Reply to reviewers and editor
soil-2015-86

1. Reviewer #1

General comments: Taking everything into account, I believe this work make a very good paper for SOIL journal and would provide a better understanding of how human interventions affect the structure and composition of soil microbial communities over time. I would like to commend the the way the authors gave a clear and concise background to their work in the introduction that even non-expert in this field can have a clear idea of what this study is about. There is, however, one or two modifications and details that would need to be added (in the specific comments below).

We thank Referee #1 for reviewing the manuscript and for the kind comments regarding the paper.

Specific comments:Page 1398, line 6-10. Provide more details on how you are going to analyse the soil properties. The reader will be interested to know what volume/mass of soil you used and how you did it.

In the revised manuscript, page 7 lines 17-23 provide this information.

Page 1398, line 24-25. On DNA extraction, how was it done? Please give details or refer the reader to a previous work where exactly the same method has been used. Page 1398, line 27. Again give the name and sequences of the primers used. State exactly what part of the ribosomal DNA you amplified. Did you use the same primer pairs for all three taxa, Bacteria, Achaea and Fungi. Please clarify. What was the composition of the PCR mix, PCR conditions used.

Technical comments: Page 1399, line 2. Life technologies is now part of ThermoFisher Scientific.

In the revised manuscript, page 8 line 12 to page 9 line 6 provide this information.

2. Reviewer #2

General Comment: Buyer et al. present the results of a field study of soil microbial community structure in a Namibian savannah that has been managed for shrub encroachment via physical removal of shrubs. The broad suite of analyses employed by the authors captures a clear picture of the effects. Microbial communities are influenced by several factors, including soil chemistry (pH) and plant cover, as would be expected from the literature on this subject. Most significant however, is the finding that these communities demonstrate recovery from shrub removal; the communities return to the state of those in control plots, where shrubs were not removed. The use of PLFA analyses to capture shifts in the biomass of specific microbial gropus was particularly appopriate for this study. This research is a valuable contribution to the journal (SOIL) and to the body of literature exploring the responses of microbial communities to land use change, particularly in critically important savannah ecosystems.

Specific Comments: I found this paper to be very clearly written and meticulously prepared (i.e., high presentation quality). All the facets of the data appear to have been clearly explored. The authors acknowledge and explore the largest flaw in their work, namely the lack of replication of field plots, such that each time point in the hronosequence is represented by only one plot, which was then sampled three times (pseudoreplication). This presents challenges in discerning whether differences among the chronosquenece plots are due to time since shrub removal or due to inherent differences between the plots. However, the changes observed are supported by comparison to adjacent, control plots.
Technical Corrections: None
We thank Referee #2 for reviewing the paper and for his/her very generous praise. We agree that the lack of replication in the chronosequence is a major difficulty. We tried to present this problem, and our statistical approach to the problem, very clearly, and are pleased that you feel we succeeded.

3. Reviewer #3
The present study intends to assess the effect of bush thinning on soil chemistry, microbial biomass and microbial community structure in a savanna and on resilience of ecosystem. The subject falls within the general scope of the journal and it is an interesting contribution. However, the design of the experiment that, in my opinion, is not valid to reach the aims planned in this study. If the authors intended to assess whether the changes promoted by a invasive plant on soil microbial communities diminish or disappear after its management using the thinning, they should have selected a noninvaded area as control of the original state of savanna. Bush encroachment is a major disturbance to the ecosystem and the recovery of soil microbial community after bush thinning should be referred to pre-invasion conditions.
We thank Referee #3 for his/her careful criticism of the paper. As stated in the paper, our hypotheses were (1) In a savanna ecosystem soil microbial community structure is different under grass than under woody plants, and (2) the soil microbial community is resilient to the disturbance caused by bush thinning. The experimental design, using control plots that had been invaded but not thinned, was adequate to test these hypotheses.
If we had been testing the hypothesis that thinning restores the ecosystem to a pre-invasion state, then the non-invaded plot would have been essential as a control. In fact, we completely agree with the reviewer that this would have been ideal. However, it was impossible as there were no non-invaded areas near the study site. The only non-invaded areas were so far from the study site that spatial variance would have rendered those areas useless as controls. We were very careful to state that we were measuring recovery from the disturbance caused by bush thinning, not recovery from bush invasion, and not recovery to a pre-invasion condition. This is, we agree, not ideal, but it does provide an experimental framework to test our stated hypothesis.
Another concern is the lack of replicates of each treatment; as only one plot by treatment was performed. Authors in- dicated in Statistical analysis section that the factor thinning was not pseudoreplicated because there were 3 pairs of thinned and control plots. However, the 3 thinned plots correspond to the three levels of factor thinning since each plot has a different time of thinning. In my opinion, only vegetation factor was replicated.
We worked very closely with a highly qualified statistician to make sure that our analysis was valid. With 3 pairs of thinned and control plots, and using a mixed model of variance to account for spatial and temporal variation, both factors

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(thinning and vegetation) were not pseudoreplicated. Any effect of the time of thinning would have been included in the spatial component of variance since there was one plot for each year of thinning. However, if we had tried to determine the effect of time of thinning in this model, using time of thinning as 3 levels as you describe, the analysis would indeed have been pseudoreplicated and entirely invalid. We would also like to point out that pseudoreplication is not always a straightforward concept to apply. In some cases it is a matter of judgment as to whether an experiment is pseudoreplicated or not, and whether a pseudoreplicated experiment is still useful or not. There has been much discussion of this in recent years, and here are two references on this point:

Heffner, R.A., Butler, M.J., Reilly, C.K., 1996. Pseudoreplication Revisited. Ecology 77, 2558-2562.

Schank, J.C., Koehnle, T.J. 2009. Pseudoreplication is a Pseudoproblem. J. Comparative Psychology 123, 421-433.

As Schank and Koehnle stated in their paper cited above, "The problem of pseudoreplication rests on the question of whether data gathered with any degree of spatiotemporal proximity is too intercorrelated and statistically interdependent to permit statistical inference." In our case the use of a mixed model of variance permits us to test hypotheses regarding thinning and vegetation without pseudoreplication, but not a hypothesis regarding time of thinning. Instead, the effect of time of thinning was inferred but could not be separated entirely from possible effects of spatial variation, as we clearly stated in section 3.1.3 and in the last paragraph of section 3.2.2.

