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Changes in Soil Microbial Community Structure and Function in an Alpine Dry Meadow Following Spring Snow Melt

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ABSTRACT

Previous work in an alpine dry meadow in the Front Range of the Rocky Mountains has shown that microbial biomass is high during winter and declines rapidly as snow melts in the spring, and that this decline is associated with changes in temperature regime and substrate availability. In this study we tested the hypothesis that the summer and winter microbial communities differ in function and composition. Shifts in species composition between pre- and post-snowmelt communities were detected using reciprocal hybridization of community DNA; DNA extracted from soils sampled at different times was significantly less homologous relative to spatial replicates sampled at the same time. Fungal/bacterial ratios, as measured by direct microscopic counts and by substrate-induced respiration experiments with specific inhibitors, were higher in winter soils. Specific activity of cellulase (absolute cellulase activity per unit microbial biomass C) was higher in the winter soils than in summer soils, while specific amylase activity was not different between winter and summer. Based on most-probable number measurements, the use of the phenolic compound vanillic acid was highest in the winter, while the use of the amino acid glycine was lowest in the winter. Winter and summer soil respiration responded differently to temperature; at 0°C, winter soils respired at a higher proportion of the 22°C rate than did summer soils.

Introduction

Our previous work in an alpine dry meadow in the Rocky Mountains showed that soil microbial biomass increases during fall and winter and rapidly declines after snow melt in the spring, and that these microbial population dynamics were important drivers of the alpine N cycle [18]: Our work on the mechanisms of the post-snowmelt decline of biomass showed that the C balance of the soil microbial biomass undergoes a critical shift between winter and summer due to an increase in temperature and a corresponding decrease in C availability [19]. The finding that the microbial community was greatly affected by changes

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in temperature regime and substrate availability led to this study's hypothesis that the summer and winter microbial communities differ in function and composition.

Because many species of bacteria and fungi can grow rapidly, and soil microbial biomass turns over many times each year, there is ample time for microbial community species composition to change dramatically in response to new seasonal conditions. Also, as many bacteria and fungi produce resistant spores or dormant structures, the seasonal community shifts could be cyclic, allowing each community to regenerate as conditions shift back in their favor. Seasonal changes in microbial community composition and function have been reported elsewhere [3, 5, 27, 34]. Seasonal changes in microbial biomass can have important ramifications for nutrient cycling and ecosystem functioning [18, 20, 26, 31, 32]. It has been observed that microbial communities can thrive during the winter in cold ecosystems [7, 8, 12, 15, 18, 19]. Spring thaw is, a dynamic time in cold ecosystems that typically produces a flush of available nutrients [9, 14, 18, 32]. If these seasonal microbial population dynamics can be explained in terms of community shifts, then this adds a more detailed understanding of how the microbial community composition affects important ecosystem processes.

The Colorado alpine dry meadow environment undergoes stark seasonal changes. Soils are frozen most of the winter after receiving plant litter in the autumn [19]. In alpine dry meadows, snow cover is sporadic and soils are frequently exposed to very low air temperatures. Eventually, a maximum snow cover of 0.5–1 m in depth accumulates. During the spring, the snow pack melts, briefly saturating soils and exposing them to a more dynamic temperature regime. During the early summer, soils warm and start to dry as plants become active. In late summer the soils become quite dry, until the monsoonal rains in August or September. Plants senesce during the fall, and soils again become frozen and exposed until late winter or early spring.

Microbial biomass increases throughout the fall and winter, apparently growing on plant litter deposited in the fall, and peaks just before snowmelt in late winter or early spring. During the first month after snow begins to melt, microbial biomass declines steadily. During the rest of the plant growing season, microbial biomass is very dynamic and appears to coincide with plant activity, being relatively high when soils are warm and moist [18]. These microbial dynamics appear to be linked to shifting availabilities of substrate and changes in temperature regime. The winter community becomes C limited as soils warm, and there may also be a direct negative effect of higher temperature [19]. We hypothesize that the winter microbial community uses more complex substrates, such as phenolics, starch, and cellulose from dead plant material, and functions well at low temperatures, whereas the summer community is more reliant on live plant root exudates, such as amino acids, and functions better at warmer temperatures.

