample has been shown to be directly related to whether P, N or C are primarily limiting in a variety of ecosystems (Sinsabaugh et al. 2009; Hill et al. 2012; Cross et al. 2015; Jiang et al. 2019), including cold soils in Antarctica and the Arctic (Zeglin et al. 2009; Schmidt et al. 2016). To compare our data to past studies we used the following enzyme ratios: BG/NAG, NAG/Phos, and BG/Phos (Sinsabaugh et al. 2009; Zeglin et al. 2009; Hill et al. 2012; Schmidt et al. 2016). Where both NAG and LAP were measured as nitrogen-related activity enzymes (2002 only), we added NAG + LAP within the enzyme ratios.

Soil microcosms

We used an incubation experiment with soil microcosms to assess limitations of phototrophic organisms. Soil samples used in the microcosm experiment were collected in 2011 from the top 4 cm of soil at four randomly chosen unvegetated sites spread across the landscape (at least 20 m from each other), and were sieved through a 2.36-mm pore-size sieve (to remove rocks) and homogenized prior to setting up microcosms. Soil moisture content, water holding capacity (WHC) and pH were determined using the approach of King et al. (2010). Microcosms for assessing nutrient limitations of soil phototrophs were constructed using 14 g of soil (dry weight equivalent) spread to a depth of 4–5 mm in each of 12 petri dishes (20 mm depth × 55 mm diameter). Treatments (three replicates per treatment) consisted of + N, + P, and + N + P and controls following the methods of Schmidt et al. (2012) and Darcy and Schmidt (2016). Briefly, nutrient amendments consisted of NaH_2PO_4 (+ P) and/or NH_4NO_3 (+ N) to deliver 75 µg of either N and/or P per gram of soil, with the pH of these solutions being adjusted to the pH of the soil. Nutrient addition levels were based on previous work and in excess of the level needed to overcome nutrient limitation and were not meant to mimic in situ conditions. Nutrient additions were added in 2.2 ml of solution and controls received equal volumes of sterile distilled water, which brought all soils up to 60% of WHC for a final water content of 0.16 (g H_2O per g dry soil). This water content had been previously determined to be adequate to support the growth of microbial phototrophs in this soil type and is representative of soil water contents during periods of active CO_2 uptake by photoautotrophs at this site (Freeman et al. 2009a, b). Microcosms were incubated at room temperature (~ 21 °C) under a photoperiod of 12 h light/12 h dark (for 55 days) and soil water content was monitored by weight every 3 days to maintain soils at 60% of WHC. These water contents and temperatures are indicative of conditions when maximum photosynthesis would occur at the soil surface at our sites (cf. Freeman et al. 2009a, b). Positions of the microcosms were also rerandomized every 3 days.

During the incubation period, microscopic (45 ×) observations of the microcosms were done every 3–4 days. A modified point-intercept method was used to estimate the percent cover of microbial phototrophs as described in detail elsewhere (Schmidt et al. 2012; Darcy and Schmidt 2016). Briefly, a total of 50 random observations ("field of view" or FOV) were made for each microcosm at each time point and the presence or absence of microbial phototrophs was

recorded in each microscopic FOV. On several dates a true point-intercept method using 100-point observations per microcosm was also employed in order to estimate total percent cover. Conversion of the FOV data to percent cover was done using a standard curve. Growth rates per day were calculated from the final and initial percent covers over the time span of 33 days.

