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Microbial Community Responses to Alterations in Historical Fire Regimes in Montane Grasslands

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Abstract: The influence of fire regimes on soil microbial diversity in montane grasslands is a relatively unexplored area of interest. Understanding the belowground diversity is a crucial stepping-stone toward unravelling community dynamics, nutrient sequestration, and overall ecosystem stability. In this study, metabarcoding was used to unravel the impact of fire disturbance regimes on bacterial and arbuscular mycorrhizal fungal community structures in South African montane grasslands that have been subjected to an intermediate (up to five years) term experimental fire-return interval gradient. Bacterial communities in this study exhibited a shift in composition in soils subjected to annual and biennial fires compared to the controls, with carbon and nitrogen identified as significant potential chemical drivers of bacterial communities. Shifts in relative abundances of dominant fungal operational taxonomic units were noted, with Glomeromycota as the dominant arbuscular mycorrhiza observed across the fire-return gradient. A reduction in mycorrhizal root colonisation was also observed in frequently burnt autumnal grassland plots in this study. Furthermore, evidence of significant mutualistic interactions between bacteria and fungi that may act as drivers of the observed community structure were detected. Through this pilot study, we can show that fire regime strongly impacts bacterial and fungal communities in southern African montane grasslands, and that changes to their usually resilient structure are mediated by seasonal burn patterns, chemical drivers, and mutualistic interactions between these two groups.

Keywords: bacteria; mycorrhizal fungi; montane grassland; fire regime; microbial community composition

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1. Introduction

Fire is present in almost all the world's grasslands [1] and is necessary for maintaining their biodiversity, soil dynamics [2], and ecosystem services such as rangeland, water production, and regulation of biogeochemical cycles [3–8]. Major grassland ecosystems such as African and Brazilian savannas and the Argentinian pampas have been well studied in terms of fire ecology, wildlife dynamics, grassland restoration, and grassland ecology [1,8–15]. The same cannot be said of montane grasslands based on the limited available literature on the topic. The impacts of fires on both soil chemical properties and soil microorganisms are widely acknowledged but have not been fully documented, especially in African ecosystems. Long-term studies on montane grasslands are few and far between, and the body of work from the Drakensberg in South Africa indicates the ecological complexity of these grasslands and their relationship with fire [16–19]. For the most part, studies to date have neglected to consider the impact of fire regimens on aboveground [20] and belowground community biodiversity [21,22], leaving soil microbial diversity profiles in soils, especially montane soils, poorly understood. These soils harbour

enormous numbers of microbial organisms including bacteria, fungi, and other unicellular and multicellular life forms [23] of which the taxonomic identity and function remain largely uncharacterised.

Belowground processes are critical for maintaining biodiversity and ecosystem services, yet the effects of fire regimes on belowground processes remain largely unexplored [21]. Fire and herbivory have been acknowledged to shape bacterial and fungal communities in southern African montane soils through edaphic properties (including Mg, pH, Ca) for bacteria, and vegetation for fungi [24]. Improving the understanding of fire regimes and the links between belowground processes and biodiversity, in addition to aboveground processes, is essential for a more complete understanding of ecosystems [22].

The influence of fire regimes on soil ecosystems, including their recovery response, is dependent on mean fire-return interval, intensity, and season of burn [25], as well as vegetation type and the soil microbial community. Fungi are generally considered to be more efficient decomposers of complex organic compounds, such as lignin and cellulose, compared to bacteria [26]. Therefore, the reduction in organic matter caused by fire can have a greater impact on the fungal community, leading to changes in their composition and abundance, while bacteria may rely on alternative carbon sources or be less affected by the changes in substrate availability. Seasonality has a direct impact on temperature profiles during a fire as soil heating remains elevated for longer periods during dry seasons [27]. Fire regimes also impact the combustion of litter, volatilisation of minerals, and deposition of pyrogenic nutrients [17], promote changes in vegetation structure, composition and associated productivity, release root exudates [18,25], and change soil surface albedo and related soil temperature and moisture [28]. Collectively, each of these parameters affects the soil environment and plant communities to which soil microbiota are closely linked [21].

Here, we present the first study assessing the impact of fire frequency and seasonality on the belowground microbial ecology of montane grasslands in the Drakensberg, South Africa, adding to the body of knowledge on this ecosystem. In this study, we aim to explore the effect of season-specific burn patterns on bacterial and fungal communities from this montane region. We hypothesise that unique microbial community profiles exist across a fire-return interval gradient subjected to seasonal burn regimes, and soil chemistry dynamics are one of the main drivers contributing to the community structure.

2. Materials and Methods

2.1. Site Description

The Brotherton trial (29°00′ S, 29°15′ E) is located on a flat to gently rolling plateau within the Cathedral Peak conservation area of the uKhahlamba Drakensberg Park World Heritage Site (South Africa), at an altitude of 1890 m. This region is relatively constant for altitude, soil type, and climatic variables [17], presenting a unique opportunity to assess the impact that a range of fire regimes have had on soil microbes, without additional external physical and environmental effects. The long-term montane site represents "pristine" Drakensberg Basalt Grasslands [29] previously described as Highland Sourveld [30]. The upper geology of the Drakensberg region comprises basaltic lava that appeared 184 million years ago when the Gondwana supercontinent broke apart [31]. This basalt has weathered to produce dark soils consisting of a mix of clay as well as coarse and fine sand which are high in organic matter [17].

From c. 1950, rigid biennial and annual burns have been implemented, with the burning regime experiment established in 1980 by E. Granger of Forestek (CSIR) at this site. Initially, the trial consisted of 44 plots (25 m × 25 m) and 21 burning and mowing treatments, 12 of which were replicated three times. A subset of the treatments which were subjected to varying burning regimes (Table 1) remain continuous to date and allowed for the exploration of the effect of season and frequency of fire on montane grasslands dynamics [17].

Table 1. Plots and the burn regimes to which they were subjected at the time of sampling.