In this study we use functional assays to measure how the soil microbial community differs seasonally in substrate use and response to temperature. We use microscopy to compare fungal and bacterial abundance, and we measure the potential activity of these groups in a respiration experiment using anti-bacterial and anti-eukaryotic inhibitors. We use reciprocal hybridization of community DNA (RCH) to verify that microbial community composition changes between winter and summer.

Methods and Materials

Site Description and Soil Sampling

The study area is located at the Niwot Ridge Long Term Ecological Research (LTER) area in the Front Range of the Colorado Rocky Mountains, United States of America (40° 03′ N, 105° 35′ W). The alpine dry meadow sites used in this study are dominated by the tussock-forming sedge, *Kobresia myosuroides* (Vill) Paol. and Fiori. The soil is classified as a skeletal-loamy pergelic Cryumbrept [10]. The site used primarily in this study (site B) [22] has an elevation of 3550 m and an eastern exposure. The soil is particularly shallow, with a 10 cm A horizon overlaying a rocky B horizon with very few *K. myosuroides* roots. The A horizon contains 81 g C kg^{-1} and 7.4 g N kg^{-1} [22]. For the RCH experiment, soil was also collected from a second dry meadow site about 2 km away at a higher elevation (3670 m), referred to as site W [22]. This soil is drier and has a southern exposure, steeper pitch, a lower organic matter (OM) content, and coarser texture. All experiments herein were performed on A horizon soil. The dry meadow is generally snow-free from May/June to October/November. On each collection date, a minimum of three soil cores (10 cm deep, 5 cm diameter) were collected from each site. To maintain consistency with respect to the effect of the proximity of plants, each sample was collected directly alongside a tussock of *K. myosuroides*. Within each site, soil samples were collected 1–10 m apart from each other. Soils were sealed in plastic bags and brought to the laboratory for analysis. Soil was coarsely sieved (4.75 mm) to remove rocks and large roots. Subsamples of soil were dried to constant weight at 100°C for determination of moisture content. Soil data are expressed per unit dry mass. For convenience, soil collected during the spring or winter before the snow pack recedes is referred to as winter soil. Soil collected within 1 week following the disappearance of

the snow pack is referred to as spring soil, and soil collected close to summer solstice is referred to as summer soil.

Response of Soil Respiration to Temperature

Two respiration experiments were performed on soil collected on 11 April 1998 (about 1 month before snowmelt) and on 18 July 1998. The experiments were performed on soil composited from the spatial replicates. In the spring experiment, 10 g soil was placed in tightly stoppered biometer side arm flasks. The flasks were incubated at either 0°C (on ice in a 3°C incubator) or 22°C. The side arm contained 5 mL (1 M) NaOH. Control flasks contained, instead of soil, a volume of water equivalent to the water content of the soil samples. All treatments contained two duplicate flasks. Soil samples were amended with glutamate (1 mg C g^{-1} soil) or an equivalent volume of water as a control (1 mL per flask). The base trap was replaced regularly and CO_2 was measured using acid titration in $BaCl_2$ [33]. Measurements were carried out for 4+ days.

Measurements of Microbial Functional Groups

Most probable number (MPN) determinations were performed as described earlier [18]. The growth medium contained 0.14 g K_2HPO_4, 0.02 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 0.10 g NH_4NO_3, and 0.05 g $CaCl_2$, per L H_2O, to which was added 1 mL soil extract (10:1 v/w, sterile filtered). Substrates were added to this media to obtain a final concentration of 1 mM. The most generally utilized substrates (glucose, potassium glutamate, and soil extract (10:1 v/w, autoclaved, sterile filtered)) all produced statistically indistinguishable results and so were combined to represent total MPN. Vanillic acid and glycine produced lower values and are expressed as a fraction of total MPN. Soil was blended (2 min on, 1 min off, 2 min on), diluted and pipetted into 96-well plates with growth media, and incubated at 22°C for 1 month. The MPN measurements were made on soils collected from autumn of 1995 to winter of 1997.