Microbial community analyses

At the end of the soil incubations, DNA was extracted using Mo Bio PowerSoil DNA extraction kits (Mo Bio Laboratories, Inc., Carlsbad, CA) and amplified as described in Nemergut et al. (2010), with a modification to the PCR amplification step. Briefly, a fragment of the 16S rRNA gene was amplified using the barcoded primer sets 27F and 338R with 454 Titanium adapters. PCR reactions were performed in triplicate and consisted of 10 µl of sterile water, 10 µl of 5-PRIME hot master mix (5-PRIME, Gaithersburg, MD, USA), 2 µl (5 µM) of the reverse primer, 1 µl (10 µM) of the forward primer, and 2 µl of the sample DNA. Samples were initially denatured for 3 min at 94°C followed by 30 cycles at 94 °C for 45 s, 50 °C for 30 s, 72 °C for 90 s and a final elongation step at 70 °C for 10 min. The three PCR reaction products per sample were quantified, pooled and then purified using UltraClean PCR Clean-up kits (Mo Bio Laboratories, Inc., Carlsbad, CA), according to the manufacturer's protocol. 16S rRNA gene amplicons were sequenced using Titanium chemistry (454 Life Sciences, Bradford, Connecticut, USA) on a GS FLX platform at the Duke Genome Sequencing & Analysis Core Resource Center (Duke University, Durham, NC). QIIME (Caporaso et al. 2010) was used to trim primer regions from sequence data, calculate an OTU table, produce rarefactions curves for all samples, construct a phylogenetic tree for representative OTU sequences, and compute a weighted UniFrac distance matrix (Lozupone et al. 2007). The OTU table was rarefied to 1200 sequences per sample (including singletons), and taxonomy was assigned to the remaining OTUs using the Greengenes database (DeSantis et al. 2006, version May 2013).

Statistical analyses

All statistical analyses were conducted in R (R Core Team 2018). We used a robust version of a paired t test, the bootstrapped M-estimator (function 'bootdpci') in the package 'WRS' (Field et al. 2012; Wilcox and Schönbrodt 2014), to compare enzyme activities collected at the same plots in both 2007 and 2015, while the 2002 survey was just used as a reference and not compared statistically as they were not sampled from the same plots and thus could not be compared over time. To compare enzyme ratios in unvegetated and sparsely vegetated soils to soils with dense and developed vegetation, we calculated a mean enzyme activity for densely vegetated areas near our field site from the 2002 data and other vegetated soils collected in 2006 (Suding et al. 2008), and report the global soil mean (Sinsabaugh et al. 2009) and tropical soil means (Waring et al. 2014). Within the 2002 survey, we used a T-test to compare between log-transformed enzyme ratios in unvegetated and vegetated soils.

Differences in final phototroph percent cover, phototroph growth rate (from an exponential curve), and Chao1 richness estimates in the soil microcosms were evaluated using an analysis of variance (ANOVA). The matrix of UniFrac distances was used in a 2-way permutational analysis of variance model (Anderson 2001) with the 'adonis' function in the 'vegan' package (Oksanen et al. 2013) to test for the effects of N and P addition on bacterial community structure (beta diversity).

Results

Stoichiometric approaches (Hill et al. 2012; Sinsabaugh et al. 2009) using exoenzyme data across 3 years (2002, 2007, 2015) indicated strong P and C limitation of microbes across the unvegetated and sparsely vegetated high-elevation landscape (Fig. 1). There was a significant difference in enzyme ratios between unvegetated and vegetated (dry meadow tundra) soils collected at the same time (September 2002) (t-test, $p < 0.0001$, Fig. 1a). The same overall pattern of enzyme stoichiometries was seen in subsequent years (2007, 2015) across the same landscape (Fig. 1b). The enzyme data also showed that unvegetated and sparsely vegetated soils deviate from stoichiometric patterns seen in alpine tundra, sub-