Fire Treatment	Plot	Last Burnt	Period since Last Fire	Fires since Start of Trial
Annual autumn burn	5	May 2014	9 months	34
Annual spring burn	12	October 2014	4 months	34
Biennial autumn burn	1	May 2014	9 months	18
Biennial spring burn	2	October 2014	4 months	18
Five-year rotation burn	6	October 2012	27 months	8
No-burn treatments	11	2007 (unplanned fire)	±180 months	2

At the inception of this trial, the plant species composition of the plots was relatively similar. Dominant species identified were *Themeda triandra*, *Tristachya leucothrix*, *Stiburus alopecuroides*, *Harpochloa falx*, *Heteropogon contortus*, *Trachypogon spicatus*, *Koeleria capensis*, and *Diheteropogon amplectens*, in addition to non-graminoid herbaceous plants and sedges [8].

2.2. Sampling and Soil Chemical Analysis

Soil samples were retrieved from six plots at the study site in February 2015. Samples were collected for the following burn seasons: annual autumn, annual spring, biennial autumn, biennial spring, after 5 years, and a no-burn control site. All samples were collected aseptically into sterile 15 mL falcon tubes from 1–3 cm below the surface of the soil for analysis. Each sample comprised 6 mL of soil collected from five individual locations within a ±1 m radius (maintaining a distance of more than 2 m from the edge of the plots) pooled to create a heterogenous soil sample. Sampling was performed in triplicate per plot, resulting in a total of 18 samples being retrieved. RNAlater™ (Ambion™) was added to all soil samples in an approximately 1:1 (w/v) ratio to stabilise the genetic material. The samples were transported at 4 °C to the laboratory where they were subsequently stored at −20 °C. A mixture of non-species-specific root and bulk soil samples from the same plots were collected in triplicate, placed in sterile zip-lock bags, and stored at 4 °C. Once at the laboratory, the roots were manually removed from bulk soil and stored in 50% ethanol for further processing. Approximately 200 g of a composite soil sample from each plot was sent for soil chemistry analysis at Eco-Analytica Laboratories (North-West University, Potchefstroom). Soil organic carbon content was determined using the Walkley–Black titration method [32]. Total available nitrogen was measured using the distillation method and the LECO combustion method. pH was measured using a Radiometer PHM 80 Portable pH meter. Anions were measured by ion chromatography using a Metrohm 761 Compact IC. The remaining soil was retained for arbuscular mycorrhizal fungal spore extraction and enumeration.

2.3. Mycorrhizal Assessment

Root samples were cleared of alkali-soluble pigments in a 5% potassium hydroxide (KOH) solution and stained in Lactoglycerol Trypan Blue using a standard protocol [33]. Roots were examined microscopically under 400× magnification using a Nikon YS 100 compound microscope. Percentage colonisation was determined using a modified grid-line intersect method [33].

Arbuscular mycorrhizal (AM) fungal spores were extracted from 50 g of air-dried soil samples using a wet sieving and decanting method. Briefly, debris from the 45 µm, 125 µm, and 250 µm sieves was washed into 50 mL tubes for purification using sucrose centrifugation. The supernatant containing AM fungal spores was filtered through filter paper (Whatman® Grade 1) using a Buchner funnel and water vacuum, and spores were counted using a stereo microscope [33]. Differences between colonisation and spore enumeration across fire frequency and burn season treatments were determined using Kruskal–Wallis tests (at a significance of $p < 0.05$) [34].

2.4. DNA Extraction, Amplification, and Sequencing

Total genomic DNA was extracted from ±0.2 g of each soil sample using the ZR Soil Microbe DNA Miniprep™ Kit D6001 (Zymo Research, Tustin, CA, USA) according to the manufacturer's protocol, and quantified spectrophotometrically (NanoDrop™ 2000, Thermo Fisher Scientific, Johannesburg, South Africa). For an in-depth profile of the bacterial community present in the no-burn control and burn-treated soils, two biological replicates from each plot were sequenced on the Illumina MiSeq platform using the bacterial primers 16Sf (5′-GTGCCAGCMGCCGCGGTAA-3′) and 16Sr (5′-GGACTACHVGGGTWTCTAAT-3′) [35]. The 16S rRNA gene region amplicon libraries were prepared using 30–50 ng template DNA and AccuPrime™ Pfx SuperMix (Thermo Fisher Scientific, MA, USA) PCR mix as per manufacturer specifications. The PCR cycling parameters were as follows: 1 cycle at 95 °C for 2 min, 30 cycles at 95 °C for 20 s, 55 °C for 15 s, 72 °C for 5 min, and a final extension at 72 °C for 10 min. Amplicon libraries were purified of excess nucleotides, primer dimers and normalised using the SequalPrep™ Normalization Plate Kit (Thermo Fisher Scientific). Sequencing was performed on the Illumina MiSeq platform as per manufacturer specifications for ~230 bp reads, and repeated on the 454 GS Junior Platform (Roche, Ricardo Rojas, AR, USA) which generated read lengths of ~420 bp. The Roche amplicon libraries were prepared as described in Matcher et al. (2011) [36] using the same template DNA used for the MiSeq analysis.

For the 18S rRNA gene region amplicon library, DNA extracted from the third biological replicate was subjected to a two-step nested PCR amplification protocol. The primary PCR reaction utilised 30–50 ng template DNA and arbuscular mycorrhizal-specific primers NS31 (5′-TTGGAGGGCAAGTCTGGTGCC-3′) and AML2 (5′-GAACCCAAACACTTTGGTTTCC-3′) [37] with KAPA HiFiHotStart ReadyMix (KAPA Biosciences, MA, USA) as per manufacturer specifications. Cycling parameters were as follows: 98 °C for 5 min, followed by 5 cycles of 98 °C for 30 s, 49.1 °C for 45 s, 72 °C for 1 min, then 25 cycles of 98 °C for 30 s, 65 °C for 45 s, 72 °C for 60 s and a final extension at 72 °C for 5 min. The amplification product was subjected to a secondary PCR using Nextera XT MID tagged primers (Illumina, CA, USA) as per manufacturer specifications. PCR products were subjected to agarose gel (1%) electrophoresis and the relevant amplicon (300 bp) was excised from the gel and purified using the Isolate II PCR and Gel purification kit (Bioline, Meridian Bioscience, Newtown, OH, USA) as per manufacturer specifications. Sequencing of the fungal amplicons was performed on the GS Junior 454 Titanium platform (Roche) as per manufacturer specifications. The sequence datasets generated in this study are deposited in the sequence reads archive (SRA) database of the National Centre of Biotechnology Information under the BioProject accession number PRJNA422406 (SRP126751).