If the Journal considers acceptable the use of pseudo-replicates, the authors should perform a statistic analysis of the data in Tables 1 and 2. The effect of treatment thinning (thinned vs. control plot) was not statistically analysed and then it cannot be concluded if soil chemistry and PLFA concentrations were more affected by the type of vegetation or by the treatment thinning.

Table 1 was statistically analyzed because, as we stated above, the effect of thinning was not pseudoreplicated. The statistical analysis was clearly presented in Table 1. However, we did not include an analysis of the main effects, only of the factorial treatment x vegetation. We have now included in the Table 1 header information on the P values of the main effects (page 26 lines 4-7). We have also included a brief discussion of the main effects on page 11 lines 6-10.
Table 2 was not and should not be statistically analyzed because the effect of time of thinning is indeed pseudoreplicated and therefore any statistical analysis would be invalid. We believe that Table 2 contains useful information, particularly when combined with the microbial community analysis presented in Figures 3-6, that should be presented despite the lack of statistical analysis. As Hurlbert said in the original paper on pseudoreplication (Hurlbert, S.H., 1984, Ecological Monographs 54, 187-211), "Because an obsessive preoccupation with quantification sometimes coincides, in a reviewer or editor, with a blindness to pseudoreplication, it is often easier to get a paper published if one uses erroneous statistical analysis than if one uses no statistical analysis at all." We believe that we have avoided this trap, providing a statistical analysis where it is valid and avoiding it where pseudoreplication renders such an analysis invalid.
Most importantly, we clearly stated which data was pseudoreplicated, and we were extremely cautious in our interpretation of it, so the issue was clearly laid out and the reader was not deceived.
Specific comments: -The application of PLFA 16:1!5 as biomarker of AMF is limited due to its presence in bacteria (Frostegård et al. (2011). Soil Biology and Biochemistry 43, 1621–1625.).
Thank you for pointing this out. The problem is actually more general than just AMF. We have added a statement to the text (page 13 lines 20-23).
-What month was carried out the thinning?. -How many times were the plots thinned each time?.
This information was added on page 6 lines 7-10.

4. Technical editor
Please provide the soil classification.
Unfortunately we do not have the capability to dig a soil pit on the site and properly determine the full soil classification. The information we have is from a soil atlas. We were able to add slightly more detail than we originally provided on page 5 line 15.
The authors have to consider seriously the recommendation of referee 3 about the statistical analysis of data in tables 1 and 2, and should perform a statistic analysis of

these data to conclude if the soil chemistry and PLFAs concentration, are affected by different factors.
Please see the reply above to Referee 3. We have added the P values of the main effects to the Table 1 header. We did not report each P value individually as they were almost all identical, and that would have made 2 highly redundant columns in the table.

LIST OF ALL CHANGES

Page 5 line 15 Cambisols changed to chromic Cambisols
Page 6 lines 7-10 exact dates of sampling added, and a statement that each plot was thinned once
Page 7 lines 17-23 details on soil analysis added
Page 8 line 12 to page 9 line 6 details on soil DNA analysis added
Page 11 lines 6-10 Significance of main effects added
Page 13 lines 20-23 Statement about PLFA biomarkers added
Page 20 lines 6-7 Reference added regarding PLFA biomarkers
Page 26 lines 4-7 Table 1 header modified to include information on main effects

Soil microbial communities following bush removal in a Namibian savanna

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Abstract

Savanna ecosystems are subject to desertification and bush encroachment, which reduce the carrying capacity for wildlife and livestock. Bush thinning is a management approach that can, at least temporarily, restore grasslands and raise the grazing value of the land. In this study we examined the soil microbial communities under bush and grass in Namibia. We analyzed the soil through a chronosequence where bush was thinned at 9, 5, or 3 years before sampling. Soil microbial biomass, the biomass of specific taxonomic groups, and overall microbial community structure was determined by phospholipid fatty acid analysis, while the community structure of Bacteria, Archaea, and fungi was determined by multiplex terminal restriction fragment length polymorphism analysis. Soil under bush had higher pH, C, N, and microbial biomass than under grass, and the microbial community structure was also altered under bush compared to grass. A major disturbance to the ecosystem, bush thinning, resulted in an altered microbial community structure compared to control plots, but the magnitude of this perturbation gradually declined with time. Community structure was primarily driven by pH, C, and N, while vegetation type, bush thinning, and time since bush thinning were of secondary importance.

Keywords: soil systems, bush encroachment, PLFA, TRFLP

1. Introduction

Savanna ecosystems are defined as grassy areas with woody plants varying from widely spaced to 75% canopy (Smit, 2004). Savannas cover more than 20% of the world's land surface and include most of the world's rangelands (Riginos et al., 2009). Bush encroachment affects rangelands worldwide (O'Connor et al., 2014), while historical photographic evidence indicates that it is occurring in certain regions of Namibia (Rohde and Hoffman, 2012). Indeed, 32% of the land surface of Namibia was estimated to be affected by bush encroachment in 2004 (de Klerk, 2004). Factors controlling the density of woody plants, the temporal dynamics of woody plant density, and bush encroachment have been heavily studied but are still not completely understood. Contributing factors include herbivory, fire frequency and intensity, soil properties, rainfall, and atmospheric carbon dioxide concentrations (O'Connor et al., 2014; Bond and Midgley, 2012; Kambatuku et al., 2013; Ripple et al., 2015).

Plant communities are closely linked to soil microbial communities, with plants providing energy to most microbes through root exudates and plant litter (Lynch and Whipps, 1990; Schenck zu Schweinsberg-Mickan et al., 2012; Singh et al., 2014). In return, soil microbial communities provide critical ecosystem services including soil formation and aggregation; plant litter degradation, humus formation, and carbon sequestration; nitrogen fixation, nitrification, and denitrification; biocontrol of pathogens; and degradation of xenobiotics (Lehman et al., 2015). The linkages between above-ground and below-ground biota are poorly understood in savanna ecosystems, where dynamic changes in C3/C4 and leguminous/non-leguminous plant communities result in

a highly complex system. However, it is clear that woody plant encroachment has the potential to alter soil microbial community biomass, structure, and diversity. Plant species composition is known to affect microbial species composition and diversity (Wardle, 2006; Maul and Drinkwater, 2010). Invasive plants have been shown to alter soil microbial communities (Batten et al., 2006), biogeochemical cycling, nutrient availability, and ecosystem function (Weidenhamer and Callaway, 2010). Soil microbial biomass has been reported to increase with increasing woody plant density and age (Liao and Boutton, 2008), while soil community composition was altered and microbial biomass increased during a reforestation experiment with *Eucalyptus urophylla* (Wu et al., 2013). Soil biota were altered along a desertification gradient (Klass et al., 2012). These changes in soil microbial communities have the potential to alter the rate of bush encroachment. Both positive and negative feedbacks (Wardle et al., 2004) can occur between plant and soil microbial communities, affecting the progress of plant invasion (Reinhart and Callaway, 2006; Shannon et al., 2012; Ke et al., 2015).