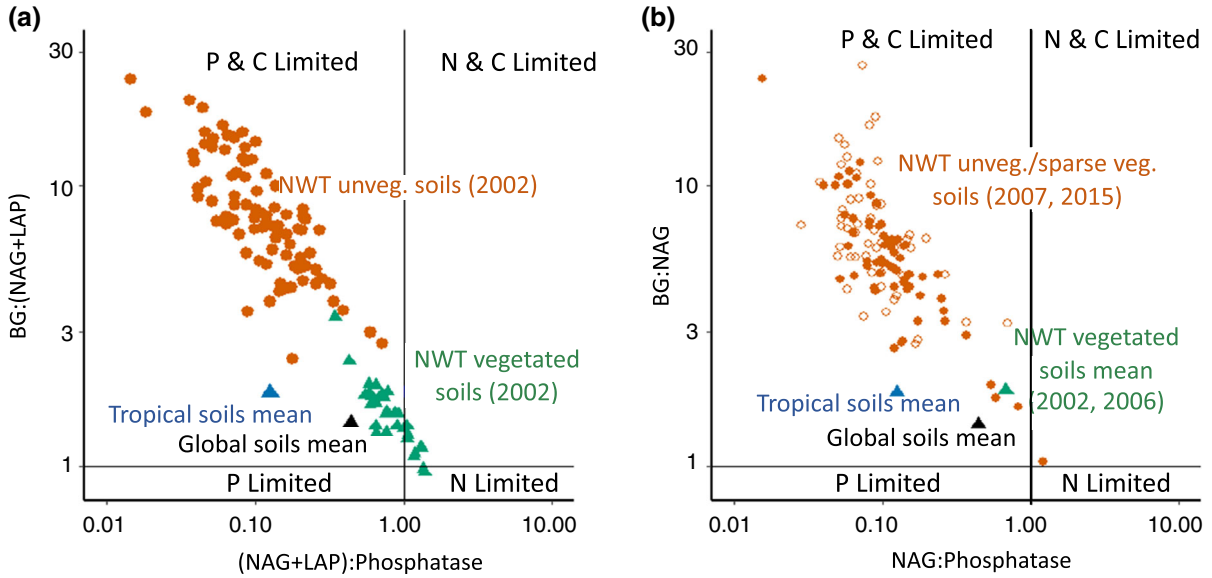

Fig. 1 Stoichiometric relationships of enzyme activity ratios (*BG* C processing, *NAG and LAP* N processing, *Phosphatase* P processing) across the landscape at Niwot Ridge. **a** 2002 survey to compare unvegetated (orange circles) and nearby vegetated soils (green triangles) indicates more P and C limitation of heterotrophic activity in unvegetated soils. Vegetated soils grouped closer to the intersection where C, P and N limitations are equally strong. **b** Unvegetated and sparsely vegetated sites sampled in 2007 (closed orange circles) and 2015 (open orange circles) demonstrate the extent of P and C limitation of heterotrophic remained similar over time. Data in the vegetated soils mean (closed green triangle) included samples from sub-alpine forests, dry meadow tundra, and moist meadow tundra at the Niwot Ridge LTER site (King et al. 2008; Suding et al. 2008; Weintraub et al. 2007). The global soils mean (closed black triangle) from a meta-analysis of vegetated soils (Sinsabaugh et al. 2009), and the tropical soils mean (closed blue triangle) from Waring et al. (2014) are provided for reference. Note the log scale on both axes. (Color figure online)

alpine forests, and other vegetated soils (Fig. 1b); that is, high elevation unvegetated sites are more P- and more C-limited than are vegetated sites, where N limitation may also exist.

A soil microcosm experiment indicated that P limits the growth of phototrophic microbes in unvegetated, high-elevation soils (Fig. 2). P addition (+ P) greatly increased the overall growth of the phototrophic microbial community compared to N and the control (ANOVA: $F_{3,8} = 22.2$, $p < 0.001$, Fig. 2). P addition (+ P and + N + P) also significantly increased the growth rate of soil phototrophs (ANOVA: $F_{3,8} = 29.6$, $p < 0.001$, Fig. 2), but there was no synergistic effect of P and N as has been seen in systems co-limited by P and N (cf. Allgeier et al. 2011). P addition did not significantly change alpha diversity (ANOVA: $F_{1,9} = 0.6$, $p = 0.46$, Fig. 3) and only marginally affected beta diversity (PERMANOVA: $F_{1,9} = 2.6$, $p = 0.08$, Fig. 4) compared to control soils. Taken together, our field and microcosm analyses show that P is the most limiting nutrient for microbial activity and growth in plant-free, high-elevation soils.

