Landscape Position Influences Microbial Composition and Function via Redistribution of Soil Water across a Watershed

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ABSTRACT

Subalpine forest ecosystems influence global carbon cycling. However, little is known about the compositions of their soil microbial communities and how these may vary with soil environmental conditions. The goal of this study was to characterize the soil microbial communities in a subalpine forest watershed in central Montana (Stringer Creek watershed within the Tenderfoot Creek Experimental Forest) and to investigate their relationships with environmental conditions and soil carbonaceous gases. As assessed by tagged Illumina sequencing of the 16S rRNA gene, community composition and structure differed significantly among three landscape positions: high upland zones (HUZ), low upland zones (LUZ), and riparian zones (RZ). Soil depth effects on phylogenetic diversity and β-diversity varied across landscape positions, being more evident in RZ than in HUZ. Mantel tests revealed significant correlations between microbial community assembly patterns and the soil environmental factors tested (water content, temperature, oxygen, and pH) and soil carbonaceous gases (carbon dioxide concentration and efflux and methane concentration). With one exception, methanogens were detected only in RZ soils. In contrast, methanotrophs were detected in all three landscape positions. Type I methanotrophs dominated RZ soils, while type II methanotrophs dominated LUZ and HUZ soils. The relative abundances of methanotroph populations correlated positively with soil water content (R = 0.72, p <0.001) and negatively with soil oxygen (R = -0.53, p = 0.008). Our results suggest coherence of soil microbial communities within and difference in communities between landscape positions in a subalpine forested watershed that reflect historical and contemporary environmental conditions.
INTRODUCTION

In the western U.S., approximately 70% of carbon sink activity is located at elevations above 750 m, where 50-85% of land is dominated by hilly or mountainous topography (1). Fluxes of carbonaceous gases such as carbon dioxide (CO₂) and methane (CH₄) significantly affect the size of the carbon sink, with soil respiration accounting for the largest terrestrial CO₂ flux to the atmosphere (2). CO₂ in soil pore spaces is primarily derived from autotrophic (root) and heterotrophic (microbe) respiration, which is mediated by environmental factors such as temperature, soil water content (SWC), O₂ availability and organic matter (3-5). The direction and intensity of CH₄ flux depends on the local balance of the CH₄ consumption by methanotrophs and CH₄ production by methanogens, both of which are also subject to such environmental influences. Because diffusive gas transport through soils is reduced with increasing SWC, hydrologic variations can strongly affect soil O₂ levels, which in turn influence the relative rates of (anaerobic) methanogenesis and (aerobic) methanotrophy. Although saturated soils (e.g. wetlands) are major terrestrial sources of CH₄ emissions (6), emission may at times occur from unsaturated soils, depending on the fine-scale heterogeneity of soil redox status (7); in some cases CH₄ source/sink switching behavior is observed with seasonal flooding or drydown (8-12).

Little is known about how soil microbial community structure is influenced by both historical and contemporary environmental conditions of subalpine forested soils (13), and how microbial community structure might correlate with soil fluxes of CO₂ and CH₄. Landscape factors that may influence occurrence and abundance of microorganisms include geographic location (14), topographic features such as drainages (15), and soil characteristics across spatial scales (16). Contemporary soil environmental conditions include organic C availability (17),
nutrient content (18), SWC and temperature (19), and vegetative cover (20). Forested subalpine
watersheds are often heterogeneous with respect to both historical and contemporary
environmental conditions. To date, a watershed wide assessment of the variability of soil
microbial communities within the context of environmental conditions imposed by landscape
heterogeneity is lacking.

Over the past decade, research efforts at the Tenderfoot Creek Experimental Forest (TCEF, Fig. 1) within the Lewis and Clark National Forest, Montana have focused on the spatial
and temporal scaling of hydrological, biogeochemical and ecological processes across the larger
Tenderfoot Creek watershed with particular focus on the Stringer Creek drainage. These studies
have included watershed hydrology (e.g. stream water sources, flow paths, and riparian
dynamics) (21, 22), relationships between hydrologic conditions and CO2 efflux across
landscape positions (23, 24), and landscape scale land-atmosphere CO2, H2O, and energy fluxes
(25, 26). This site is characteristic of vast extents of forests in the Northern Rocky Mountains,
and continues to be the focus of studies aimed at generating models that accurately describe and
explain the biotic and abiotic processes that contribute to subalpine ecosystem function.