One technique for restoring bush-encroached savannas is to remove the woody plants, thus promoting grass growth and improving the grazing capacity of the savanna (Smit, 2004). In this study we report on a chronosequence of bush thinning. Excess *Senegalia mellifera* (formerly *Acacia mellifera*) and other woody vegetation were removed from three different plots (one plot each year) in 2003/4, 2007, and 2009, and a paired “control” plot was established adjacent to each thinned plot. We analyzed soil taken from the bush and grass environments for soil chemistry, microbial biomass, and microbial community structure in order to test the following hypotheses: (1) In a savanna ecosystem soil microbial community structure is different under grass than

under woody plants, and (2) the soil microbial community is resilient to the disturbance caused by bush thinning. Soil microbial biomass, the biomass of individual taxonomic groups, and soil microbial community structure were measured by phospholipid fatty acid (PLFA) analysis. A more detailed analysis of bacterial, archaeal, and fungal community structure was provided by terminal restriction fragment length polymorphism (TRFLP) analysis of soil DNA.

2. Materials and methods

2.1 Site description

All samples were taken from the Elandsvreugde farm at the Cheetah Conservation Fund International Research and Education Centre, Otjiwarongo, Namibia (Figure 1). The Elandsvreugde farm (20º 25' S, 17º 4' E) is 7,300 hectares in size. Soils are classified as chromic Cambisols based on a soil atlas (http://spatial-web.nmsu.edu/flexviewers/NamibiaSpatialData/). The vegetation is characterized as thornbush savanna, with woody species such as *Senegalia mellifera* (formerly *Acacia mellifera*) predominating. Understory vegetation is sparse except for forbs which are briefly present following rainfall. The major grass species is hairyflower lovegrass (*Eragrostis trichophora*). The area receives an average annual rainfall of 400-500 mm, thus classifying it as a semi-arid zone. The wet-hot season is January to April, followed by a dry-cold season from May to August, with September to December intermediate. The vegetation is utilized by wild game including kudu (*Tragelaphus strepsiceros*), oryx

(*Oryx gazella*), red hartebeest (*Alcelaphus buselaphus subsp. caama*), eland (*Tragelaphus oryx*), warthog (*Phacochoerus africanus*), steenbok (*Raphicerus campestris*), and duiker (*Sylvicapra grimmia*).

2.2 Experimental treatments

In 2003-4 (approximate dates November 15 2003 – February 28 2004), 2007 (February 2-22), and 2009 (August 1 2009 – January 31 2010) three plots (one each year) were thinned of excess *Senegalia mellifera* and other bushy species. Each plot was thinned once. The sizes of the thinned plots were 25 ha, 21 ha, and 20 ha, respectively. A control plot adjacent to and similar in size to each thinned plot was not thinned. Samples were taken from both the thinned plots and the control plots at three sampling times starting in 2012, so the plots are referred to as 9 year (bush thinned in 2003), 5 year (bush thinned in 2007), or 3 year (bush thinned in 2009) plots. In the thinned plots bush density was reduced up to 70%, almost entirely by manual cutting, while a small amount of thinning was accomplished using a hydraulic cutterhead. Tree and shrubs were cut aboveground (±30cm) with roots left intact.

2.3 Sampling

Samples were taken on May 2-8, 2012; August 18-22, 2012; and November 7-9, 2013. Six plots were sampled at each date: the 9 year, 5 year, and 3 year thinned and corresponding control plots, as described in Sect. 2.2. Within each plot three geo-

referenced sampling locations were selected using Hawth's random selection tool for ArcGIS (http://www.spatialecology.com/htools). Paired soil samples were collected at each sampling location, with one sample taken under bush (*Senegalia mellifera*) and the other under adjacent grass (*Eragrostis trichophora*). A free-standing bush was selected in open areas, whereas a bush within a cluster was selected in dense areas. Samples under bush were collected halfway between the trunk and the edge of the canopy. Samples were taken at the same geo-referenced sampling locations on all 3 sampling dates. All samples were taken at 0-15 cm in depth after removing surface litter. Samples were stored at -20 ºC until analysis. A total of 108 samples were collected: 3 years of thinning (9, 5, and 3 years) x 2 treatments (thinned and control) x 3 geo-referenced sampling locations x 2 vegetation types (bush and grass) x 3 sampling dates (May 2012, August 2012, and November 2013). The experimental design is summarized in Fig. 2.

2.4 Soil analysis

Soil pH was measured with a combination electrode after shaking 1 g soil in 10 ml 0.01 M $CaCl_2$ for 1 hour and letting solids settle for 15 min. Total C and N were measured on an Elementar VarioMax CNS analyzer (Elementar Americas, Mt. Laurel, NJ, USA), using duplicate 0.5 g samples. No inorganic C was detected by reaction with acid, so the soil was noncalcareous, and total C equaled organic C (Nelson and Sommers, 1996). Soil texture was analyzed on 50 g samples taken from a single sampling date using the hydrometer method (Gee and Bauder, 1986).

Phospholipid fatty acids (PLFA) were analyzed as previously described (Buyer and Sasser, 2012). Briefly, lipids were extracted and phospholipids separated by solid-phase extraction. The fatty acids present in the phospholipids were converted to fatty acid methyl esters by transesterification and analyzed by gas chromatography. Quantification was performed relative to an internal standard. Identifications were confirmed on a random subset of samples by gas chromatography-mass spectrometry. PLFA's were summed into biomarker categories as follows: Gram-positive bacteria, iso and anteiso saturated branched fatty acids; Gram-negative bacteria, monounsaturated fatty acids and cyclopropyl 17:0 and 19:0; actinomycetes, 10-methyl fatty acids; fungi, 18:2 ω6 cis; arbuscular mycorrhizal fungi, 16:1 ω5cis; and protozoa, 20:3 and 20:4 fatty acids (Buyer et al., 2010).