2.5. Bioinformatic Analysis

Data processing was conducted using Mothur software (version 1.35.1) [38]. All sequences with a quality score lower than 20 over a qwindowsize of 50 were removed. In addition, all sequences shorter than 200 nucleotides, with ambiguous nucleotides, or with homopolymeric runs longer than 8 were removed. Chimeric sequences were identified using de novo-based VSearch [39] and removed from the dataset. All datasets were normalised by random subsampling to compensate for differences in overall dataset sizes so that overestimation of species numbers in larger datasets may be avoided. Sequences were classified using the Naïve Bayesian approach [40] against the SILVA reference dataset (v123) [41] to genus level and to remove non-specific amplifications. Operational taxonomic units (OTUs) were determined in Mothur using the Maarjam database [42] for fungal identification and NCBI BLASTn against Reference RNA sequences (refseq_rna) database [43] for bacterial identification at a distance value of 0.03, and singletons were removed from the dataset as an added stringency measure against sequencer errors. Whilst the MiSeq platform generated many reads, allowing for robust statistical analyses, >50% of the sequences were poorly classified against the SILVA reference database (v123) [41],

due to the inability to classify short sequence reads with any confidence, resulting in the primary use of the Roche data for classification purposes.

2.6. Statistical Analysis

PCO and statistical analysis results for bacteria were consistent for both datasets generated on the MiSeq and Roche sequencing platforms, confirming the validity of using both platforms.

Principle coordinates analysis (PCO), analysis of similarity (ANOSIM) tests, and rank correlation (RELATE and BEST) analyses were performed on square root transformed Bray Curtis dissimilarity matrices in PRIMER (v7) and PERMANOVA+ [44]. To ascertain details on the impact of the soil chemistry on the microbial community, distance-based linear modelling (distLM) was performed on resemblance matrices of both bacterial and fungal data using a stepwise selection procedure. The influence of the bacterial community on the fungal community, and vice versa, was explored by overlaying the datasets of the bacterial and fungal communities using distLM in PRIMER (v7) and PERMANOVA+. Additional statistical analyses were performed in RStudio [34] using ANOVA (at a significance of $p < 0.05$), followed by a post-hoc Tukey test to determine significant differences between samples.

3. Results

3.1. Soil Chemistry

Soil nutrient chemistry of a single pooled sample per plot (Table 2) revealed a distinct seasonal trend within the burn plots, whereby autumn and spring components clustered together regardless of burn frequency, and further, clearly separated from the no-burn control and five-year burn interval plots (Figure 1).

Table 2. Soil properties of the sample sites.

Soil Characteristic	Control	Five-Year	Biennial Autumn	Biennial Spring	Annual Autumn	Annual Spring
Ca (mg/kg)	228.5	183.0	330.0	245.0	303.5	224.5
Mg (mg/kg)	85.0	77.5	103.5	86.0	97.0	78.5
K (mg/kg)	216.5	222.5	240.5	261.0	233.0	290.0
Na (mg/kg)	27.5	43.0	45.0	35.5	51.5	36.5
P (mg/kg)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
pH (H20)	5.10	5.10	5.16	5.15	5.13	5.16
pH (KCl)	4.38	4.34	4.40	4.43	4.37	4.42
EC (mS/m)	14	10	12	12	12	13
% Carbon (Walkley Black)	11.17	13.32	11.56	14.10	10.97	12.93
% Nitrogen (LECO)	0.6	0.85	0.68	0.76	0.65	0.77

Overall, burned soils had a high content of calcium (Ca, 183–330 mg/kg) and potassium (K, 216.5–290 mg/kg). The fluctuations show that Ca peaked in autumn burn plot soils, decreased in soils burned during spring and no-burn control soils, and was lowest in five-year burn interval soils. Furthermore, biennial burn plot soils showed higher Ca than annually burned soils. Magnesium (Mg, 77.6–103.6 mg/kg) and sodium (Na, 27.5–51.5 mg/kg) showed the same seasonal trend as Ca but a contrasting burn frequency trend. K showed a clear divergence from Ca, as it was higher in spring burn plots compared to autumn burn plot soils, and higher in annual burn plots, but exhibited similar lower levels in no-burn control and five-year burn interval soils. Carbon (C, 10.97–14.1%) and nitrogen (N, 0.6–0.85%) were higher in spring burn plots. C peaked in the biennial burn plot soils (14.10%) and five-year burn interval soils (13.32%), while nitrogen was highest in five-year burn interval soils (0.85%). p was consistently low (<0.01) for all samples. Soils were identified to be weakly acidic (pH_{H_2O} = 4.2–4.4 and pH_{KCl} = 5.10–5.16); electrical

conductivity (EC) peaked in no-burn control soils (14 mS/m); neither measurement showed any distinct seasonal trends.