Amylase and cellulase activities were measured using modifications of the methods of Tabatabai [29] and Schinner and von Mersi [23]. Starch and carboxymethylcellulose (CMC, a soluble cellulose analog) were added (0.5% final concentration) with citrate buffer (50 mM, pH 6.0, 10 mL final volume) to 3–5 g soil, with 0.2 mL toluene as an inhibitor of microbial growth. Tubes were shaken at 22°C for 24 h, centrifuged, and placed on ice, and glucose was measured using the ferricyanide-reducing sugar assay [23]. To express enzyme activities on a biomass basis, chloroform fumigation-extraction (CFE) measurements were performed. Chloroform (1 mL/g soil) was added directly [17] to 3–5 g soil samples in tightly capped 15 mL polyethylene tubes and incubated for 72 h. Unfumigated and fumigated soils were shaken in 10 mL of 2 M KCl for 1 h (120 rpm) and centrifuged, and then total organic C in (0.5 M) K_2SO_4 extracts of fumigated and unfumigated soil was measured using a Mn(III)-reduction assay [4]. The specific enzyme activity measurements were made in winter and summer of 2001.

To compare the abilities of bacteria and fungi to respond to inputs of labile substrate in summer vs. winter, we performed substrate-induced growth response (SIGR) measurements [13, 25] on soils that had been incubated for 12 h with factorial combinations of antibacterial and antieukaryotic/antifungal compounds (no inhibitors, streptomycin (3 mg/g soil) + erythromycin (0.15 mg/g soil), cycloheximide (1 mg/g soil) + nystatin (0.2 mg/g soil), both sets of inhibitors) [24]. Potassium glutamate was added in sufficient quantities to induce growth (2.5 mg C/g soil) along with a ^{14}C-tracer to soils, then placed in a side-armed flask with base trap (0.5 M NaOH), and respiration of the substrate was followed over time by liquid scintillation counting. The respiration data were fit with the SIGR model to estimate biomass C capable of responding in each treatment. Previous work showed that glutamate is a good general substrate for microbial growth [1] and that glutamate SIGR is highly correlated to other measures of microbial biomass [18]. The experiment was first performed using soil collected 27 April 1997, from under the snow pack, incubated at 3°C. It was repeated for summer soil 1 July 1997, at 22°C. The incubations were carried out at temperatures close to the ambient temperatures of the collected soils to more realistically describe the potential activity of each community.

We compared the seasonal abundance of bacteria and fungal cells using light and epifluorescence microscopy [6]. "Active" bacteria and fungal hyphae were estimated using fluorescein diacetate; total bacteria were stained with SYBR Green I (Molecular Probes, Eugene OR); and total fungi were stained with lactophenol–trypan blue. The active counts were done in 1998 and the total counts were done in 2001.

Reciprocal Community Hybridization (RCH)

We tested the similarity of community DNA extracted from different soil samples using the RCH technique [28]. In this technique one measures the hybridization of probe DNA and target DNA from different samples. DNA was extracted from soil in relatively large samples (3–5 g) to include within-sample variation, as gently as possible, so that relatively large DNA fragments survive the extraction process. Soil was subjected to 5 freeze–thaw cycles (liquid N_2 for 5 min, 65°C for 5 min), then shaken (100 rpm) at 37°C for 2 h in TE/0.2% SDS with proteinase K and lysozyme. DNA was crudely purified by two washes of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with isopropanol. Humic substances were removed on Sepharose 4-D columns (Sigma Chemical, St. Louis, MO) [30] and DNA was further purified using QiAmp tissue columns (Qiagen). DNA was quantified using PicoGreen (Molecular Probes) on a Storm 860 Phosphoimager (Molecular Dynamics). Digoxigenin (DIG)-labeled probes were made from soil DNA using random priming (DIG HighPrime, Roche Biochemicals). Soil DNA (200 ng per dot) from each spatial replicate and date was blotted onto a positively charged nylon membrane (Hybond+, APB), using a dot-blotting manifold (Roche). The blots were hybridized with 10 mL of 10 ng/mL probe in APH buffer [2] at 68°C overnight. After