Soil DNA was extracted and purified using 0.25 g samples and the PowerSoil-htp 96 Well Soil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA). Multiplex terminal restriction fragment length polymorphism analysis was performed for Bacteria, Archaea, and fungi as previously described (Singh et al., 2006). Briefly, bacterial, archaeal, and fungal ribosomal DNA sequences were amplified by polymerase chain reaction using fluorescent dye-labelled primers. Each PCR reaction contained:1X Amplitaq Gold® 360 master mix (ThermoFisher Scientific, Waltham, MA), 0.2 µM eubacterial forward (63fVIC) and reverse (1087r) primers, 0.4 µM archaeal forward (Ar3f) and reverse (Ar927rNED) primers, 0.4 µM fungal forward (ITS1fFAM) and reverse (ITS4) primers, 10 ng of template DNA, and nuclease-freewater to adjust to a total reaction volume of 50 µL. Primer sequences are given in Singh et al. (2006). Amplification was accomplished on a Techne thermal cycler (Bibby Scientific US,

Deleted: Soil pH was measured using 1:10 dilutions in 0.01 M $CaCl_2$. Total C and N were measured on an Elementar VarioMax CNS analyzer (Elementar Americas, Mt. Laurel, NJ, USA). No inorganic C was detected by reaction with acid, so the soil was noncalcareous, and total C equaled organic C (Nelson and Sommers, 1996). Soil texture was analyzed on samples taken from a single sampling date using the hydrometer method (Gee and Bauder, 1986).

Burlington, NJ, USA) according to the following protocol: initial denaturation at 95ºC for 5 min; 30 cycles of denaturation at 95ºC for 45 s, annealing for 45 s at 55ºC, and extension at 72ºC for 1 min; and final extension at 72ºC for10 min. The amplicons were restricted with the enzymes MspI and HhaI and purified using a Performa® DTR Edge Plate (Edge BioSystems, Gaithersberg, MD). The dye-labelled restriction fragments were analyzed on an ABI 3730 Prism Genetic Analyzer (ThermoFisher Scientific).

Deleted: Multiplex terminal restriction fragment length polymorphism analysis was performed for Bacteria, Archaea, and fungi as previously described (Singh et al., 2006). Briefly, soil DNA was extracted and purified. Bacterial, archaeal, and fungal ribosomal DNA sequences were amplified by polymerase chain reaction using fluorescent dye-labelled primers. After purification the amplicons were restricted with the enzymes MspI and HhaI and the dye-labelled restriction fragments were analyzed on an ABI 3730 Prism Genetic Analyzer (life technologies, Grand Island, NY, USA).

2.5 Statistical Analysis

The analysis of treatment (thinned vs. control) and vegetation (bush vs. grass) effects was accomplished using a repeated measures split-split-plot design. PLFA concentrations were analyzed in SAS (version 9.2, SAS Institute, Cary, NC, USA) with a general linear mixed model. C, N, pH, and texture values were analyzed in SAS with a generalized linear mixed model utilizing a Beta distribution and a logit link function (Stroup, 2015). The covariance structure was optimized for each variable and compensated for spatial (3 sampling locations within each plot) and temporal (each sampling location sampled 3 different times) covariance. While the analysis of treatment and vegetation effects was not pseudoreplicated, as we had 3 pairs of thinned and control plots and compensated for covariance as described above, the analysis of recovery of thinned plots with time was inherently pseudoreplicated, as only one plot was thinned each year. Therefore, for the analysis of the effect of time since bush thinning, we calculated means for each plot and compared them without attempting to assess statistical significance.

TRFLP data was processed using the online software package T-REX (http://trex.biohpc.org/) to remove noise and align peaks (Culman et al., 2009). For both PLFA and TRFLP data, redundancy and canonical correspondence analysis were performed in CANOCO (version 5, Microcomputer Power, Ithaca, NY, USA). The proportional data was square-root transformed, and the length of the gradient relative to the standard deviation determined the choice between linear (redundancy analysis) and unimodal (canonical correspondence analysis) models, as advised by the CANOCO software.

3. Results and discussion

3.1 Soil microbial biomass and soil properties

3.1.1 Treatment and vegetation effects

The effects of treatment (thinned vs. control) and vegetation (bush vs. grass) on soil texture, pH, C, N, and PLFA concentration are summarized in Table 1. The soil was noncalcareous, so total C was equivalent to organic C. There were small but statistically significant effects on soil texture, with silt higher in control plots than thinned plots and higher under bush than grass. This may be explained by wind erosion preferentially removing silt over sand and clay from thinned plots and grassy locations (Colazo and Buschiazzo, 2015).

The effect of landscape vegetation (bush vs. grass) was far greater than the effect of treatment (thinned vs. control plot) on soil chemistry and PLFA concentrations. Soils under bush had significantly higher pH, C, N, and total PLFA, which corresponds to microbial biomass, than soils under grass. The concentration of each PLFA biomarker group was also greater, indicating that the biomass of the large taxonomic groups analyzed by PLFA all increased in bush compared to grass environments. While there were no statistically significant overall treatment effects on soil chemistry or PLFA concentrations, there was a statistically significant treatment effect under grass, where the pH was higher in control plots than in thinned plots. There were, however, significant overall treatment effects on microbial community structure (Section 3.2.2, below). These results are consistent with the concept of woody plants as 'islands of fertility' with higher soil organic matter and nutrients than under grass (Okin et al., 2008), and are similar to those observed under *Vachellia tortilis* (*Acacia tortilis*) in Tunisia (Fterich et al., 2014).

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3.1.2 Sampling date effect

The sampling date (May 2012, August 2012, November 2013) had no effect on pH, C, or N. However, there were large and statistically significant differences in PLFA concentrations between the first two samplings and the final sampling. The total PLFA concentration was 40.23 nmol/g, 40.35 nmol/g, and 28.36 nmol/g for May, August, and November samplings, respectively. Similarly, a decrease in the concentration of each and every PLFA biomarker group occurred from August 2012 to November 2013 (data

not shown). Annual rainfall totaled 669 mm in 2012 and 223 mm in 2013, which likely explains the difference in microbial biomass over this time period.