In this study, we investigated soil microbial community structure and function across the
Upper Stringer Creek watershed in relation to the variability of major topographical features,
environmental factors, and soil gas composition. Specifically, the objectives of this study were
to: 1) characterize and compare the microbial communities in drier upland soils and wetter
riparian meadows; and 2) investigate the potential relationships among Bacteria and Archaea,
environmental factors, and soil gas measurements. Data to address these objectives included soil
CO2 efflux, CO2 concentration, CH4 concentration, as well as the SWC, temperature, pH and O2
content of soils.
MATERIALS AND METHODS

Site description and sample collection

TCEF is located in the Little Belt Mountains of central Montana (46° 55’ N; 110° 54’ W). It is a subalpine forest of the northern Rocky Mountains, which are believed to contribute significantly to the North American carbon sink (1). Mean annual precipitation at the site is 880 mm with 70% falling as snow. The site is subject to a steady seasonal dry down in SWC following snowmelt (27). Mean annual temperature is 0 ºC and the growing season typically lasts from early June to the end of August. The watershed land cover is largely composed of upland forests, interspersed with riparian meadows. Vegetation in riparian meadows consists primarily of Calamagrostis canadensis (bluejoint reedgrass), whereas upland forests consist primarily of Pinus contorta (lodgepole pine) and to a lesser extent Abies lasiocarpa (Subalpine fir) and Picea engelmannii (Engelmann Spruce). Vaccinium scoparium (Whortleberry) is the predominant upland understory species (28). The geology is characterized by granite gneiss, shales, quartz porphyry, and quartzite (29). The hillslopes are mainly composed of loamy skeletal, mixed Typic Cryochrepts, whereas the riparian zones are composed of highly organic clayey, mixed Aquic Cryoboralfs (30).

Three years (2005-2007) of measurements of soil CO₂ efflux, soil temperature, and SWC were previously collected at 62 sites within the Stringer Creek watershed (24, 31, 32). These prior studies established selection criteria for the nine sites that were included in this study and are referred to as: NWD1, NWD6, SW5, T1E2, T1E3, T1W1, T2W1, T2W3, and T2W4 (Fig. 1A). These sites were selected on the basis of terrain analysis and site assessment and are characteristic of the different soils, slope, aspect, topographic positions, and hydrologic regimes.
Based on hill slope positions, sites NWD1, NWD6, SW5, and T2W4 were defined as “high upland zone” (HUZ), sites T1E2, T1E3, and T2W3 “low upland zone” (LUZ), and sites T1W1 and T2W1 “riparian zone” (RZ, Fig. 1). Soil samples at each site were collected on July 10th, 2012 at three soil depths (5, 20 and 50 cm) from hand-dug pits (~ 50 cm diameter). At each depth, two soil subsamples were scraped from the wall of the pit into sterile 50 mL centrifuge tubes. All soil samples were transferred to the laboratory on dry ice and stored at -80°C until analysis.

Soil Environmental Measurements

Soil environmental measurements were conducted at all nine sites between July 8th and 11th, 2012 (Table S1). Volumetric SWC was measured using a portable time domain reflectometry meter (Hydrosense, Campbell Scientific, Logan, UT) that reports volumetric soil water content of the upper 12 cm of soil at each location. Soil temperature in the top 12 cm was measured using a 12-cm soil thermometer (Reotemp Instruments, San Diego CA). Soil temperature and volumetric SWC data are presented here as means of triplicate measurements made within two meters of gas wells and soil sampling locations.

Soil gases were collected from nested gas wells previously augered and installed at three depths (5, 20, and 50 cm) and left in place since 2005 (24). A hand-held infrared CO2 analyzer with an integral air pump (GM-70, Vaisala, Woburn, MA; 0-5% CO2 working range) was connected to two sampling ports on each gas well. The air from the well was circulated through the instrument and returned, creating a closed loop and minimizing pressure changes during sampling. Factory calibration of the GM-70 was validated in the laboratory using air-CO2 mixtures. Soil O2 concentrations were measured using a galvanic oxygen sensor (MO 200,
Apogee Instruments, Logan UT) plumbed in line within the closed sampling loop; the MO200 was field calibrated to ambient air assumed to contain 20.95% O₂. Using a syringe, ~50 ml of soil gas were extracted from the circulation loop through a septum tee fitting and injected into a 180-ml laminated foil gas sampling bag (FlexFoil®, SKC Inc., Eighty Four, PA). These bags were returned to Montana State University for analysis of CH₄ by gas chromatography with flame ionization detection. Certified CH₄ mixtures (Scotty™, Air Liquide America, Houston, TX) were used to calibrate the gas chromatograph.

Surface soil CO₂ efflux was measured using a portable infrared gas analyzer (EGM-4, accuracy within 1% of calibrated range [0 to 2,000 ppm], PP Systems, MA) connected to a soil respiration chamber (SRC-1, footprint = 78 cm², PP Systems). All CO₂ efflux measurements are reported as means of triplicate measurements made on undisturbed ground within two meters of the gas well nests. Additional details on the soil CO₂ efflux measurements were reported by Pacific et al. (2011).

DNA extraction and sequencing

DNA was extracted from 1 g soil subsamples using the FastDNA SPIN Kit for Soil (MP BIO Biomedicals, Santa Ana, CA) following the manufacturer’s instruction. DNA extracts were purified using a desalting procedure and using the OneStep PCR Inhibitor Removal Kit (Zymo Research Corporation, Irvine, CA). Purified DNA extracts were quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA) and PCR tested prior to submission for sequencing. DNA extracts were then overnight express shipped to the Institute for Genomics & Systems Biology Next Generation Sequencing Core at Argonne National Laboratory for PCR amplification using primers 515F and 806R targeting the V4 region of the 16S rRNA gene in the
domains Bacteria and Archaea (33). Amplicons were sequenced using the Illumina MiSeq sequencing platform.