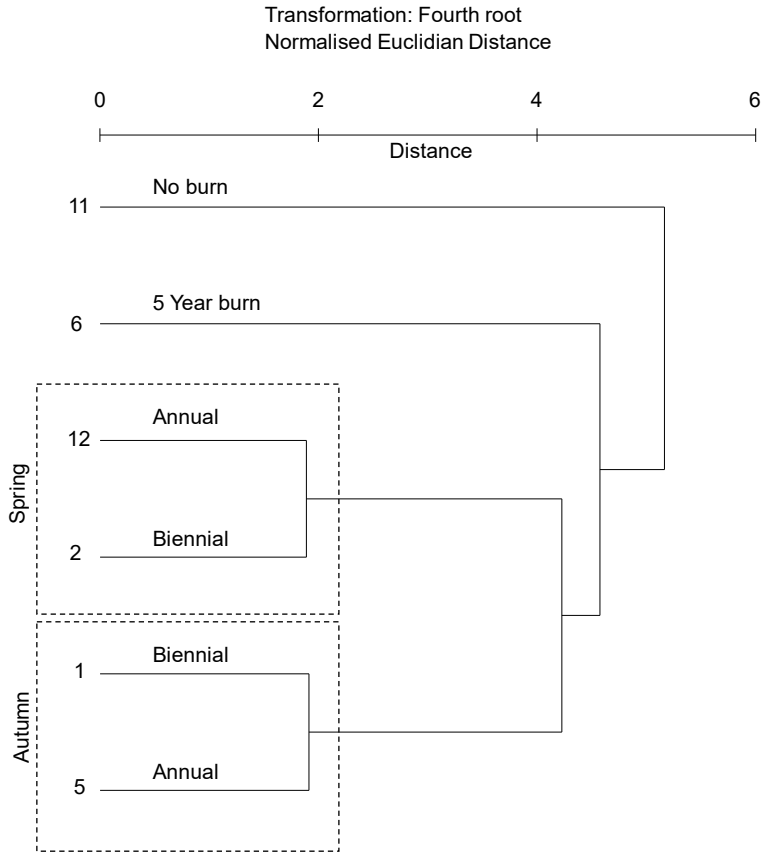

Figure 1. Sampling plots of the 5 sample types and control site from the Brotherton trial, Cathedral Peak, uKhahlamba Drakensberg Park, including the cluster analysis of environmental chemistry.

3.2. Microbial Community Composition of Burn-Treated Soils

3.2.1. Soil Bacterial Community Profile

A total of 1,654,946 16S rRNA gene region sequences remained after data curation and quality control. After the removal of singleton reads and subsampling of each sample to a final number of 10,000 sequence reads per sample (i.e., a cumulative 120,000 across the dataset), a final count of 22,631 OTUs was observed. Species diversity measures (as determined at a distance level of 0.03 for OTU selection) showed an even distribution of OTUs between all soil samples (Pielou's evenness: 0.89 to 0.90), as well as high OTU diversity (Shannon index: 6.76 to 6.86, Simpson index: 0.99) that was consistent between no-burn control and burn treated plots (Supplementary Table S1). Examination of the phylogenetic composition of the bacterial data indicated a dominance of Actinobacteria within the soils across all burn plots (19.4% to 32.4% relative abundance (RA), $p = 0.015$, $F = 7.48$) (Figure 2, Supplementary Table S2). Within this phylum, the dominant classes were identified as Acidimicrobia (5.3% to 9.5% RA to the total number of reads assigned to the phylum Actinobacteria), Thermoleophilia (28% to 34% RA), and Actinobacteria (53% to 64% RA to the total number of reads assigned to the phylum Actinobacteria). The Proteobacteria ($p = 0.001$, $F = 23.86$) (Supplementary Table S2) present in each of the soil burn plots were predominantly Alphaproteobacteria (50.8% to 60.5% RA) and Gammaproteobacteria (21.2% to 29.5% RA). At the order level, the Alphaproteobacteria showed an increased representation of Rhizobiales (25.7% to 26.2% RA of the total Proteobacterial reads) in the no-burn control and five-year burn interval plots, as well as in the annual spring burn plots.

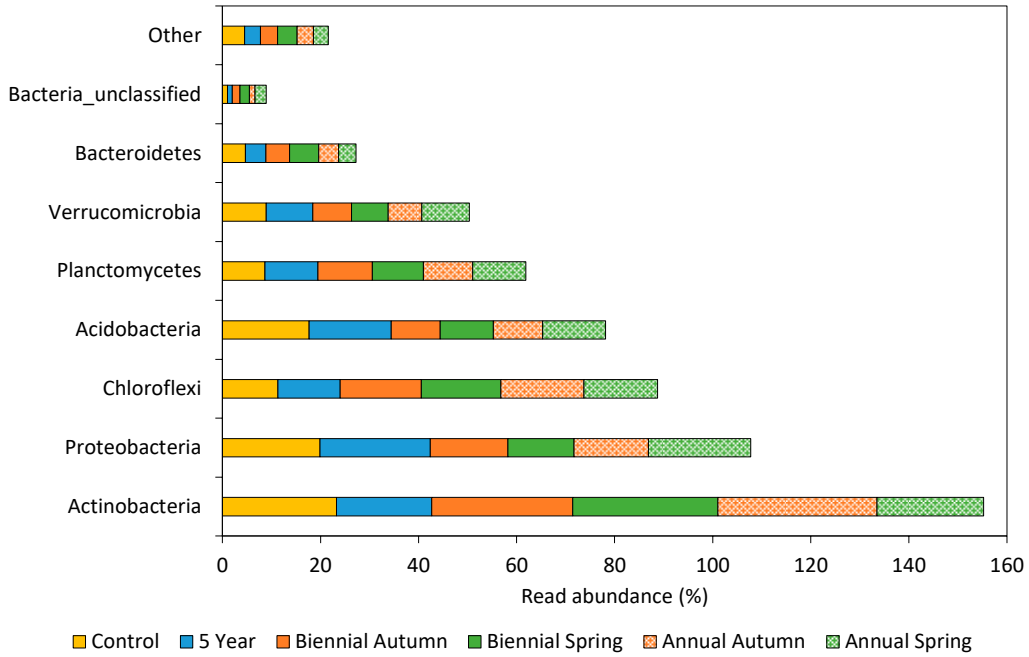

Figure 2. Absolute abundances of bacterial phyla from the various soil treatments classified to phylum level against the Silva database (version 128).

In contrast, soils from the biennial spring, biennial autumn, and annual autumn burn interval plots were dominated by Rhodospirillales (39.8%, 44.8%, and 49.4% RA of the total Proteobacterial reads, respectively). Of the Rhizobiales reads, between 69% and 83% were associated with the families Bradyrhizobiaceae, Xanthobacteriaceae, and unclassified Rhizobiales. There was a significant change in abundance of bacteria from the phyla Actinobacteria ($p = 0.021$, F = 10.97), Bacteroidetes ($p = 0.043$, F = 7.22), Chloroflexi ($p = 0.045$, F = 7.07), Planctomycetes ($p = 0.038$, F = 7.76) and Proteobacteria ($p = 0.001$, F = 57.64) when comparing to the spring/autumn burn plots overall. However, upon pairwise analysis of these taxa, it was noted that Actinobacterial communities were significantly lower for annual spring burn plots ($p = 0.003$, F = 376.18) when compared to annual autumn burn plots, as were Bacteroidetes ($p = 0.019$, F = 50.82), while Proteobacteria was significantly higher ($p = 0.006$, F = 177.70) for the same. Annual spring burn plots showed significantly lower bacterial community changes for Actinobacteria ($p = 0.014$, F = 67.87) and higher abundances for Planctomycetes ($p = 0.036$, F = 26.38) when compared to biennial spring burn plots. Planctomycetes and Proteobacteria were also significantly higher in abundance in annual autumn versus the biennial spring burn plots ($p = 0.030$, F = 32.21 and $p = 0.0255$, F = 38.54, respectively). Chloroflexi ($p = 0.044$, F = 24.45) and Proteobacteria ($p = 0.104$, F = 69.77) showed significantly lower abundance in biennial spring burn plots compared to biennial autumn burn plots. Proteobacteria additionally showed a significantly higher abundance in annual spring versus biennial autumn burn plots ($p = 0.003$, F = 340.61) and lower abundances for annual autumn burn plots when compared against biennial autumn burn plots ($p = 0.001$, F = 1119.34). Acidobacteria and Verrucomicrobia populations did not significantly vary in RA irrespective of the burn status of the plots.