Analyses of microbial community diversity and structure in the microcosms also demonstrated an apparent negative effect of added N on microbial communities. Richness (Chao1 richness estimator) of the microbial community was significantly decreased (ANOVA: $F_{1,9} = 42.9$, $p < 0.001$, Fig. 3) in microcosms receiving added N, whereas microcosms receiving just P maintained high levels of alpha diversity (ANOVA: $F_{1,9} = 0.6$, $p = 0.46$, Fig. 3). In terms of beta diversity, N addition also caused a significant (PERMANOVA: $F_{1,9} = 18.2$, $p < 0.001$) shift from a more phototrophic bacterial community to a more heterotrophic community (Fig. 4). N addition (+ N and + N + P) resulted in decreases in the relative abundances of cyanobacteria and diatoms within communities compared to controls whereas these organisms remained relatively more abundant in microcosms that received just P (+ P).

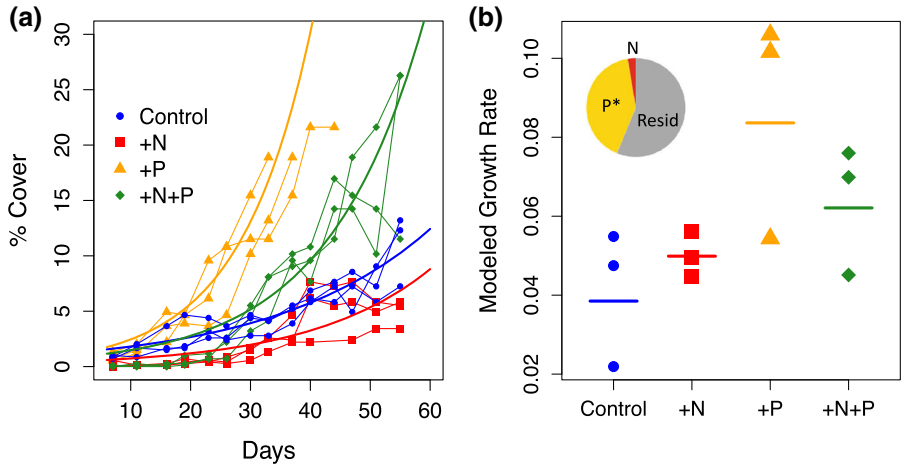

Fig. 2 **a** Growth of microbial phototrophs in soil microcosms demonstrating that P addition dramatically accelerated the growth of phototrophic microbes. **b** Growth rates of soil phototrophs calculated from exponential curves. Only microcosms receiving added P (+ P, + N + P) showed an increase in growth rate compared to the control. Horizontal lines represent the mean of three replicates, which are shown as points. The pie chart shows the effect sizes of the factors and residuals

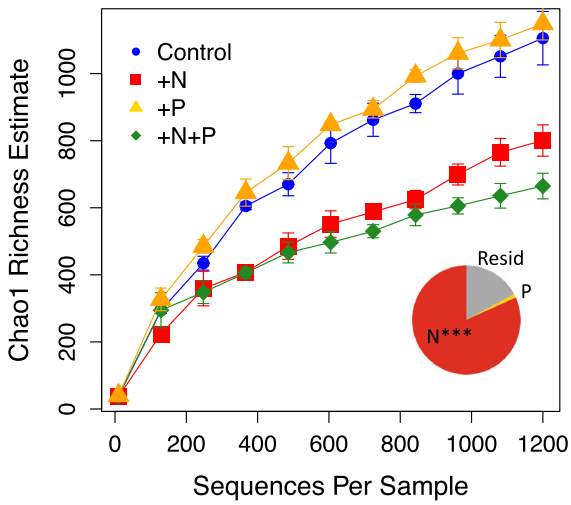

Fig. 3 Alpha diversity of the microbial community (Chao1 richness estimates) at the end of the nutrient addition experiment depicted in Fig. 2. Treatments receiving N (i.e. + N and + P + N) were significantly different from controls and the + P treatment (ANOVA, $p < 0.0001$). The pie chart shows the effect sizes of the factors and residuals