The 16S rRNA gene was used as a molecular marker for estimating the relative abundance of methanotrophs (34) because the 16S rRNA gene and functional gene pmoA cover nearly identical similarity for methanotrophic populations in environmental samples (35), though we note that it does not track the forest soil methanotroph thus far only known by its pmoA sequence (36). The following genera were considered methanotrophic bacteria in this work based on previous studies (37, 38): Methylobacter, Methylomonas, Methylomicrobium, Methylocaldum, Methylococcus, Methylosoma, Methylosarcina, Methylothermus, Crenothrix, Clonothrix, Methylosphaera, Methylocapsa, Methylocella, Methylosinus, and Methylocystis.

Sequencing and analysis

A total of 3.14 gigabytes of sequence was generated and later processed using Quantitative Insights Into Microbial Ecology (QIIME) version 1.7.0 (39). Chimera sequences were identified and removed using USEARCH 6.1 (40), which detected chimeras using reference operational taxonomic units (OTUs) in GreenGenes defined at 97% identity and performed de novo chimera detection based on abundances of input sequences (41). Low quality sequences were removed using the default filter parameters in QIIME: quality score <25, minimum/maximum length = 200/1000, maximum number of homopolymer runs (n= 6), no ambiguous bases allowed and no mismatches allowed in the primer sequence. The sequences are deposited at NCBI Sequence Read Archive under the accession number SRP052862.

Phylotypes were determined with UCLUST at a default sequence similarity level of 97% (Edgar, 2010). The representative sequences for each phylotype were aligned against the
GreenGenes core set using PyNAST (42). The sequences were then classified using the BLAST taxonomy assignment (43). Alignments were filtered to remove uninformative data and sequence gaps using Greengenes alignment Lane mask file (44), and subsequently phylogenetic trees were built with FastTree (45). Based on the OTU summary, sequence libraries containing less than 10,000 sequences were considered low quality and excluded from further analyses. The smallest library included in this study contained 17,699 sequences. OTU tables were rarefied to a sampling depth of 15,000 sequences per library. Alpha diversity (diversity of microbial communities found within individual samples) was estimated with rarefied OTU tables using Faith’s phylogenetic diversity (PD) metric (46), Shannon Index, Chao1 index, observed species, and richness. Beta diversity (diversity of microbial communities found between different samples) was estimated with rarefied OTU tables by weighted-Unifrac distances (47).

Statistical analysis

Sequence libraries from duplicate DNA extracts (i.e., technical replicates) were merged prior to the Mantel tests and ADONIS analyses. Mantel tests were conducted in QIIME to test the significance of correlations between weighted UniFrac distances between soil bacterial communities and the normalized Euclidean distances in environmental factors and soil carbonaceous gas measurements. Pearson correlations were performed using the software R (R Foundation for Statistical Computing, Vienna, Austria) to identify correlations between relative abundances of major bacterial phyla or methanotrophs as a function of environmental factors and carbonaceous gas measurements.

RESULTS
Soil Environmental Measurements

In situ soil environmental measurements were conducted within a maximum of 1-2 days before or after soil samples were collected for microbial analyses. Volumetric SWC ranged from 6.0% to 12.4% in the HUZ soils, from 6.3% to 49.4% in LUZ sites, and from 48.9% to saturation in RZ soil profiles (Table S1). Soil temperature ranged from 11.2 to 15.9°C across all sites. At HUZ and LUZ sites, most of the soil O₂ levels were within a narrow range (20.2-21.4%) near that of the atmosphere (20.95%) and varied little across depths (Table S1). In contrast, soil O₂ declined with depth at the RZ sites (Table S1). Soil pH ranged from 4.22 to 5.64 in HUZ sites, from 5.63 to 6.86 in LUZ sites, and from 5.41-6.30 in RZ sites. Soil CO₂ and CH₄ concentrations, and surface CO₂ efflux were consistently higher in RZ sites than in LUZ and HUZ sites (Table S1), in agreement with previous reports on CO₂ from this site (23, 24, 26, 31).

General analyses of the sequencing libraries

Four DNA extracts (duplicate DNA extractions for each of the two soil subsamples for each depth) were used to establish four sequence libraries for each of the 27 soil samples (9 sites × 3 soil depths). Nine of the 108 DNA extracts failed to yield quality libraries; these included one extract each from T1E2-5cm, T1E2-20cm, T1W1-5cm, T1W1-5cm, T2W1-20cm, T2W1-50cm, and T2W3-5cm, and two extracts from NWD1-5cm. The remaining 99 libraries consisted of a total of 5,572,763 sequences, ranging from 17,699 to 155,603 sequence reads per library, and were rarefied to 15,000 reads each. Rarefaction curves (Fig. S1) suggested the sequencing effort recovered the dominant taxa at the genetic distance of 3%. At RZ sites, the OTU counts at 5 cm were higher than at 20 cm ($p=0.009$) and at 50 cm ($p=0.003$). In comparison, the trends at HUZ and LUZ sites were less pronounced. With two replicate libraries for each soil subsample,
analysis of similarities (ANOSIM) tests showed that the two subsamples were similar ($p > 0.05$ for all soil samples tested). Consequently, diversity and richness assessments were conducted based on the average of replicate libraries.