3.1.3 Chronosequence effect

The recovery of the soil microbial biomass in the years following bush thinning was examined by comparing thinned and control plots separately for each year of thinning. We only performed this analysis for samples under grass because the purpose of bush thinning was to restore the ecosystem to one dominated by grass. In the 9 year plots, the thinned and control plots were nearly identical in total PLFA, all PLFA biomarker groups, C, N, and pH (Table 2). In the 5 year plots, the control plot was slightly higher than the thinned plot in pH, C, total PLFA, and most of the PLFA biomarkers. In the 3 year plots, total PLFA was much higher in the thinned plot than the control plot. PLFA biomarker groups were also higher in the thinned plot, although the amount of increase varied somewhat between biomarker groups. C and N were also higher in the thinned plot, but the pH was much lower. Only one thinned plot was established each year, so we cannot control for spatial variation among the different years of the chronosequence, but the general trend in the chronosequence was for soil properties, microbial biomass, and the biomass of each taxonomic group in thinned plots to become more similar to control plots with time, indicating ecosystem recovery from the disturbance created by bush thinning. These results may be explained by the death and decomposition of woody plant roots following harvesting, thus temporarily

raising C, N, and microbial biomass, and by indirect effects of the regrowth of hairyflower lovegrass (*Eragrostis trichophora*) in the bush removal area.

3.2 Soil microbial community structure

3.2.1 PLFA analysis of community structure

PLFA analysis was also used to analyze microbial community structure. The ordination plot is presented in Fig. 3. The horizontal axis separates samples primarily according to vegetation type (grass vs. bush), while the vertical axis separates primarily by sampling date. All November 2013 samples have positive Axis 2 values, while all May and August 2012 samples have negative values on Axis 2. Soil samples under bush were associated with higher proportions of AMF, protozoa, and Gram-negative bacteria, while soils under grass were associated with higher proportions of actinomycetes and Gram-positive bacteria. The higher proportions of actinomycetes and Gram-positive bacteria under grass may be a response to lower carbon availability and a more oligotrophic environment, as described in Sect. 3.1.1, and are consistent with results in an agricultural system (Buyer et al., 2010). Samples taken in 2012 had higher proportions of Gram-positive bacteria, AMF, and protozoa, while samples taken in 2013 had higher proportions of actinomycetes and Gram-negative bacteria. These biomarkers should be interpreted very cautiously, as there is ample evidence in the literature to suggest that these groupings are not entirely specific (Frostegård et al., 2011).

3.2.2 TRFLP analysis of community structure

Soil microbial community structure was analyzed in greater detail by TRFLP. Ordinations are presented in Figures 4, 5, and 6 for Bacteria, Archaea, and fungi. Vectors indicate correlations to treatment and environmental variables. For Bacteria and Archaea, the samples taken in November 2013 had very different TRFLP profiles than the samples taken in 2012, so they were analyzed in a separate ordination. For fungi, each sampling had different profiles, so they were all analyzed separately. Bush and grass samples were generally different in microbial community structure, while thinning and years since thinning also had some effect. Soil pH, C, and N also affected the TRFLP profiles. Since pH, C, and N were all greater in samples taken under bush than under grass, the vectors for these factors were all partially aligned with the vector for bush.

Decomposition of variance of the ordinations is presented in Table 3. Soil pH, C, and N explain a greater proportion of the total variance than vegetation, thinning, year of thinning, or soil texture. These results indicate that soil chemistry is more important than soil texture, vegetation type, bush thinning, or years since bush thinning in determining soil microbial community structure in this experiment. There is some shared variance between vegetation and pH, C, and N (data not shown), suggesting that some but not all of the effect of vegetation on the soil microbial community is through alterations in soil chemistry. This is consistent with the results presented in Table 1 which demonstrate the effects of vegetation on pH, C, and N. pH is believed to be the primary

driver of soil microbial community structure at continental scales (Lauber et al., 2009) while soil C has been identified as another factor contributing to soil microbial community structure (Fierer et al., 2007).

Bush thinning and years since bush thinning also had some effect on soil microbial community structure which was not attributed to shared variance with soil chemistry. Bush thinning may have perturbed the soil ecosystem through death and subsequent decay of roots, through changes in root exudates as the ecosystem shifted from bush to grass, or both. We interpret the effect of year of bush thinning as ecosystem recovery, but recognize that it could represent spatial variation since each thinned plot was at a different site within the Elandsvreugde farm. However, if this is a location effect, it is not entirely attributable to differences in soil pH, C, or N, since not all variance was shared with soil chemistry. Furthermore, the thinned and control plots represent a stark contrast in terms of plant community composition and potential ecosystem function.

3.3 Soil community recovery

Ecosystem recovery was also assessed by calculating the Euclidean distance, in ordination space, between control and thinned plots for each year since thinning. For Bacteria in 2012, the distance between control and thinned samples under grass was 0.11 for 9 years, 0.11 for 5 years, and 0.49 for the 3 year plot. For Bacteria samples taken in November 2013, the same three distances were 1.8, 0.9, and 2.0. Similarly, the analysis for Archaea indicated that the 3 year plots had the greatest distance between

control and thinned under grass in samples taken in both 2012 and 2013 (data not shown). The pattern for fungi was more complex. In May 2012 and November 2013 the 3 year plots had the greatest distance between control and thinned, while in August 2012 the distance was 9 years<3 years<5 years.

These results indicate that the microbial communities that were perturbed by bush thinning partially recovered over a time-span of 3-9 years to a state similar to that of undisturbed grass in a bush-encroached area, which is consistent with other findings (Marchante et al., 2009). The recovery was partially obscured by seasonal variations in microbial community structure. Recovery may have been more complete for Bacteria and Archaea than fungal communities, as previously demonstrated with the response of soil microbial communities under different land-use systems to drought (de Vries et al., 2012), or else the seasonal variation had a greater obscuring effect on recovery in fungal communities than in bacterial and archaeal communities. The recovery of the 5 year plot was not always intermediate between the 3 year and 9 year plots, which may reflect unstable temporal variations in community structure during recovery or transitional microbial community structures that are dissimilar to both the 9 year and 3 year plots.

Our results demonstrate that the soil microbial community is sensitive to bush thinning but is also resilient, with some recovery over a time-span of several years. The sensitivity to disturbance and the time-scale of resilience are consistent with other studies (Allison and Martiny, 2008). However, a recent study (Ke et al., 2015) found no effect of plant invasion on soil microbial community structure, suggesting that certain

soil communities are resistant to plant invasion and that sensitivity or resistance may depend on factors such as climate and site history as well as the specific plant species.