When comparing changes in the enzyme ratios over time (2007 to 2015), there was a significant increase in BG:NAG ratios (bootstrapped M-estimator: n = 61, p = 0.03), but not in NAG:PHOS (bootstrapped M-estimator: n = 58, p = 0.14), or BG:PHOS (bootstrapped M-estimator: n = 56, p = 0.86, Fig. 5), in plots that remained unvegetated and sparsely vegetated, suggesting more stringent limitation of the microbial community by C than N over time. Interestingly, in the more densely vegetated plots across the same landscape (> 32 stems m^{-2}, n = 9) the results were the opposite, with significant decreases in BG:NAG ratios (bootstrapped M-estimator: p = 0.02), significant increases in NAG:PHOS ($p < 0.01$), and no changes in BG:PHOS (bootstrapped M-estimator: p = 0.60). In plots where plant density increased over time (from < 32 stems m^{-2} in 2007 to > 32 stems m^2 in 2015, n = 21), there were no significant changes in any of the ratios (bootstrapped M-estimator: $p > 0.05$).

Discussion

Both the microcosm experiment and enzyme stoichiometric data indicate that P limits activity and growth of both heterotrophic and phototrophic components of the soil ecosystem, and that C also limits activity of heterotrophic microbes in these soils. It is important to note, however, that this is a limitation on growth and activity, not on richness or community composition. In particular, P and C are much more limiting in unvegetated and sparsely vegetated soils compared to nearby more developed soils vegetated by alpine plants. We observed a shift in nutrient limitations in unvegetated and sparsely vegetated soils following a