**Taxonomic diversity**

Microbial community composition varied between the three landscape positions in the watershed; i.e., HUZ, LUZ and RZ. *Bacteria* were more abundant than *Archaea* in all libraries (Table S2). A total of 25 bacterial phyla were identified across the entire sample set; some phyla were undetectable in some soils/depths (Table S3). In all sequence libraries, *Proteobacteria* (18.15% – 45.59%), *Acidobacteria* (3.92% – 28.62%), *Verrucomicrobia* (0.94% – 27.93%), and *Actinobacteria* (2.51% – 21.69%) were dominant (Fig. 2, Table S3). Other phyla that were consistently detected included *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes*, *Nitrospirae*, and *Planctomycetes*. Rare phyla, defined as those with a relative abundance less than 1%, were clustered together in the “other” category (Fig. 2). Because methanotrophs are a functional group of interest in this study and belong to *Alphaproteobacteria* and *Gammaproteobacteria*, these two subphyla were examined in greater detail (Fig. S2). Both subphyla, particularly the *Alphaproteobacteria*, were most abundant at the 5 cm depth and typically declined with depth (Fig. S2).

*Archaea* made up small portions of the communities, ranging in relative abundance from undetectable to 4.85% (Table S2). In most locations, they were more abundant at the 20 and 50 cm depths than at 5 cm, and were lowest in HUZ sites and highest in RZ sites (Table S2). *Crenarchaeota* and *Euryarchaeota* were the dominant phyla (Table S4), with *Crenarchaeota* dominating in all locations except the two RZ sites (T1W1 and T2W1). The relative abundance
of these phyla was similar at T1W1, while the *Euryarchaeota* were more abundant than *Crenarchaeota* at T2W1 (Table S4).

Shannon and Chao1 indices were calculated to estimate and compare the microbial richness and diversity among different depths and locations (Table S5). In general, $\alpha$ diversity did not pattern with depth in the HUZ soils, whereas it decreased with soil depth in the LUZ and RZ soils (Table S5 and Fig. S3). Additionally, the communities in the HUZ soils exhibited lower diversity than those at LUZ and RZ (Fig. S3).

Principal coordinate analysis (PCoA) was then employed to examine the relative relatedness of the various microbial communities (Fig. 3). The two coordinates accounted for 40.39% and 21.50% of the total variation, respectively. The microbial communities could be distinguished in a manner that clearly related community structure with landscape positions. For the most part, replicate libraries clustered closely, consistent with the above-mentioned ANOSIM analysis. In general, additional ADONIS comparisons were largely consistent with the PCoA clustering, primarily delineating communities to within the HUZ, LUZ and RZ landscape positions ($R^2 = 0.3379-0.4752$, $p < 0.001$; Table S6) as illustrated in Fig. 3. One location of particular interest was T1E3, which represents a transition zone between the HUZ and LUZ landscape positions (Fig. 1B). More specifically, the T1E3 5cm libraries clustered distinctly away from the other, deeper T1E3 communities (20cm and 50cm, Fig. 3). ADONIS analysis of the T1E3 5cm community agreed with its PCoA separation from the T1E3 20cm and 50cm communities, albeit the distinction was only marginally significant ($p=0.066$, Table S6), and implied relatively weak similarity to either the HUZ or LUZ groups (Table S6).

**Correlation with environmental factors and soil carbonaceous gases**
In order to better understand the community composition and diversity patterns (Fig. 2, Fig. 3), Mantel tests were conducted to examine the relationships between community composition and soil environmental measurements (Table 1). Statistically significant (all *p*-values < 0.001) correlations of varying strength were observed for SWC, soil O2, and soil pH (Table 1). Soil CO2 efflux, CO2 concentration, and CH4 concentration also correlated with community structure (Table 1). Phylogenetic diversity, a measure of alpha diversity, was positively correlated with both SWC and soil CO2 efflux (Fig. 4A and 4B). SWC and soil CO2 efflux also exhibited a strong positive correlation (Fig. 4C). Pearson correlation analysis was conducted to individually examine the relative abundance of major bacterial phyla relative to environmental factors and soil carbonaceous gas measurements (Table 2). Correlations varied in direction, strength, and pattern. For example, the relative abundance of *Chloroflexi* was positively correlated with SWC, pH, and all soil carbonaceous gas measurements, but was negatively correlated with soil O2 (Table 2). In contrast, the relative abundance of *Acidobacteria* was positively correlated with soil O2 and negatively correlated with SWC, pH, CO2 efflux, and CO2 concentrations (Table 2).