Clear clustering of the bacterial communities present in the biennial spring burn interval plots and the annual and biennial autumn burn plots were evident (Figure 3a). The remaining burn plot communities appeared distinct from each other, demonstrating a moderate yet significant degree of dissimilarity between the annual and biennial burn plots versus the no-burn control (ANOSIM, $p = 0.022$, $R^2 = 0.603$) and the annual and biennial burn plots versus the five-year burn interval plots ($p = 0.022$, $R^2 = 0.677$).

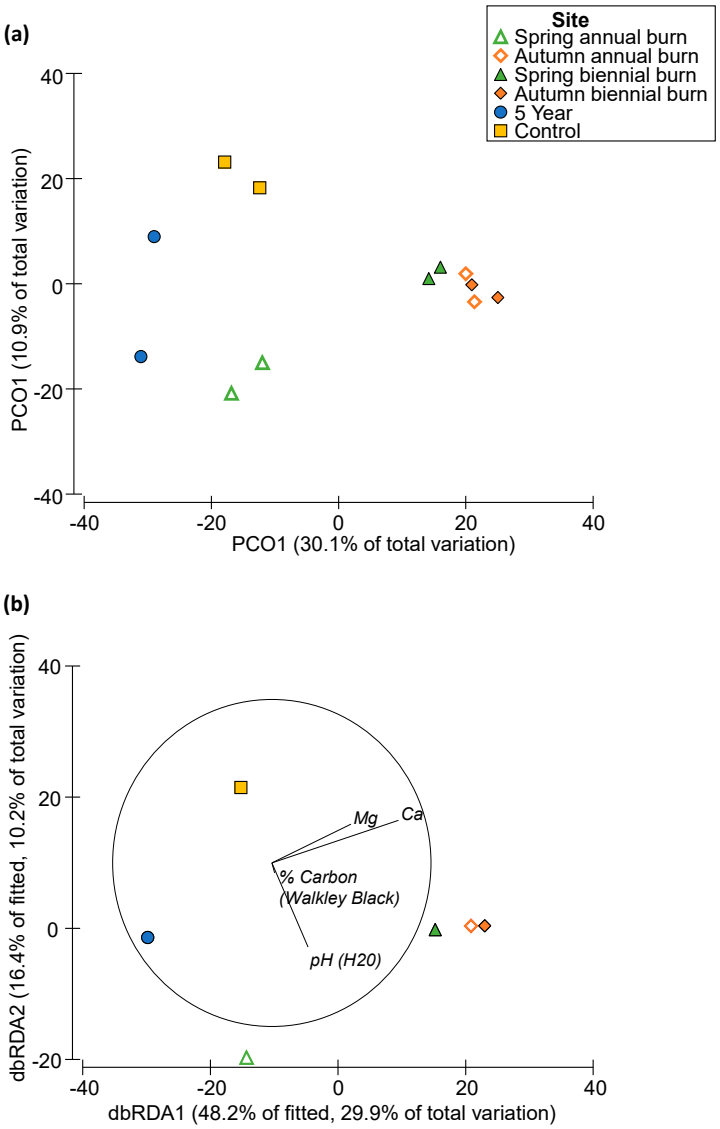

Figure 3. Ordination using Bray Curtis similarity of non-transformed bacterial abundances revealed (**a**) clustering of communities present in the annual and biennial autumn burn plots as well as the biennial spring burn plots. All other sites appeared disparate. (**b**) Distance-based RDA ordination of this bacterial data with vectors indicating the effect of soil chemistry parameters.

3.2.2. Fungal Community Profile of the Soil

Mycorrhizal colonisation was confirmed by the presence of arbuscules within the plant root material (Supplementary Figure S1) by microscopic examination. Roots from all burn-treated soils had over 60% mycorrhizal colonisation, with annual autumn, biennial autumn, and five-year burn interval plots being significantly lower than the no-burn control plot (Table 3). AM spore counts for each burn plot ranged from 100 to 600 spores per 100 g of soil. The annual spring burn plot had significantly higher numbers of AM spores when compared to the other plots.

Table 3. Arbuscular mycorrhizal root colonisation and spores in soils under different burn regimes.

Site	% Mycorrhizal Colonisation	AM Spores/100 g Soil
Control	87.7 ± 6.1	198 ± 131.2
Annual spring	65.6 ± 21.3	575 ± 114.0
Annual autumn	67.9 ± 7.9	128 ± 72.1
Biennial spring	73.3 ± 13.4	94 ± 60.1
Biennial autumn	66.6 ± 5.6	289 ± 162.3
Five-year rotation	67.6 ± 4.5	287 ± 54.0
Chi-square value	11.595	12.135
p value	0.041	0.033

Values represent means ± standard deviation (n = 3).