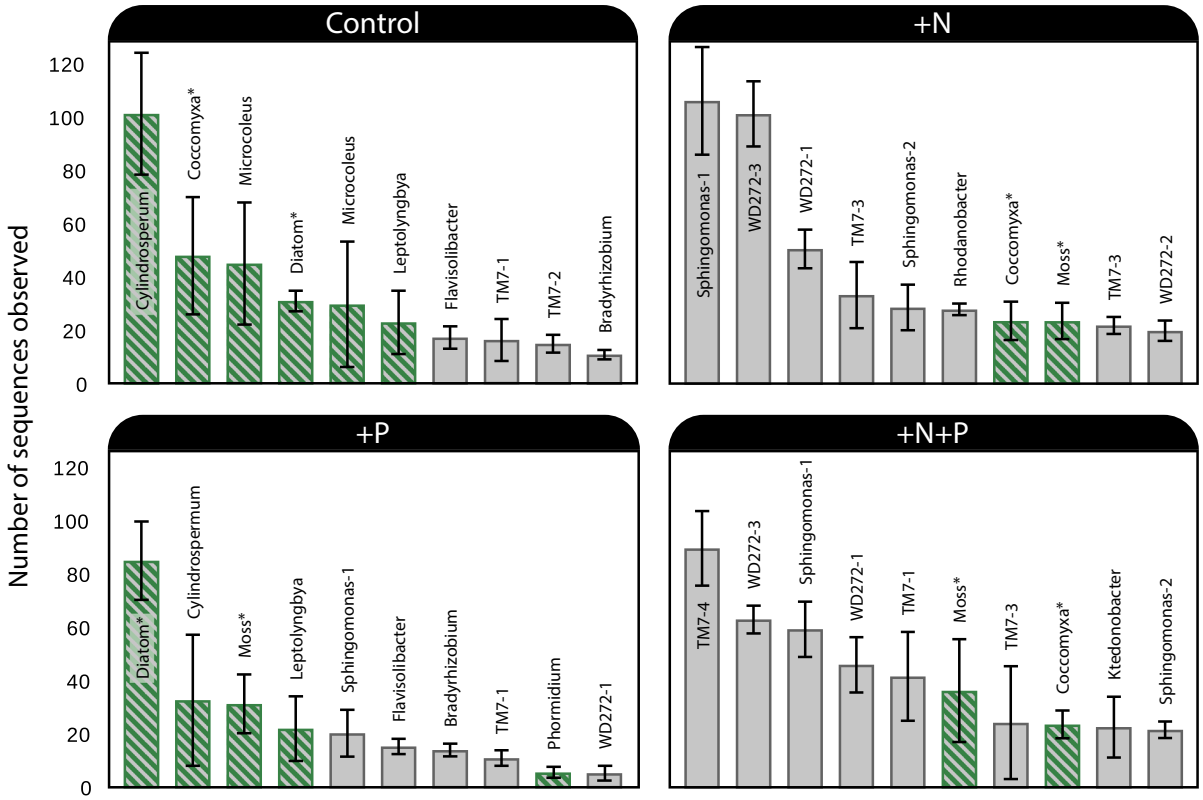

Fig. 4 Rank abundance plots of the top ten most abundant phylotypes in each treatment. Note that all samples were rarefied to 1200 sequences per sample. Treatments receiving N were significantly different from controls and the + P treatment, in terms of beta diversity, and showed a decrease in the relative abundances of most phototrophs. Photosynthetic phylotypes are shown in green (hatched) and asterisks indicate eukaryotic phototrophs. All other phototrophs were cyanobacteria. Repeating names signify different operational taxonomic units within that group. (Color figure online)

resampling of the same sites after eight years (2007–2015) such that heterotrophs invest more in C acquisition relative to N. Conversely, the investment in C acquisition relative to N declined in more densely vegetated soils over that same time period as would be expected as plants deplete N-stocks and add more C to the soils.

Nutrient limitations of the microbial community

As hypothesized, the landscape survey of enzyme stoichiometries demonstrated P and C co-limitation of the microbial community (Fig. 1). Since phototrophs are not likely to be limited by C, the co-limitation by C is driven by the heterotrophic portion of the microbial community. The finding of P limitation is in line with a previous microcosm study at this site (King et al. 2008) and limitations found at other plant-free ecosystems at high elevation sites in the Andes and Alaska Ranges (Schmidt et al. 2012; Darcy and Schmidt 2016; Darcy et al. 2018). All of these studies contrast with the paradigm that N primarily limits early successional ecosystems while P primarily limits later successional ecosystems, an idea that was developed from studies conducted in humid coastal and island ecosystems (Walker and Syers 1976; Vitousek 2004). In contrast, P limitation in plant-free soils at our site and other early successional in colder/inland sites is partially due to the fact that most P in early successional soils is tied up in parent-material rock and is not biologically available (Schmidt et al. 2011). In addition, many cyanobacteria in early successional, plant-free soils can overcome low C and N conditions because they are able to fix both atmospheric C and N (Chapin et al. 1994; Nemergut et al. 2007; Schmidt et al. 2008), which in turn may reinforce P limitation. Increased N-deposition is also likely driving these unvegetated sites to a state of P