**Methane cycling microbes**

With the exception of the 20 cm sample at NWD6 (6 of 15,000 reads), methanogens were only detected in soils from RZ sites (Fig. 5A). At the genus level, *Methanobacterium*, *Methansaeta*, and *Methanosarcina* were detected in all riparian libraries, whereas *Methanocella* and *Methanospirillum* were less abundant and only detected in the deeper RZ soil horizons (Fig. 5B). The 16S rRNA signatures of various known methanotroph genera were detected in 21 of the 27 soil samples (Fig. 6A). RZ sites T1W1 and T2W1 had the highest relative abundance (up
to ~0.96%) of methanotrophs (Fig. 6A). Type II methanotrophs (annotated to the genera *Methylosinus* and *Methylocella* of the *Alphaproteobacteria*) dominated in the HUZ topographies, whereas Type I methanotrophs (*Methylomonas*, *Methylocaldum*, and *Crenothrix* of the *Gammaproteobacteria*) were most prevalent in the RZ soils (Fig. 6A and B). *Crenothrix* was the most abundant methanotroph (0.07% ~ 0.72%), whereas *Methylomonas* and *Methylocella* were the least abundant (<0.01%). Relationships between the relative abundances of methanotrophs and environmental factors and soil carbonaceous gases were also examined using Pearson correlations (Table 3). The relative abundance of methanotrophs (especially Type I) was positively correlated with SWC (R=0.72, p<0.001). Both types of methanotrophs were negatively correlated with soil O2 levels (particularly Type II). All soil carbonaceous gas measurements exhibited statistically significant positive correlations with methanotroph relative abundance (Table 3).

**DISCUSSION**

Given the extensive distribution of subalpine forests, a better global understanding of how these ecosystems contribute to C exchange with the atmosphere is critical (1, 48). Surprisingly, there is little information regarding the soil microbial communities involved. This experimental forest has been extensively studied in efforts to quantify soil CO2 production and surface efflux as a function of hydrology at the landscape scale (23, 24, 26, 31, 32, 49, 50). The current study aimed to continue these landscape scale efforts by assessing potential linkages between different soil environments within this watershed and the microbial drivers of greenhouse gas exchanges. The nine sampling sites were selected based on prior research that had identified landscape positions in this drainage that differed with respect to soil
environmental variables and gas fluxes (26, 31). This sampling strategy allowed us to identify how community structural patterns differed among landscape positions and how they might be correlated with key soil environmental factors (e.g. SWC, temperature, O₂, and pH) and ecosystem function (carbonaceous gas fluxes/concentrations).

Landscape position in this watershed was important in shaping microbial communities (Fig. 3), with the differences observed at the phylum level (Fig. 2). Riparian zones and upland zones often exhibit different rates of microbially mediated soil processes due to the distinct soil moisture regimes (51) and differing microbial community compositions (52). The distinct microbial community structural patterns revealed in this study suggest that deterministic processes associated with habitat specialization are important. Snowmelt events offer significant annually repeated opportunities for the downslope redistribution of microbes from HUZ to LUZ or to RZ positions, yet distinct community structure and diversity patterns were evident (Fig. 2 and Fig. 3, Table 2). There are likely several contributing factors. Soil temperature appeared to have little effect on most phyla, likely due to the narrow range at the time of sampling (11.2 - 15.9°C, Table S1). However, SWC stands out as major deterministic selector. Strong correlations of SWC with community structure were shown in both Mantel tests (Table 1, R = 0.68) and canonical analysis of principal coordinates (Fig. S4). This is consistent with prior investigations demonstrating similar SWC relationships with microbial biomass and soil respiration (53-55). The relative abundance of a particular microorganism is often influenced in real time by the prevailing moisture in the soil pore environment (a contemporary environmental factor). Topography can significantly influence water movement and thus can influence relationships between landscape position and microorganisms (Fig. 2, Fig. 3, and Fig. 4). Major bacterial phyla differences between upland and riparian zones most noticeably involved
Acidobacteria, Actinobacteria, Chloroflexi, and Verrucomicrobia (Fig. 2). Considering the entire growing season in the TCEF watershed (May-August), HUZ soils are the first to dry down and SWC in LUZ soils tends to be higher than HUZ soils for longer periods due to the downslope redistribution of snowmelt. Perhaps it is not surprising that the relative abundance of Acidobacteria and Actinobacteria was lower in riparian soils than in upland soils. Riparian soils are generally less aerated due to high SWC (they can be saturated much of the growing season); hence they are not optimum for phyla such as Acidobacteria and Actinobacteria that include a substantial number of obligate aerobes.

Soil pH was likely another deterministic factor in this ecosystem. The pH range in the HUZ soils was largely outside that of the LUZ or RZ soils (Fig. 3, Table S1), and is differentially correlated with the various phyla (Table 2) so as to contribute to the distinct clustering of HUZ communities from those in the LUZ and RZ. Global studies of soils (56-58), as well as comparisons within the same soil profile (59), have similarly found strong connections between microbial community structure and pH.