Soil fungal diversity was lower in the spring biennial plot (Supplementary Table S1) when compared to that of the no-burn control and other burn plots. Diversity estimates for these plots demonstrated a moderate distribution of fungal OTUs (Pielou: 0.51 to 0.66), low diversity of all representative OTUs (Shannon: 2.25 to 2.97), and high diversity of the dominant OTUs (Simpson: 0.76 to 0.90). Sequence analysis of the 18S rRNA gene region yielded 134,430 collective sequence reads from all soil treatments. Classification of curated reads revealed that >90% of the total mycorrhizal 18S rRNA gene region sequences generated belonged to the phylum Glomeromycota. Within Glomeromycota, the most well-represented order in all burn treatments was that of Glomerales (Figure 4a). The no-burn control and five-year burn interval plots exhibited a slightly higher proportion of Archaeosporales (1.9 to 2.7% RA) and Diversisporales (4.9 to 7.5% RA) compared to the remaining burn regimes (<3.3% RA). The overall dominant AM fungal genera belonged to classified and unclassified Glomerales (Figure 4b), followed by *Rhizophagus*, which was only present in the annual and biennial burn plots. *Septoglomus* featured prominently in the five-year burn interval plot (5.6% of total reads) (Figure 4b).

Analysis of the prominent OTUs within the fungal community structure revealed OTU 1 (*Glomus* sp.) was the dominant taxon in the no-burn control plot (Supplementary Table S3) and was present at lower abundance in the remaining plots. OTU 1, 3, and 5 (also identified as *Glomus* spp.) were present in all soil samples irrespective of the burn treatment. Four other Glomus OTUs were unique to the biennial and annual burn plots (OTUs 6, 7, 8, and 9), while OTUs 2 (*Glomus* sp.) and 4 (*Archaeospora* sp.) were isolated to the rarely burned plots.

3.3. Effect of Soil Chemistry on Microbial Community Composition

A clear correlation was observed between soil chemistry and bacterial composition (p = 0.004, Rho = 0.371), where Ca (mg/kg), Mg (mg/kg), Na (mg/kg) and pH_{KCl} sufficiently explained the observed bacterial community structure (BEST, Global test statistic: p = 0.01, Rho = 0.743). The dbRDA ordinations again revealed an association between the bacterial communities of the biennial spring burn plot, the annual autumn burn plot, and the biennial autumn burn plot. Minimal association to environmental variables could be deduced from the ordination (Figure 3b); however, Ca (in marginal and sequential tests), Mg, pH (in marginal tests), and %C (in sequential tests) were identified as factors significantly influencing the community structure (p = 0.001–0.025) (Supplementary Table S4).

The fungal dataset revealed no pattern and a distinct lack of correlation between the soil chemistry and biological data (Supplementary Figure S2, p = 0.428, Rho = 0.024). The chemistry variables that best explained the observed fungi patterns were pH_{H2O} and %C; however, these trends were not significant (BEST, Global test statistic: p = 0.7, Rho = 0.321). The fungal dbRDA model showed that %N was associated with the annual spring burn plot and %C with the biennial spring burn plot (Supplementary Figure S2); however marginal and sequential tests showed no significant soil chemistry-related impacts (Supplementary Table S5).

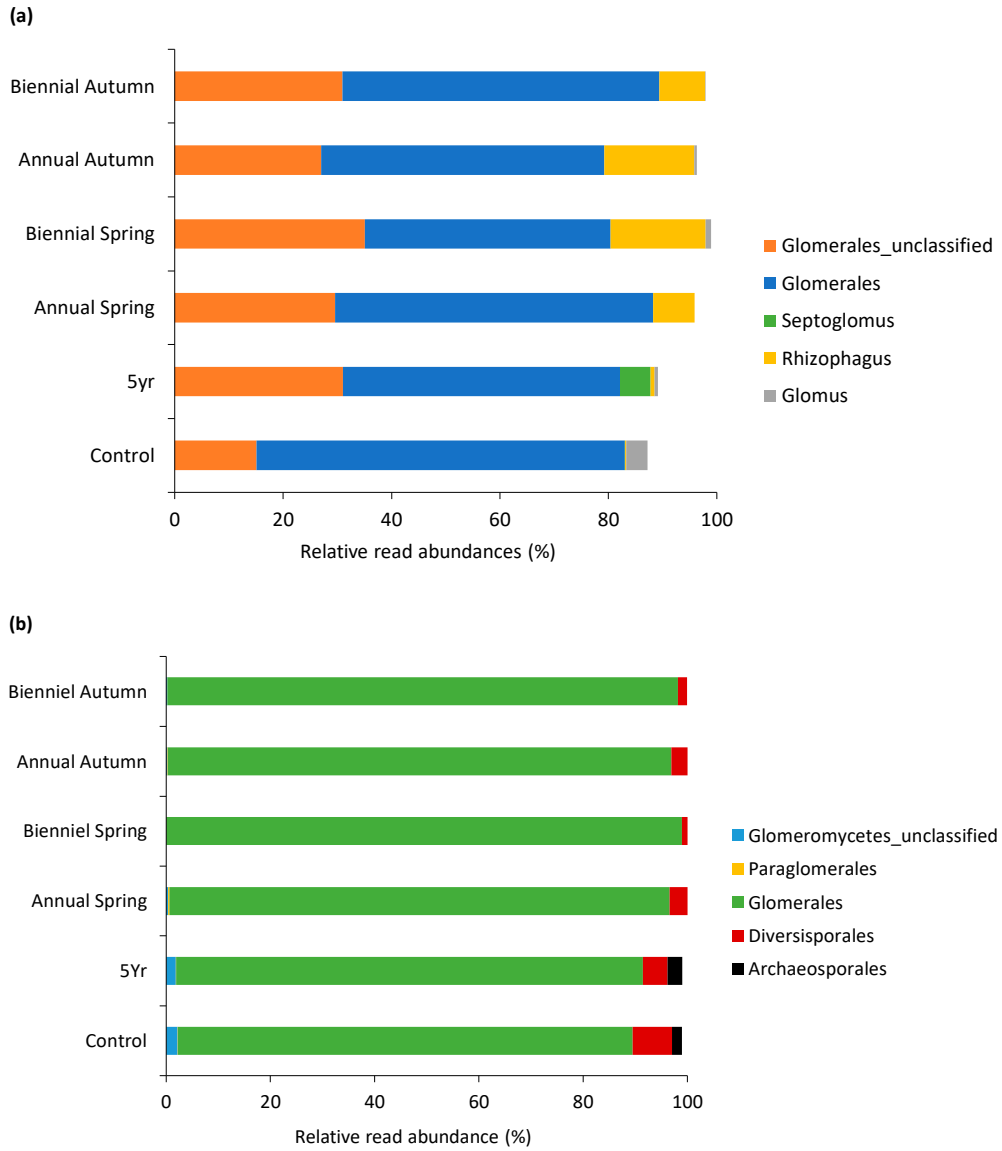

Figure 4. Abundance of the reads from all sites within the dominant Glomeromycota division showing (**a**) the genera and (**b**) the order of the *Glomerales* relative to that of the total number of fungal reads.