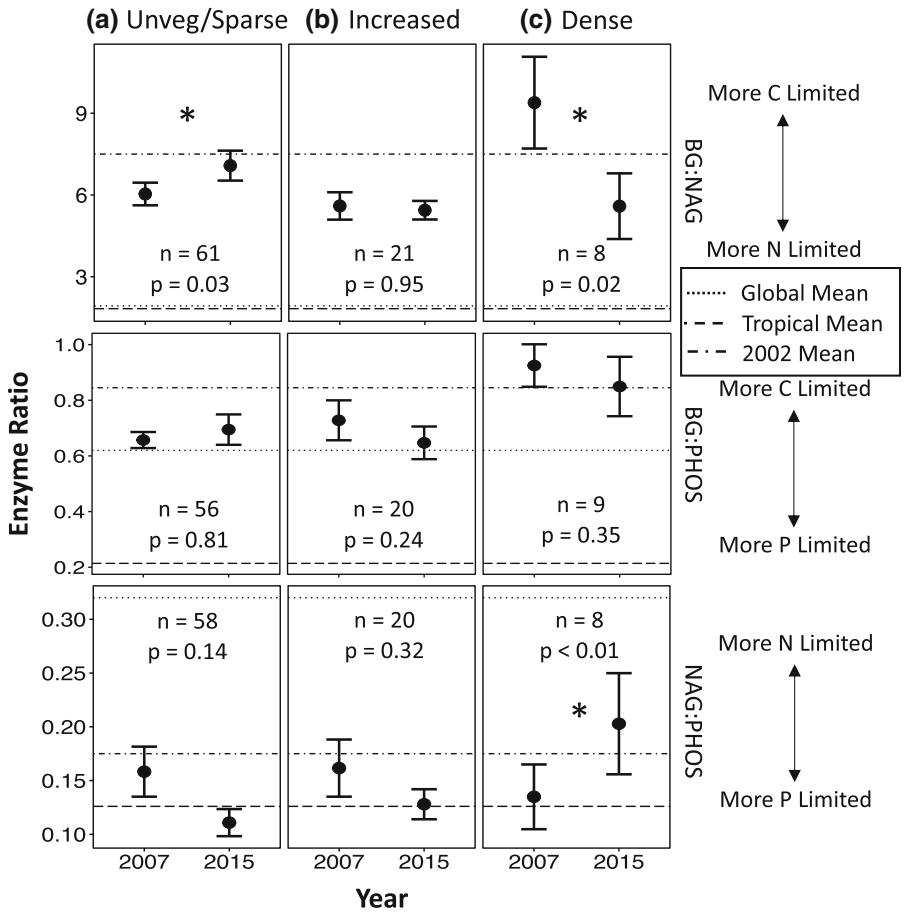

Fig. 5 Mean (± SE) ratios of activity of C-processing enzyme (BG) to N-processing enzyme (NAG), NAG to P-processing enzymes (PHOS), and BG:PHOS in **a** mostly unvegetated plots ("Sparse", < 32 stems/m^2), **b** plots that increased from sparse to dense over the 8 years ("Increased"), **c** densely vegetated plots ("Dense", > 32 stems/m^2). For the "Increased" plots, the mean increase in stems per plot was 124 (SE = 16.78). Also shown for reference are the global soil mean (Sinsabaugh et al. 2009) tropical soil mean (Waring et al. 2014) and means from unvegetated soils collected at our site in 2002. p values for comparisons are from bootstrapped M-estimator tests

limitation by further alleviating any hint of N-limitation (Li et al. 2016).

Nutrient limitations in unvegetated vs. vegetated soils

Not only are these unvegetated and sparsely vegetated soils co-limited by C, but they are also relatively more C limited than nearby vegetated soils, which supports our hypotheses. This finding is not surprising, at least for C limitation of heterotrophs, since plants are the main source of C for heterotrophic microbes in most ecosystems. By contrast, the unvegetated soils that we focus on in this study are dependent on C fixation by phototrophic microbes during a very short growing season (Freeman et al. 2009b) or limited aeolian inputs of plant material (especially pollen) from lower elevation ecosystems (Freeman et al. 2009a; Mladenov et al. 2012). In addition, much of these C inputs to this system are metabolized under the snow during the long winter months, resulting in perennially low levels of total and available C in these soils (Ley et al. 2004, Freeman et al. 2009a). Thus, it is expected that as the growing season lengthens (due to earlier snow melt out) and as plants invade these soils, they will become less C limited and will display exoenzyme stoichiometric patterns more in line with vegetated soils (Bueno de Mesquita et al. 2017). However, plots that shifted from unvegetated or sparsely vegetated to more densely vegetated plots experienced no

alteration in enzyme stoichiometries, suggesting that this shift may take longer or require an even greater increase in vegetation density. In addition to greater C limitation, we also found greater P limitation in unvegetated and sparsely vegetated plots compared to more developed soils and vegetationed plots. This is likely due to plant roots and mycorrhizae being able to access more pools of P in vegetated soils (Lambers et al. 2012; Mullen and Schmidt 1992; Peace and Grubb 1982) and to P being locked up in parent material in early successional ecosystems with low weathering rates.