Phylogenetic diversity was also correlated with landscape position, with the RZ and HUZ tending to represent the end-members (Fig. 4, Fig. S3). The RZ and HUZ represent very different environments and so differences in this regard are not surprising and are consistent with previous studies, which found that soil bacterial phylogenetic diversity differed by ecosystem type (56, 59). In addition to SWC, the type and extent of vegetation also varied substantially between the riparian zone and the upland zones. As noted by Prober et al. (60), plant diversity can be a good predictor of the beta diversity of soil microbes in grassland. The full extent of this effect remains as a topic for future research efforts.
The relation between ecosystem type and β-diversity reported in other studies (61, 62) was also clearly observed here (Fig. 3), although our current study is focused on a single geographical location and aimed to compare communities across landscape positions. Dispersal barriers between landscape positions can be important contributors to the β-diversity (13). Distance can in some cases act as a dispersal barrier, however, physical separation did not appear to be a factor shaping β-diversity in this drainage. Despite the significant spatial separation (up to ~1000 meters), the HUZ microbial communities were more closely related to each other than the microbial communities in the LUZ or RZ soils that were only separated by 5-20 m (Fig. 1 and Fig. 3). This observation is consistent with Wang and coworkers’ finding that β-diversity among habitat types was significantly higher than within habitat types (61).

Soil depth effects on microbial community structure have been observed previously in Colorado montane soils (59) and grasslands in Germany (63). In the present study, phylogenetic diversity decreased with soil depth in the riparian soils, while the trend was not as evident or consistent in the upland soils (Fig. S3). Effects of soil depth on β-diversity and composition in the LUZ and RZ soils were also more evident than that in the HUZ soils (Fig. 3). During the July sampling dates for this study, the soil pits for both RZ sites revealed root-bound conditions at the 5 cm depth and, depending on the site, saturated conditions at the 20 cm and/or 50 cm depths. Roots were less prevalent but still conspicuous at 20 cm, but were far less abundant at 50 cm. This rooting pattern together with the SWC profile could provide a general explanation for soil depth effects observed in the RZ soils. Though not saturated or as heavily rooted, soil horizons were apparent in the toe-slope LUZ locations. Changes in chemistry and physical properties associated with the horizonation (31) could have influenced the depth patterning observed in the LUZ soils (Fig. 3 and Fig. S3). In a forested montane watershed, the microbial
communities at various soil depths significantly differed from each other, irrespective of the sampling locations within their watershed study site (59, 64). While Bacteroidetes and Verrucomicrobia were found to be the primary drivers of the distinction in microbial composition along soil profiles, no such drivers were evident in the Stringer Creek watershed. Surface soils exhibited greater β-diversity than deep soil in the montane watershed study in Colorado (59), and the organic matter composition at different soil depths was considered responsible for the vertical distinction. In contrast, the relationship between β-diversity and soil depth was not consistent across three landscape positions within the forested watershed in this study (Fig. 3).

Of the different sampling sites, the T1E3 location proved to be particularly interesting. This sampling site represents a transition point with respect to topography (changing from upland to riparian). Previous research has indicated that the hydrology and CO₂ efflux patterns of this and other Stringer Creek transition sites (21, 24, 31, 65) have characteristics of both riparian and upland zones that could affect the soil microbes. For example, saturated conditions have been observed to persist for days to weeks per year in the deeper portions of the soil profile of T1E3 (65, 66), but have not been observed in the shallow portions of the soil profile (e.g. 5cm). β-diversity analysis suggested the T1E3 5cm community may be more closely related to the HUZ soils than the deeper soils within the same soil profile (T1E3 20cm and 50cm) as well as the rest of the LUZ soils (Fig. 3). ADONIS analyses shows that when the T1E3 5cm community was included with either the HUZ or LUZ communities, the resulting statistics suggested this site/depth can fit with either HUZ or LUZ soil communities (Table S6). Difficulties in clearly assigning the T1E3 5cm community led to it being considered as a separate, transitional community, consistent with important soil selectors such as pH and
moisture. The pH of the T1E3 5cm soil (5.6) was borderline between the soils in the HUZ (4.2-5.6) and LUZ (5.7-6.9) soils.

Determining how environmental effects drive microbial function can be elusive at the phylum level because of the broad range of physiologies represented in each phylum. Growing season soil CO2 efflux has been shown to vary spatially across this subalpine forest landscape by as much as seven fold (26). Correlating soil CO2 (concentration and flux) with community composition (Table 1) and phylogenetic diversity (Fig. 4C) contributes to the ongoing discussion of the role of microbial diversity on soil respiration, a topic that has been vigorously debated and investigated (67). Studies have reported negative (68), positive (69-72), or no (68, 71, 73-76) correlations between microbial species richness and soil respiration. In this study, phylogenetic diversity exhibited a positive correlation with soil CO2 efflux ($R^2 = 0.38, p < 0.001$; Fig. 4B).