The bacterial community was significantly influenced (replicate 1: $p = 0.017$, pseudo f = 2.5117; replicate 2: $p = 0.003$, pseudo f = 2.1413) by specific fungal OTUs (R-squared = 0.89), with sequential tests revealing fungal OTU, JNELW1S01AEE5W (99.6% similarity to *Glomus* sp. VTX00191), as the OTU of importance. Bacteria also showed a high influence on the fungal community structure (R-squared = 0.86131). Four OTUs were identified as having a significant influence on the fungal community structure according to sequential tests, namely M01143_10_000000000-ALHRW_1_1102_26440_23805 (93% identity to *Skermanella* sp.) (replicate 1: $p = 0.008$, pseudo f = 2.5728; replicate 2: $p = 0.005$, pseudo f = 2.5728), M01143_10_000000000-ALHRW_1_1102_8905_17907 (96% identity to *Gemmata massiliana*) (replicate 1: $p = 0.025$, pseudo f = 3.3486; replicate 2: $p = 0.034$, pseudo f = 3.486), M01143_10_000000000-ALHRW_1_1101_25464_12694 (99% identity to *Reyranella soli*) (replicate 2: $p = 0.041$, pseudo f = 11.567) and M01143_10_000000000-ALHRW_1_1105_15990_22090 (91% identity to *Stella vacuolata*) (replicate 2: $p = 0.041$, pseudo f = 11.567).

4. Discussion

This pilot study showed that the fire regime in Brotherton did not affect the observed bacterial richness, while fungal communities were more diverse in intermediate fire return plots (two- to five-year fire-return intervals). Furthermore, bacterial and fungal communities showed a strong correlation with each other, implying a potential mutualistic or symbiotic relationship between fire-exposed soil bacteria and fungi that contribute to the soil community structure in the Drakensberg montane grasslands. This could be tied to complementary resource utilisation, nutrient exchanges, or successional dynamics from season to season [45]. Fungal communities tend to be more sensitive to ecosystem changes brought about by frequent fires since fungal spores, hyphae, and mycelia are relatively more susceptible to damage or mortality when compared to bacteria [46–48]. The response of soil microbiota to a fire disturbance event is dependent on several factors, the most critical of which are the species of microbiota present, the intensity of the fire, and the physical properties of the soils [49,50]. In soil ecosystems, as well as numerous others, the complex interaction between fungi and bacteria is critical to driving biochemical cycles and contributing to the health of plants and animals [51], as well as agricultural, forestry, and environmental sciences. Further investigation of bacterial–fungal complexes can enhance our ability to utilise the interaction for practical benefits in montane habitats, as well as reshape the current understanding of bacterial and fungal microbiomes.

4.1. Correlation of Bacterial Community Composition with Fire Event Frequency

It was clear that the seasonality of the annual fires has a marked impact on the community composition of the bacterial microbiome. Annual spring burns (plot 12) show a community that was distinct from that of the remaining treatments (Figure 3). In contrast, the autumn burn treatment (plot 5) has a far greater microbiome similarity to biennial burn plots, irrespective of whether they were burned biennially in spring (plot 2) or autumn (plot 1).

As expected, in this study, the absence of frequent fire events (an anomalous state in these ecosystems) resulted in significant alterations in the bacterial community structure (Figures 2 and 3). There was a consistent trend in the differentiation between microbial populations exposed to regular fire events compared to rare or absence of fire events, irrespective of the vegetation or ecosystem type, fire intensity, or frequency [50,52–56]. The high degree of similarity between the bacterial community profiles in the no-burn control soils and those from soils five years post-fire reflects a considerable shift in the bacterial community profiles upon removal of annual or biennial burn events which are typical for the Drakensberg region. When comparing the no-burn control and five-year fire interval samples with those from the more frequently burned plots, significant variances in the relative abundances of Actinobacteria, Proteobacteria, Chloroflexi, and Acidobacteria were noted (Figure 2); these taxa are commonplace in global montane regions, including the Lesotho highlands [57–60]. Preliminary studies indicate a broad ability of these bacteria to degrade organic materials, thereby playing a vital role in organic matter turnover and carbon cycling [61]. They may also contribute via the formation of persistent soil organic matter via their necromass (dead microbial biomass) [62]. The overall OTU richness and diversity of the bacterial populations under different burn regimes were very similar (Supplementary Table S1), with community composition and clustering patterns observed (Figures 2 and 3) paralleling the findings of Khodadad et al. (2011) [63]. This suggests that the majority of the bacterial populations in these montane grassland ecosystems may have adapted to their respective fire exposures, e.g., through improved dispersal and thus recolonization rates or with selection of fire-tolerant species in frequently burned plots [64]. It is also important to mention the potential role of bacterial drivers on the fungal community as observed by the presence of *Skermanella*, *Gemmata*, *Reyranella*, and *Stella* genera. These bacteria are known to be present in the soil [65,66], plants [67], and rhizosphere [68], some of which have nitrogen-fixing and carbon cycling abilities.

4.2. Fungal Communities and Fire Event Frequency

Colonisation assessed in this study decreased by 17% (biennial spring) to 23–26% during annual and biennial autumn burns, when compared to the no-burn control plots. AMF colonization has been shown to be higher in the roots of plants grown in spring compared to autumn [69], as a consequence of higher soil temperatures and increased plant growth during spring. It is possible that due to reduced soil moisture and lower plant activity in autumn, AMF colonization significantly decreased due to reduced activity brought on by reduced plant growth as plants often senesce during autumn [70]. It is also important to note that in ecosystems where fires occur frequently, recovery time for AMF may be insufficient, resulting in the significant decrease in AMF colonization seen in this study, while less frequent fires allow AMF populations to recover and re-establish [71,72].