3.4 Further questions

Two other questions arise from this research that we cannot answer at this time. First, does soil ecosystem function change with the changes in microbial community biomass and structure reported here? While high levels of functional redundancy and soil microbial diversity suggest that changes in community structure may not affect function, there is evidence indicating that changes in microbial biomass and community structure may alter ecosystem processes (Reeve et al., 2010). Invasive plants have been shown to change soil ecosystem function (Weidenhamer and Callaway, 2010; Wolfe and Klironomos, 2005), which in one case was attributed to bacterial endophytes (Rout et al., 2013). The fact that *Senegalia mellifera* is leguminous and fixes nitrogen through symbiotic rhizobia strongly suggests that nitrogen cycling will be altered. Several soil enzyme activities were reported to be higher under the canopy of *Vachellia tortilis (Acacia tortilis)* than in adjacent open areas (Fterich et al., 2014).

Second, are there positive or negative feedback loops (Wardle et al., 2004) between grass and soil following bush removal? There is strong experimental evidence supporting the role of plant-soil feedback in driving plant community composition (Pendergast et al., 2013). The changes in soil chemistry and microbial communities following bush removal could either promote grass establishment (positive feedback) or bush regrowth and encroachment (negative feedback). Both soil nutrients and soil

microbial communities have been shown to be involved in plant-soil feedback (Perkins and Nowak, 2013). Further work is required to answer these questions and fully assess the role of soil microbes in restoration of savannas altered by bush encroachment.

4. Conclusions

We found that bush thinning initially perturbs the soil ecosystem, but over 3-9 years the system recovers to a state resembling that of undisturbed grass in a bush-encroached savanna. Bush thinning may provide a way to restore both the above-ground and below-ground components of bush-encroached savanna ecosystems to a more grass-dominated state.

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Figure Captions

Figure 1. Map of study area.

Figure 2. Experimental design. B, Bush; G, Grass; M, May 2012; A, August 2012, N, November 2013.

Figure 3. Redundancy analysis of soil PLFA. Vectors indicate the correlations between each factor and the axes. Very small vectors were eliminated for the sake of clarity.

Figure 4. Redundancy analysis of bacterial TRFLP. A: May and August 2012. B: November 2013. Vectors indicate the correlations between each factor and the axes. Very small vectors were eliminated for the sake of clarity.

Figure 5. Redundancy analysis of archaeal TRFLP. A: May and August 2012. B: November 2013. Vectors indicate the correlations between each factor and the axes. Very small vectors were eliminated for the sake of clarity.

Figure 6. Canonical correspondence analysis of fungal TRFLP. A: May 2012. B: August 2012. C: November 2013. Vectors indicate the correlations between each factor and the axes. Very small vectors were eliminated for the sake of clarity.

Table 1. Soil pH, C, N, texture, and PLFA concentrations. PLFA are reported as nmol/g dry weight. All values are least square means ± standard deviation, and values within a row with different letters are significantly different (p =0.05). N=27 except for soil texture values where N=9. The main effect of treatment (control vs. thinned) was not significant except for silt (p=0.0007). The main effect of vegetation (bush vs. grass) was significant (p=0.001) in all cases except for sand and clay which were not significant. The interaction of treatment x vegetation was not significant in all cases.

	Control		Thinned	
	Bush	Grass	Bush	Grass
pH	5.70±0.58 A	5.07±0.44 B	5.32±0.59 AB	4.53±0.43 C
Total C (%)	0.61±0.29 A	0.38±0.08 B	0.58±0.19 A	0.39±0.12 B
N (%)	0.04±0.02 A	0.02±0.01 B	0.04±0.01 A	0.02±0.01 B
Sand (%)	85±2 A	85±2 A	85±2 A	86±2 A
Silt (%)	7±1 A	5±1 B	5±1 B	4±1 C
Clay (%)	9±2 A	9±2 A	10±2 A	10±2 A
Total PLFA	42.68±14.23 A	26.05±10.08 B	47.21±16.67 A	29.31±13.74 B
Gram-negative	11.06±4.04 A	5.79±2.27 B	12.25±4.66 A	6.08±2.37 B
Gram-positive	10.49±3.45 A	7.38±3.27 B	11.07±3.51 A	8.02±3.57 B
Actinomycetes	4.74±1.30 A	3.37±1.41 B	4.47±1.08 A	3.45±1.18 B
Fungi	2.09±1.40 AB	0.85±0.80 B	3.43±3.02 A	1.16±1.54 B
AM Fungi	1.52±0.63 A	0.82±0.38 B	1.65±0.79 A	0.82±0.33 B
Protozoa	0.35±0.17 A	0.10±0.09 B	0.44±0.26 A	0.13±0.13 B

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Table 2. Soil pH, C, N, and PLFA mean concentrations by year of harvest under grass (N=9). PLFA are reported as nmol/g dry weight. Means are reported without standard deviation or statistical significance as this data is pseudoreplicated.

	9 years since thinning		5 years since thinning		3 years since thinning	
	Control	Thinned	Control	Thinned	Control	Thinned
pH	4.82	4.69	5.11	4.87	5.28	4.02
Total C (%)	0.34	0.36	0.42	0.39	0.35	0.40
N (%)	0.02	0.02	0.02	0.02	0.02	0.03
Total PLFA	24.3	24.4	28.55	27.37	25.3	36.16
Gram-negative	5.39	5.28	6.23	6.02	5.76	6.95
Gram-positive	6.74	6.94	8.42	7.56	6.97	9.55
Actinomycetes	2.82	3.07	3.77	3.50	3.51	3.77
Fungi	0.68	0.81	1.03	1.03	0.86	1.63
AM Fungi	0.76	0.77	0.92	0.88	0.79	0.79
Protozoa	0.08	0.09	0.12	0.17	0.11	0.12

Table 3. Decomposition of variance of TRFLP ordinations.