Most aspects of soil microbial heterotrophic C metabolism cannot be linked with specific phylogenetic signatures generated by Illumina sequencing. However, organisms involved in methane cycling can be distinguished at the genus level. The distribution of recognizable methanogen signatures in the Stringer Creek drainage was clear: they were below detection in all but one upland soil as opposed to comprising up to ~0.3% of the total community in the RZ soils (Fig. 5A). Identified methanogenic genera were most prevalent in the deeper RZ soil horizons (Fig. 5B), which were saturated at the time of sampling and over the course of nearly a decade of study have been found to generally remain so through most of the year (21, 65, 66). Therefore, the relative abundances of methanogens likely correlated with anaerobic RZ environments, which constitute only ~1.8% to the total land area in this ecosystem (21) but account for most of the CH4 efflux (77).
Occurrence of methanotrophs is common in the range of environments represented in this study. Recognizable type I methanotrophs were most prevalent in the RZ, while type II methanotrophs dominated in upland zones (Fig. 6A). These data strengthen and support the observations that type II methanotrophs dominate in mature, upland forest soils (38), whereas type I methanotrophs dominate in littoral wetland environments (78) and wet arctic soils (79).

Environmental factors such as pH, vegetation type, and soil temperature can influence methanotroph populations in forest soils (37, 80, 81). In the current study, the relative abundances of the detectable methanotrophs (total, type I or type II) did not appear influenced by pH nor by temperature (Table 3). However, they were positively correlated with soil CH₄ concentrations (Table 3) and SWC, but negatively correlated with soil O₂ (Table 3). While known bacterial methanotrophs are aerobes, the majority of CH₄ oxidation in riparian-like environments (e.g. rice paddies) occurs at the oxic-anoxic interface in the rhizosphere (82-88).

Rahalkar and co-workers reported that no oxygen could be detected in the sediment zone that had the highest abundance of methanotrophs and highest level of methane oxidation activities (89). A major caution in assessing the relative importance of such observations in the context of a forest ecosystem function is that the upland soil clusters α and γ [identified based on distinct pmoA clades (90-93)], which are known to be important to CH₄ consumption in forest soils (38, 94, 95), are not represented in this study since their 16S rRNA gene signatures are not yet known.

For all RZ soils, the abundance of Crenothrix was considerable (Fig. 6B). Here we present it as a methanotroph (96) and as such it represents 68%–94% of methanotrophs in these soils. However, this microorganism may be capable of growth on other carbon compounds (96), hence its role in methane cycling in this particular environment cannot necessarily be assumed.
In particular, its potential for utilizing acetate might correlate well with these environments, which presumably favored anaerobic conditions conducive to fermentation leading to the synthesis of acetate and other organic acids (96).

In conclusion, we characterized the soil microbial community from different positions within a subalpine forested watershed and correlated the microbial communities with historical and contemporary environmental conditions. Our results show that the composition, α-, and β-diversity of the microbial communities varied across the three landscape positions tested: HUZ, LUZ, and RZ. SWC, an environmental factor closely related to landscape position within the watershed, appeared to have the highest correlation with the structure of the overall microbial communities as well as the relative abundance of methanotrophs. Methanogens essentially only occurred in riparian soils, while methanotrophs occur in both upland and riparian soils.

ACKNOWLEDGEMENTS

The authors thank Kendra Kaiser, Erin Seybold, Tim Covino, and Liyin Liang for helping collect field environmental data. We would also like to thank the USDA National Forest Service for site access and logistic support. This project was supported by the US Department of Agriculture (2012-67019-21711) and the National Science Foundation (EPS-1101342 and EAR-1114392). Any opinions, findings and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of USDA or NSF.


Figure 1. The Stringer Creek research site located within the Tenderfoot Creek Experimental Forest, Montana USA. (A) shaded relief of elevation within the Stringer Creek watershed. Sampling sites examined in this study illustrated as color-coded dots (to match the sites shown in Fig. 3). (B) Cross section (note vertically exaggerated soil and elevation) depicting two of the transects illustrating the sampling sites relative to the creek and their topographic position. Each sampling site was located adjacent to previously installed gas well nest set at 5 cm, 20 cm, and 50 cm (inset shown accompanying T2W3) that allowed for sampling of soil O₂, CO₂ and CH₄.

Figure 2. The relative abundance of dominant bacterial phyla at the three soil depths (5 cm, 20 cm, and 50 cm) at the nine sites sampled within the Stringer Creek watershed.

Figure 3. Principal coordinate analysis of b-diversity observed in the Stringer Creek soil microbial communities. Grouping of the sampling sites into high upland (referred to as HUZ in the text), low upland (LUZ), and riparian (RZ) zones are shown by black circles and supported by ADONIS analysis (Table S6). The T1E3 5cm depth community (red diamonds) is distinguished by the gray dashed circle because its composition appears transitional between the HUZ and LUZ communities (see Table S6).