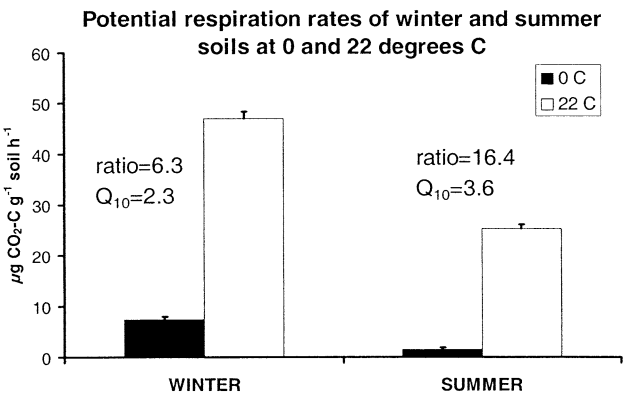

Fig. 1. Differential responses of soil respiration to temperature between winter and summer soils. Soils were brought to optimal water content and given unlimiting potassium glutamate as a C and N source. Error bars are standard errors for two experimental duplicates for each treatment. The experiment was performed on soil composited from 3 spatial replicates.

a preliminary wash (2× SSC/0.1% SDS 22°C, 2 × 5 min), consecutive washes of increasing stringency were done: mild (0.2× SSC/0.1% SDS, 22°C, 2 × 5 min), medium (0.1 × SSC/0.1% SDS, 42°C, 2 × 15 min), and stringent (0.1× SSC/0.1% SDS, 68°C, 2 × 15 min). Blots were shaken in blocking solution (5% casein in TBS) for 1 h, then alkaline phosphatase (AP)-conjugated anti-DIG Fab fragments (Roche) were added at 5 μL/25 mL and shaken for 1 h. The blot was rinsed in TBS (4 × 12 min) and the blot was visualized using AttoPhos (JBL, Sunnyvale CA), a fluorogenic AP substrate [11]. The blot was allowed to develop overnight, and fluorescence was measured using a Storm 860 (Molecular Dynamics), and quantitated using ImageQuant software (Molecular Dynamics). Data were normalized by expressing the signal strength of each dot as the percent of the maximum signal (obtained from hybridizing the probe against DNA from the same date as the probe). Fresh blots were used with both seasonal probes. The RCH experiment was done on soils collected in 1999.

Statistical Analysis

The spatial replicates were analyzed separately in the measurements of MPN, specific enzyme activity, total bacterial and fungal counts, and RCH. In these cases, comparisons of data between dates was done with analysis of variance (ANOVA), with date coded categorically. The RCH experiment was also analyzed using analysis of covariance (ANCOVA), with date coded continuously as Julian day. For the temperature response experiment, the inhibitor SIGR and the fluorescein diactetate-active counts of fungi and bacteria, the spatial replicates were combined to make these experiments more tractable and to allow a greater number of experiments to be run on a limited amount of soil, in an attempt to minimize disturbance of the field site. In these cases, a measure of spatial variability is not produced, but the means produced by these experiments are essentially spatially balanced seasonal means of three or more spatial replicates. In the case of