	Bacteria		Archaea		Fungi		
	May + Aug	Nov	May + Aug	Nov	May	Aug	Nov
Total Variance Explained	0.223***	0.369***	0.169***	0.245***	0.328***	0.326***	0.356**
Total Variance Due to:							
Vegetation	0.058***	0.051***	0.036***	0.030*	0.049***	0.049***	0.040***
Thinning	0.023***	0.043*	0.027***	0.021	0.025	0.039***	0.045***
Year of Thinning	0.045***	0.078*	0.030*	0.046	0.077***	0.070**	0.076**
pH, C, and N	0.100***	0.166***	0.071***	0.103***	0.109***	0.105*	0.124***
Texture	0.055**	0.107	0.038	0.080*	0.095	0.088	0.107

Significance of results: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.005$

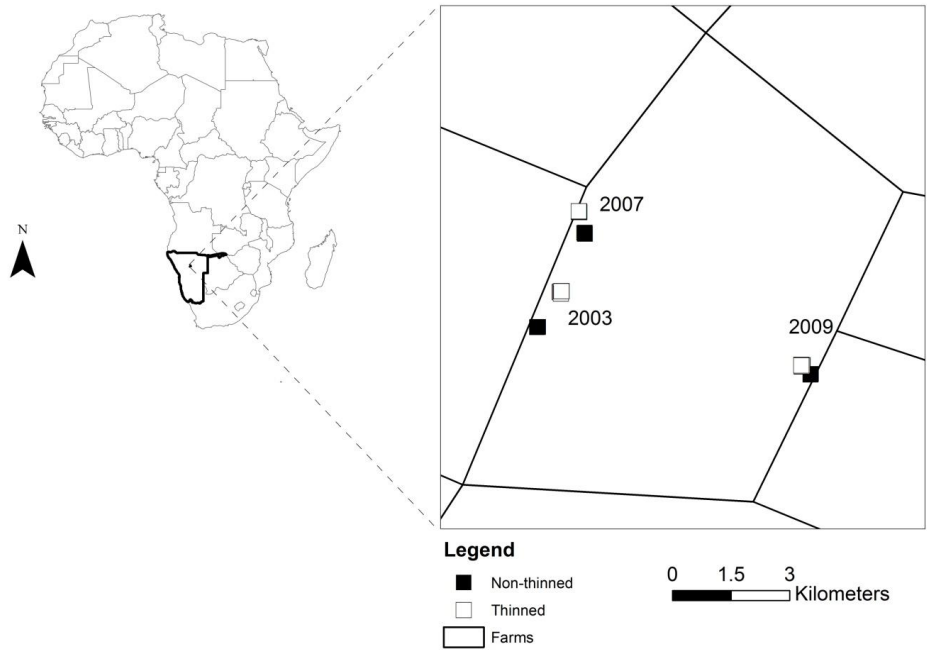

Figure 1. Map of study area.

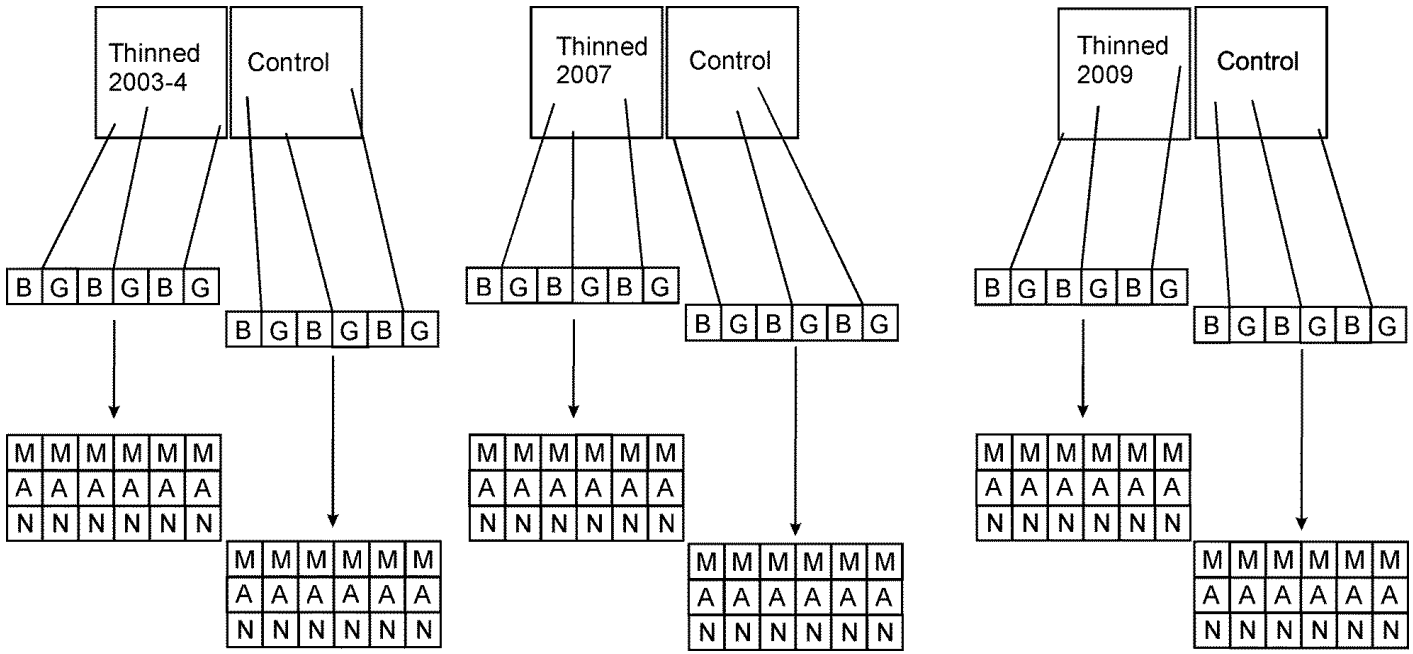

Figure 2. Experimental design. B, Bush; G, Grass; M, May 2012; A, August 2012, N, November 2013.