In contrast, spore counts show significantly higher density during annual spring; this mirrors observations of both the bacterial community and vegetation composition. This plot was sampled four months after the burn event indicating a response to the most recent disturbance. AM fungal spores, which are resilient propagules, serve in dispersal as well as fulfilling a survival role in response to ecosystem disturbances, in this case, fire [47,73]. It should also be noted that soil K concentrations observed in the annual spring plot were higher when compared to the no-burn control plot, suggesting a synergistic effect of this nutrient. A similar increase in AM spore densities was observed after a prescribed spring fire in a semi-arid rangeland in Iran and was theorised to be a result of the increase of soil nutrients from plant ashes [74]. In contrast, the biennial spring plot, which was disturbed at the same time, did not show corresponding results. However, the reason for these anomalies may also be related to vegetation types and coverage, which was not assessed in this study.

Examination of the mycorrhizal diversity profiles in the different burn regimes in the Drakensberg revealed a dominance of Glomeromycota (Figure 4). Whilst they are not as diverse as other soil fungi, with less than 200 species recognised, their importance as cosmopolitan root symbionts has great ecological significance [75]. Two representative sequences detected in this study, OTU 5 and OTU 9, have previously been isolated from plant roots [76] and may aid in plant nutrient uptake. The Glomerales, comprising *Glomus* group A and B species, along with the genera *Septoglomus* and *Rhizophagus* [77], are dominant in the burn plots. Representatives of the rarer Diversisporales, Archaeosporales, and Paraglomales are present in all burn treatment plots, albeit to a lesser extent in the annual and biennial datasets. The dominance of *Glomus* OTUs reflects the level of disturbances such as tillage and fallow periods [78] (Supplementary Table S3). Species richness declined in the more frequently burned plots, with fewer OTUs represented, particularly in the biennial spring burn plot. Dove and Hart (2017) [79] conducted a meta-analysis on the effect of fire on fungal species richness. They analysed 68 records from 29 studies across fungal guilds, including mycorrhizal fungi; these indicated an average species richness reduction of 28% post-fire. This is similar to the present study, where there was a difference of 25–59% in species richness between no-burn control and frequently burned plots, with no-burn control plots having higher richness. The reason for the lower diversity indices for the biennial spring burn plot is unclear, however, records show that this particular Brotherton site was dominated by a single grass species in 2018 (exhibiting a low Shannon–Weaver diversity index), corresponding with the microbial community indicators. The similarity between no-burn control and five-year burn interval plots indicates that prolonged periods with an absence of fire events increase AM fungal species richness.

4.3. Impact of Soil Chemistry on Microbial Communities

The strong correlation highlighted by distance-based modelling of the soil chemical components observed in the Brotherton microbial community showed micronutrients and N and C content are strong drivers of the microbial community, similar to Australian forest ecosystems [80]. While the effects of fire regimes may be difficult to predict due to their complexity and interaction with other environmental factors [25], knowledge of

how microbial communities respond to their environment is a step forward in predicting their response to the potential effects of fire regimes. Bacteria are favoured at low pH and low C:N ratios, while the response of fungi is inverse [81]. The complex nature of soil microbial communities is clearly ecosystem specific and depends on how particular fire regimes interact with vegetation and soils. Furthermore, when regarding differences in soil properties associated with burn season, Manson et al. (2007) [17] found that annual autumn burn plots had lower C:N ratios and pH, and their bulk density was greater when compared to other frequently burned treatments. Microbial communities in Brotherton are strongly controlled by the soil environment particularly by soil pH, and soil nutrients [24]. While the soil chemistry results described in this study are limited, the study firmly highlights the need for long-term monitoring processes in soil microbiome studies of this nature.

5. Conclusions

This study addresses the knowledge gap surrounding Southern Hemisphere ecology, specifically in South Africa where recurrent fire is a critical feature of grasslands. We demonstrate that bacterial and fungal communities from this region demonstrate a significant response to season-specific burn patterns and that these seasonal patterns have a unique temporal aspect, resulting in varying impacts of burn event frequency on the corresponding soil microbial community profiles. Most studies performed to date on below-ground processes which influence the plant–soil environment are in non-burned systems. The bacterial and mycorrhizal populations in the Drakensberg grassland soils diverge from their original configuration when subjected to annual fires. With return intervals longer than two or more years, they demonstrate an ecological shift in the population that resembles a no-burn control plot. Given that the bacterial and mycorrhizal populations in the montane grasslands in the Drakensberg do not alter significantly with biennial and annual burn events (apart from our anomalous finding associated with the annual spring burn plot), it may be indicative of these microbiota having adapted to regular fire events which are the norm in these grasslands. While a significant correlation between the bacterial community profiles and the soil chemistry was observed, this did not extend to the fungal community and did not highlight the role of carbon and nitrogen in the microbial community as originally expected. Instead, mutualistic interaction between fungi and bacteria as well as cations, calcium, and magnesium, appeared to be the main drivers of the observed microbiome. However, shifts in bacterial and mycorrhizal community profiles in soils subjected to frequent burn events may also be attributed to the selection of taxa with preference/tolerance to the altered physical and chemical state of the soil as a result of fire (e.g., water retention, nutrient availability, soil porosity, mineral content, etc.) or temperature-sensitive species mortality. To determine if the divergence of microbial populations from the norm in the absence of fire is at both the level of metabolic functionality as well as species composition profiles, the degree of functional redundancy of the microbial populations in the different sample types needs to be determined through further functional metagenomic studies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15070818/s1, Figure S1: Mycorrhizal colonisation structures visualized by light microscopy (1: vesicle, 2: intercellular hyphae, 3: arbuscule); Figure S2: Distance-based RDA ordination of fungal data. Vectors indicate the direction of the soil chemistry parameter effect in the ordination plot; Table S1: Diversity indices generated for bacteria and AM fungi on Roche and MiSeq platforms; Table S2: Statistical analysis at phylum level for the soil bacterial community in burn-treated soils where statistical significance is indicated by $p \leq 0.05$; Table S3: BLAST analysis of select dominant fungal OTUs against the NCBI nucleotide database from which the uncultured/environmental sequences were omitted; Table S4: Marginal and sequential tests from distance-based redundancy analysis of the bacterial community; Table S5: Marginal and sequential tests from distance-based redundancy analysis of the fungal community.

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