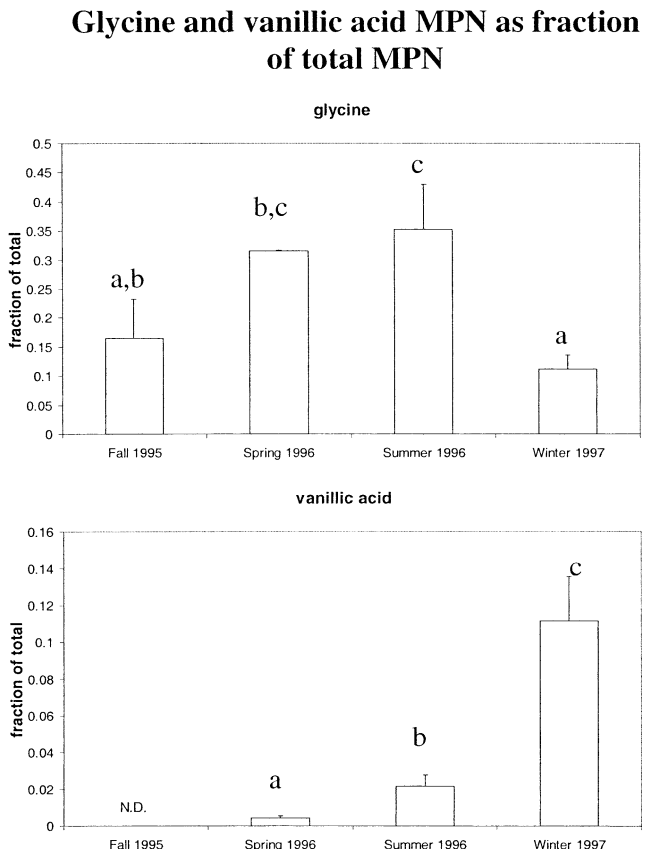

Fig. 2. Seasonal changes in substrate use, measured using MPN method. Presented as fraction of total cells measureable with MPN using the most generally used substrates (glucose, potassium glutamate). Within each panel, bars with the same letter are not significantly different by Fisher's test (ANOVA run on log-transformed data). N.D. indicates that no data are available.

the inhibitor SIGR and the active counts, these results complement the results of a fully replicated experiment (total counts) done in different years and using different methods. In the inhibitor SIGR and temperature response experiment, two experimental duplicates were taken from the pooled soil for each treatment so that the effect of season can be compared to intraexperimental variability. The active bacterial and fungal counts made on pooled spatial replicates were analyzed by regressing counts on Julian day. This is similar to performing a repeated measures analysis. MPN data were log-transformed before analysis. Analyses were performed using Statview software (SAS Institute, Cary, NC). The errors presented in figures and tables are standard errors of the mean.

Results

Respiration in winter and summer soils responded differently to temperature (Fig. 1). Respiration proceeded rapidly at 0°C during the winter, but respiration in the

Table 1. Seasonal changes in soil amylase and cellulase activities, expressed as absolute rates (μmol glucose released g^{-1} soil h^{-1}) and as specific rates (absolute value/MBC, units = nMol h^{-1} μg^{-1} MBC)[a]

	Absolute rates		Specific rates		
	Amylase	Cellulase	Amylase	Cellulase	MBC
Winter (4/30/10)	4.27 ± 0.59	2.80 ± 0.44	6.46 ± 0.69	4.27 ± 0.48	668 ± 89
Summer (6/18/01)	2.87 ± 0.36	0.55 ± 0.09	8.54 ± 1.83	1.69 ± 0.42	354 ± 44
P-value	0.090	0.003	0.372	0.009	0.021

[a] MBC shown for reference (μg C/g soil)

summer soil was almost completely halted at 0°C. The Q_{10} for respiration over this temperature range were markedly different: 2.3 in winter and 3.6 in summer. The microbial communities of different seasons also show distinct patterns of substrate usage (Fig. 2). A larger proportion of organisms from the winter soils could grow on the phenolic compound vanillic acid, compared to the spring and summer communities, while the amino acid glycine was used by a larger portion of the spring and summer communities than by the winter community. The microbial biomass showed differential expression of amylase and cellulase activities during winter and summer (Table 1). When expressed as specific rates (enzyme activity per unit microbial biomass C), specific amylase rates were not significantly different between winter and summer, but specific cellulase rates were lower in the summer. Microbial biomass, as measured by chloroform fumigation–extraction, was significantly lower in summer than in winter, as has been observed previously [18].