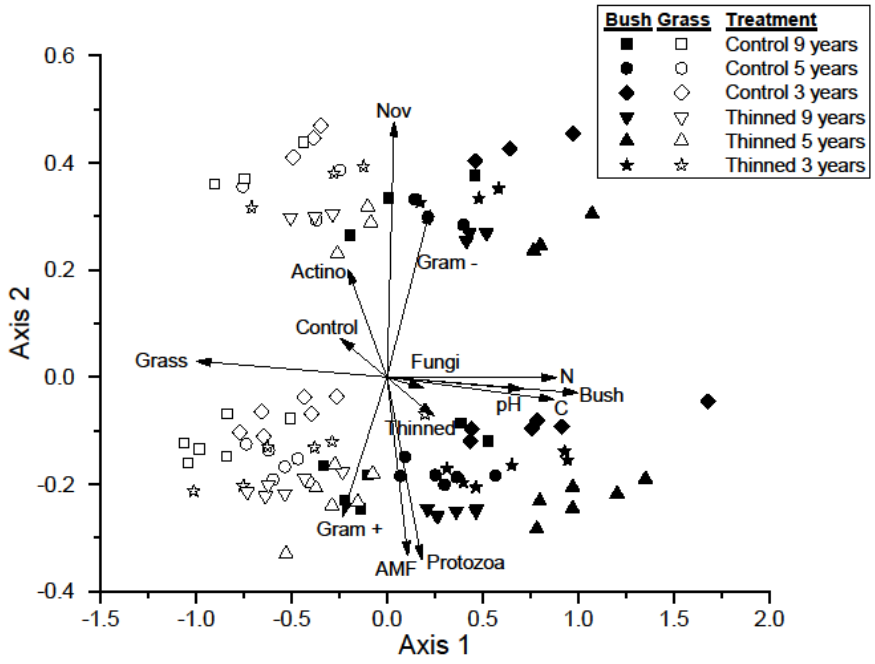

Figure 3. Redundancy analysis of soil PLFA. Vectors indicate the correlations between each factor and the axes. Very small vectors were eliminated for the sake of clarity.

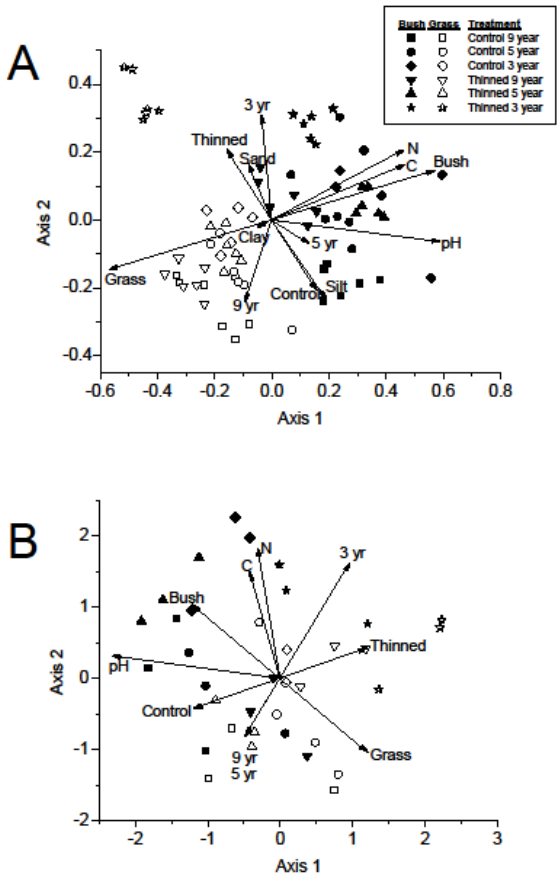

Figure 4. Redundancy analysis of bacterial TRFLP. A: May and August 2012. B: November 2013. Vectors indicate the correlations between each factor and the axes. Very small vectors were eliminated for the sake of clarity.

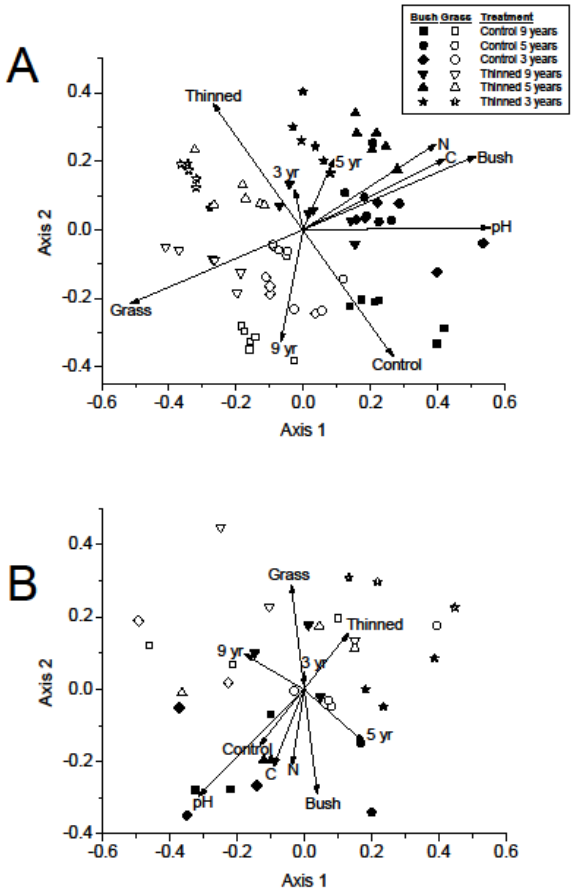

Figure 5. Redundancy analysis of archaeal TRFLP. A: May and August 2012. B: November 2013. Vectors indicate the correlations between each factor and the axes. Very small vectors were eliminated for the sake of clarity.

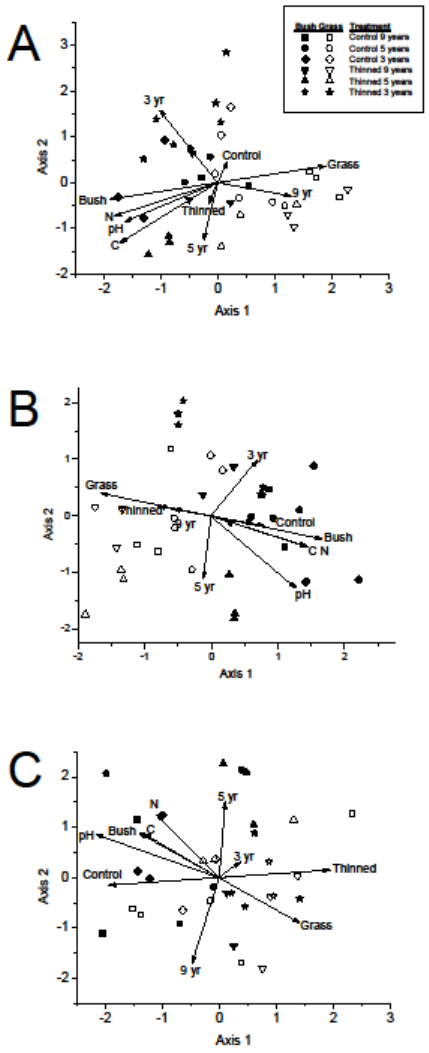

Figure 6. Canonical correspondence analysis of fungal TRFLP. A: May 2012. B: August 2012. C: November 2013. Vectors indicate the correlations between each factor and the axes. Very small vectors were eliminated for the sake of clarity.