Figure 4. Soil water content exhibited positive correlation with both microbial phylogenetic diversity (A) and CO₂ effluxes (B). Panel (C) shows the correlation between the
phylogenetic diversity and CO$_2$ effluxes. Solid lines are linear regression lines, while the dash lines illustrate the 95% confidence intervals. Phylogenetic diversity was measured for each soil depth, while CO$_2$ flux was measured for each site yielding only 9 data points. SWC, CO$_2$ efflux, and PD values for two HUZ sites, SW5 and NWD6, are very similar and hence early overlap in the figure.

Figure 5. The relative abundance of total methanogens in soil microbial communities (A), and the relative abundance of different methanogenic genera at the two RZ sites T1W1 and T2W1 (B).

Figure 6. The relative abundance of methanotrophic bacteria in sampled soils. (A) Total methanotrophs delineated as Type I (white bars) and Type II (black bars). (B) Relative abundance of identified methanotroph genera in the two RZ sites, T1W1 and T2W1.
Table 1. Mantel correlations relating bacteria community composition and environmental factors and soil carbonaceous gas measurements.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mantel correlation</th>
<th>p-value</th>
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</thead>
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<tr>
<td>Environmental factors</td>
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</tr>
<tr>
<td>SWC (%)</td>
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<td>&lt;0.001</td>
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<tr>
<td>Soil T (°C)</td>
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<td>0.002</td>
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<td>Soil O₂ (%)</td>
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<td>Soil pH</td>
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<td>Soil carbonaceous gases</td>
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<tr>
<td>CO₂ Efflux (g m⁻²h⁻¹)</td>
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<td>CO₂ (ppm)</td>
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<td>CH₄ (ppm)</td>
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Table 2. Pearson correlation analysis of environmental parameters with main phyla of all soil samples. Dark grey shaded entries highlight statistically significant (p-value < 0.05) positive correlations, whereas light grey shaded entries denote significant negative correlations.

<table>
<thead>
<tr>
<th>SWC (%)</th>
<th>soil T (°C)</th>
<th>Soil O₂ (%)</th>
<th>Soil pH</th>
<th>CO₂ efflux (g m⁻²h⁻¹)</th>
<th>CO₂ (ppm)</th>
<th>CH₄ (ppm)</th>
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<td>R</td>
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<td>R</td>
<td>p* value</td>
<td>R</td>
<td>p* value</td>
<td>R</td>
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<tr>
<td>AD3</td>
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<td>0.01</td>
<td>0.974</td>
<td>0.30</td>
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<td>0.21</td>
<td>3</td>
<td>0.47</td>
<td>0.022</td>
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<td>Actinobacteria</td>
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<td>0.523</td>
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<td>0.015</td>
<td>0.10</td>
<td>0.635</td>
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<td>0.535</td>
<td>-0.29</td>
<td>0.165</td>
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<tr>
<td>Chloroflexi</td>
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<td>0.08</td>
<td>0.49</td>
<td>0.014</td>
<td>0.61</td>
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<td>Firmicutes</td>
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<td>9</td>
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<td>0.08</td>
<td>0.49</td>
<td>0.015</td>
<td>0.46</td>
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<tr>
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<td>0.270</td>
<td>-0.27</td>
<td>0.196</td>
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<td>Verrucomicrobia</td>
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<td>1</td>
<td>0.25</td>
<td>3</td>
<td>0.44</td>
<td>0.031</td>
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</table>

| WS3     | 0.33       | 0.091       | 0.26     | 0.189       | -0.15    | 0.486    | 0.57    | 0.002     | 0.28     | 0.164   | 0.21    | 0.320   | 0.10    | 0.646   |
Table 3. Pearson correlation analysis of methanotroph relative abundance as a function of environmental parameters measured in this study.

<table>
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<th>Parameters</th>
<th>Total</th>
<th>Type I</th>
<th>Type II</th>
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<td>R</td>
<td>p-value</td>
<td>R</td>
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<tr>
<td>Environmental factors</td>
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<tr>
<td>SWC (%)</td>
<td>0.72</td>
<td>&lt;0.001</td>
<td>0.72</td>
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<td>Soil T (°C)</td>
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<td>0.071</td>
<td>-0.35</td>
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<tr>
<td>Soil O₂ (%)</td>
<td>-0.53</td>
<td>0.008</td>
<td>-0.51</td>
</tr>
<tr>
<td>Soil pH</td>
<td>0.22</td>
<td>0.263</td>
<td>0.2</td>
</tr>
<tr>
<td>Soil carbonaceous gases</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CO₂ Efflux (g m⁻²h⁻¹)</td>
<td>0.54</td>
<td>0.004</td>
<td>0.54</td>
</tr>
<tr>
<td>CO₂ (ppm)</td>
<td>0.53</td>
<td>0.007</td>
<td>0.51</td>
</tr>
<tr>
<td>CH₄ (ppm)</td>
<td>0.68</td>
<td>&lt;0.001</td>
<td>0.65</td>
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</tbody>
</table>
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