The results of the inhibitor SIGR experiment (Table 2) showed that the glutamate substrate-induced growth response was dominated by fungi in the winter, but that both bacteria and fungi grew in the summer soil. In both winter and summer experiments 60% of the biomass was not inhibited by the presence of both anti-bacterial and anti-fungal compounds together. This implies either that the antibiotics are not completely effective in this high OM soil due to interaction with the soil matrix, or that a large antibiotic-resistant component exists. Microscopic counts of fluorecein diacetate-active (Table 3) and total (Table 4) bacteria and fungi show that bacterial cells increase in abundance after snow melts. There was a marginally significant decrease in total fungal hyphae after snowmelt, as well (Table 4).

Both winter and summer soil community DNA probes hybridized most strongly with soil DNA collected on the same date, and the signal decreased with increasing time between sampling dates (Fig. 3). The seasonal changes were larger than the variations between spatial replicates. The two-way ANOVA showed a significant probe date/target date interaction ($p = 0.04$), meaning that probes from different seasons hybridize differently with target

Table 2. Percent of antibiotic-susceptible microbiol biomass made up by bacteria and eucaryotes during winter and summer[a]

	Bacteria	Eukaryotes
Winter (4/27/97)	0 ± 0	95 ± 13
Summer (6/17/97)	41 ± 4	66 ± 10

[a] In both summer and winter, 40% of the total biomass was susceptible to the addition of antibacterial (erythromycin and streptomycin) and antifungal/eukaryal (cyclohexamide and nystatin) agents in combination.

Table 3. Microscopic counts of flourescein acetate-active bacteria and fungal hyphae[a]

	Bacteria	Fungi	F/B ratio
Winter (5/11/98)	3.68	107.4	14.9
Spring (5/27/98)	6.57	90.1	6.99
Summer (6/21/98)	9.36	120.2	6.55

[a] Active bacteria are expressed as 10^8 cells/g soil, active fungi as m/g soil. Active fungal/bacterial biomass ratio is based on conversion from biovolume to biomass. Three spatial replicates were composited for these measurements. Regression analysis shows that active bacteria increase after snowmelt ($P = 0.09$).

Table 4. Microscopic counts of total bacteria and fungal hyphae in winter and summer of 2001[a]

	Bacteria	Fungi	F/B ratio
Winter (4/30/01)	6.2 ± 0.34	192.8 ± 16.2	1.62 ± 0.12
Summer (6/18/01)	21.6 ± 1.72	126.8 ± 34.3	0.32 ± 0.11
P-value	0.0001	0.114	0.0002

[a] Bacteria are expressed as 10^9 cells/g soil, fungi as m/g soil. Total fungal/bacterial biomass ratio is based on conversion from biovolume to biomass. *P*-value is for the ANOVA testing differences between seasons ($n = 7$).

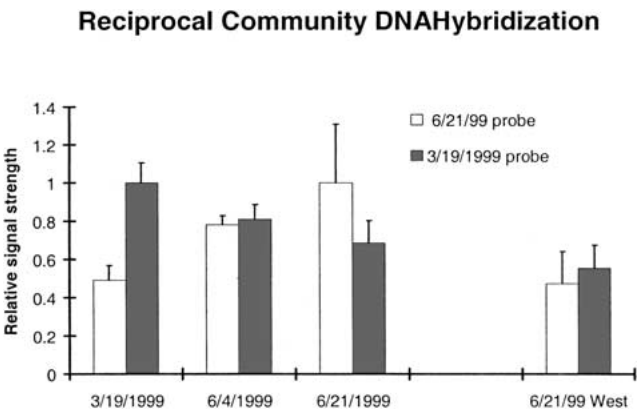

Fig. 3. Reciprocal community hybridization (RCH) experiment. The signal strength of probes made from soil DNA from either winter or summer hybridized against soil DNA collected on three dates from the main study site, and once in summer from a different alpine dry meadow soil. The signal is normalized to the maximum signal (from soil of the same date as the probe). The error bars represent variability within 3 spatial replicates for each sample.