Nutrient limitations of phototrophs

The growth response of phototrophs in microcosm fertilization experiments demonstrated P limitation (Fig. 2). In comparison with heterotrophs, microbial phototrophs are less likely to be limited by C and N due to the capability to fix these nutrients. In addition to demonstrating P limitation of phototrophic growth, our microcosm experiment has implications for understanding how N deposition affects the microbiota of high-elevation unvegetated soils. Microbial community data from the soil microcosms show a significant community shift with a decrease in alpha diversity and a significant change in beta diversity caused by nitrogen, which is similar to findings in other studies (Ramirez et al. 2010; Zeng et al. 2016). N addition had a disproportionately negative effect on cyanobacterial taxa such as *Cylindrospermum*, two *Microcoleus* taxa, and *Leptolyngbya* which were abundant in the controls and declined significantly in the N addition. In contrast, eukaryotic phototrophs such as *Coccomyxa* and moss were still in the top ten most abundant taxa in the N addition treatment. Since N addition did not affect total cover of phototrophs, it is likely that changes in beta diversity are driven by increases in the abundance of heterotrophic organisms (Fig. 4).

Nutrient limitations over time

Over the 8 years between 2007 and 2015, unvegetated to sparsely vegetated plots became more C limited relative to N, meaning either N limitation decreased, or C limitation increased. Given a longer growing season and hence more time to fix C and deposit this into the system, it is unlikely that C limitation is increasing. This suggests that a decrease in N limitation is the driving force behind the temporal change. This finding is interesting in light of the recent decline in N deposition at our site from 17 kg ha^{-1} in 2007 to 9 kg ha^{-1} in 2015 (Litaor et al. 2018) and NO_3^- export from alpine lakes from 0.32 to 0.27 mg l^{-1} in 2015, but is in line with the idea that the effects of N deposition are cumulative and may be long lasting despite declines in deposition (Bowman et al. 2018).

Conclusions

Overall, our results indicate that microbial activity and growth in the mostly plant-free, talus ecosystem of the Niwot Ridge LTER site is primarily limited by P. This conclusion is supported both by our soil incubation studies and by landscape-scale patterns of enzyme stoichiometries that have been shown to be indicators of nutrient limitations in both aquatic and terrestrial ecosystems (Sinsabaugh et al. 2009; Zeglin et al. 2009; Hill et al. 2012; Cross et al. 2015; Schmidt et al. 2016). P additions in microcosms significantly increased the growth rate and percent cover of microbial phototrophs, and stoichiometric analyses of landscape-scale patterns in enzyme activities also supported the conclusion of P limitation along with C limitation of heterotrophs in this low-C environment. The lack of N limitation suggests that additional inputs of N to the system (e.g. via N deposition) will be mostly exported, which is confirmed by disproportionately high levels of nitrate runoff found here compared to lower elevation ecosystems (reviewed in Williams et al. 2015). It is important to note that nitrate running off from the sparsely vegetated slopes in our study could still be cycled by microbes as it percolates through talus soils above alpine lakes or by the very diverse microbial communities in the inlet of these lakes (Gendron et al. 2019), which may explain why there are differences between nitrate isotopes in lakes compared to precipitation (Nanus et al. 2008). Nitrate export has declined in recent years, however, likely due to declines in deposition and increases in plant cover and uptake. Our work also demonstrates that N deposition may have negative effects on some microbial guilds, as evidenced by decreased phototroph richness in soil microcosms. Also, this study indicates that the movement of plants into formerly plant-free areas will likely decrease C-limitation and result in

more balanced limitation of microbes by C, N, and P. Together, these results provide context for understanding the consequences of global change on plant and microbial succession and potential nutrient exports from this high elevation ecosystem.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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