DNA from different dates. The two-way ANCOVA produced a significant Julian day/probe date interaction ($p = 0.01$), demonstrating that hybridization of the summer probe to target DNA increases from winter to summer, and that the winter probe does the opposite. Hybridization between winter and summer soil DNA from the same site (49–68% of maximum signal) was roughly as weak as hybridization between soils collected from two distinct dry meadow sites several kilometers apart on the same (47%) or different (55%) dates. The one-way ANOVA showed significantly lower hybridization of probes from site B to soil DNA from site W compared to target DNA from the same site and date ($p = 0.025$).

Discussion

The microbial community clearly undergoes a shift in structure and function between winter and summer. The winter community has a higher fungi/bacteria ratio relative to the summer community, is more adapted to cold temperatures, and utilizes the complex substrates, cellulose and vanillic acid, to a larger extent, but utilizes the simple amino acid, glycine, to a lesser extent than does the summer community. These differences in substrate utilization could reflect the different substrates available seasonally; in winter dead plant material is available, whereas in summer live roots and their exudates are present. The sudden change in temperature and substrate availability at snowmelt that was previously shown to reduce winter microbial biomass [19] is the most likely cause of the observed community shift.

DNA hybridization studies show that there is a clear change in the genetic composition of the microbial community between winter and summer. This seasonal change is greater than the variation on a spatial scale of several meters on a single date. However, the microbial communities from two alpine dry meadows separated by several kilometers were as distinct as the seasonal variation within one dry meadow soil. Thus, other factors such as soil texture, elevation, and aspect, the factors by which the two sites differed, can be more important in determining the microbial community composition than season and dominant plant species, the factors that the two sites shared.

Although this study shows that fungi are dominant in the winter, there is a significant active bacterial population at this time, and this bacterial community also is likely to change its species composition seasonally (Lipson, unpublished data). Fluorescein acetate-active bacteria were microscopically visible during the winter, yet antibiotics had no effect on the utilization of glutamate. Either the bacterial population in the winter is uniquely resistant to antibiotics, or the high OM content of the soil protects bacteria from these compounds, preventing the antibiotics from being fully effective. In the summer, there was a bacterial component that was sensitive to antibiotics. This could indicate either that the bacterial community changed, or that the higher bacterial numbers in the summer caused the bacterial community to be more susceptible to the antibiotics (e.g., fewer protected, occluded sites within the soil matrix). The total bacterial count increased significantly in the summer, while the chloroform-labile biomass C decreased. This indicates that the marginally significant decrease in total fungal hyphal length was probably a real effect.

The physical environment of the Colorado alpine produces an inevitable annual cycle of plant senescence, snow accumulation, snowmelt, and plant growth, and the microbial biomass has been observed to follow a predictable seasonal pattern over many years [9, 16, 18]. Because similar conditions are repeated every year and consistent responses are seen at the microbial biomass level every year, it is likely that there are consistently recurring patterns in the microbial community each year. In three separate years, fungi appeared to dominate the winter community, while bacteria show increased populations in

the summer. However, whether identical seasonal communities are re-established every year is beyond the scope of this study. This study includes experiments conducted over a 6–year period and represents a progressive understanding of the microbial community changes that occur each year after snow melts in the spring. The experiments herein showed that changes in function and structure occur every year, but it cannot be concluded whether all the observed changes are consistent annual patterns.

This study has taken the first step toward linking the previously observed microbial biomass and nutrient availability dynamics with specific characteristics of the microbial community. The microbial community shift at snowmelt coincides with a period of rapid microbial biomass turnover and increased N availability for plants [18, 21]. We can now begin to understand this important event in terms of the microbial community. The data presented here, however, are still quite general. Future studies should include more detailed information on seasonal changes in abundance of specific microbial taxa, so that the physiology of these groups can be linked to the ecosystem level, in terms of the causes and consequences of their seasonal abundance.

Acknowledgments

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